# Inhibition of crotoxin binding to synaptosomes by a receptor-like protein from *Crotalus durissus terrificus* (the South American rattlesnake)[☆]

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## Abstract

Crotoxin (Ctx) is a potent neurotoxin of the venom of *Crotalus durissus terrificus* (the South American rattlesnake). Ctx is a heterodimer composed of CB, a toxic $PLA_2$ subunit, and CA, a non-toxic and non-enzymatic subunit, that potentiates the neurotoxicity of CB in vivo. The deleterious action of Ctx upon *C. d. terrificus* snakes themselves is known to be prevented by a $PLA_2$ inhibitor (CNF) present in their blood serum. CNF acts by replacing CA in Ctx, thus forming a new stable complex CNF–CB. This complex no longer interacts with the target receptor (TR) to deliver CB to cause its lethal effect. Furthermore, CNF–CB seems to be reminiscent of the interaction Ctx–TR at the pre-synaptic site. In the present work, the binding competition between rat brain synaptosomes (TR) and CNF for Ctx was investigated. Radiolabeled Ctx, made of CA and one isoform of CB (CA−$^{125}$ICB$_2$), was used as ligand. The competition by unlabeled Ctx was taken as a reference. The potency of CNF as a competitor was evaluated under different incubation conditions with varying time scale addition of reagents (CA−$^{125}$ICB$_2$, synaptosomes and CA−CB$_2$ or CNF). CNF was able to inhibit the binding of the toxin to synaptosomes as well as to partially displace the toxin already bound to its membrane target. The mechanisms of competition involved were discussed and a previous schematic model of interactions between Ctx, TR and CNF was updated.
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*Keywords:* Crotoxin; Phospholipase $A_2$; Phospholipase $A_2$ inhibitor; Synaptosome; CNF; Crotalus

## 1. Introduction

*Crotalus durissus terrificus* (the South American rattle-snake) venom is mainly composed of a potent neurotoxic protein, crotoxin (Ctx), which is known to contain the bulk of the lethal toxicity of the crude venom. Crotoxin is a β-neurotoxin consisting of a heterodimer of a non-toxic, non-enzymatic acidic protein CA and a basic protein CB with phospholipase $A_2$ activity [1]. CA and CB are tightly linked in the Ctx complex by non-covalent, electrostatic forces [2]. The pharmacological action of Ctx is enhanced in vivo by CA that acts as a chaperone, thus preventing the non-specific adsorp-tion of CB to membrane sites other than its target receptor on pre-synaptic membranes [3]. Several isoforms of CA and CB have been isolated, each one displaying slightly different enzymatic and pharmacological activities [4,5]. The ultimate properties of Ctx are determined by the isoform of CB present in the heterodimeric complex [2].

It has long been noted that *C. d. terrificus* snakes, as well as other snake species, are resistant to envenomation by their own venom, due to the presence of toxin inhibitors in their blood. A primary function ascribed to these inhibitors has been the prevention of the deleterious action of toxins upon the snakes themselves, in case of an eventual leaking of the contents of the venom gland or a bite by another snake. In the case of *C. d. terrificus*, it was shown that the lethal activity of the crude venom and of Ctx in mice can be neutralized by a protein inhibitor present in the homologous blood. The whole *C. d.*

*terrificus* plasma inhibits the phospholipase $A_2$ enzymatic activity of Ctx in vitro as efficiently as a commercial antiserum, used in the treatment of victims of *C. durissus* snakebites [6]. The antiserum is produced by hyper immunization of horses with the whole venom and needs further processing aiming at the concentration of the immunoglobulin fraction and, consequently, the enhancement of its neutralizing potency. An acidic glycoprotein, present in the $\alpha_1$-globulin fraction of the snake blood plasma, was purified and characterized, later on, as a Ctx inhibitor [7]. The native protein, named CNF [8] or CICS [9], exists as an oligomer of molecular mass around 140 kDa. The aggregate is formed by 6 to 8 single-polypeptide-chain subunits of one type [8]. The exact number of subunits in the oligomer has not been clearly determined yet. The primary structure of the monomer was deduced from the cDNA nucleotide sequence. It is composed of 181 amino acid residues with a calculated mass of 20.06 kDa and contains a putative N-glycosylation site. When CNF is incubated with Ctx, it displaces CA in the toxin complex and binds to CB. The exchange reaction between CA and CNF leads to the formation of a new stable complex, CNF–CB that no longer delivers CB to its target receptor. Quantitative analysis of the CNF–CB complex demonstrated that it is most likely formed by one CB per subunit of CNF and it is completely devoid of CA [8].

During the last 15 years, a series of natural $PLA_2$ inhibitors (PLIs) have been isolated and characterized from the blood plasma of snakes from different families ([10–12] for reviews). The increasing number of isolated PLIs led to the proposal of three different structural classes of blood inhibitors ($\alpha$, $\beta$ and $\gamma$) [13]. More recently, the sub classification of the $\gamma$-type PLIs in two subclasses, I and II, according to their heteromeric or homomeric character, respectively, was proposed [12]. CNF was placed in the subclass II of the $\gamma$-type inhibitors, which comprises homomeric PLIs with two structural units of highly conserved three-finger motifs. CNF inhibits $PLA_2$s from other snake venoms but no such effect was observed on the mammalian $PLA_2$ tested so far [14,15]. This property would seem to be advantageous if CNF is considered as a model for the development of alternative drugs for the treatment of snake bites.

It has been suggested that the interaction between CNF and CB may be reminiscent of the interaction of Ctx with its target receptor (TR) at the neuromuscular transmission site in the presynaptic cells [8,9]. Based on that, we decided to investigate more closely the effect of CNF on the interaction between Ctx and TR, here represented by rat brain synaptosomes. Particular attention was devoted to this action on the Ctx already bound to TR, a condition that simulates the human envenomation by *C. d. terrificus* snake bite.

## 2. Materials and methods

### 2.1. Crotalus Neutralizing Factor (CNF)

CNF was purified from the plasma of *C. d. terrificus* snakes in a two step procedure. First, a preparative isoelectric focusing using ampholytes within pH range 3–10 was performed. CNF-containing fractions were then loaded on a CB-agarose column, previously equilibrated with 0.1 M Tris–HCl/0.5 M NaCl pH 8.0 buffer. This column was incubated overnight at 4 °C, with periodical and automatic inversion, to assure the best contact between the matrix and the sample solution. Then, the column was successively washed with 0.15 M phosphate-citrate buffer of decreasing pH (7.0, 6.0, 5.0 and 4.0). Fractions (1.5 ml) containing CNF were eluted with the same buffer at pH 3.0 and collected in tubes containing 150 µl of 1.0 M Tris–HCl pH 8.0 buffer. The final CNF preparation was analyzed by SDS-PAGE in a 12.5% minigel (Bio-Rad Laboratories, Inc.) according to Laemmli [16], before and after deglycosylation. Deglycosylation was performed by incubating 2.5 µg of CNF with 1 mU of PNGase F (2.5U/ml, Bio-Rad Laboratories, Inc.) in 50 mM $Na_2HPO_4$ buffer, pH 7.5, at 37 °C for 24 h.

### 2.2. Crotoxin

*C. d. terrificus* snake venom was used for crotoxin purification [17,18]. Further fractionation into CA and CB isoforms was performed by reverse phase chromatography [8].

Briefly, 0.5 mg of Ctx were loaded per run on a Sephasil Peptide C18 5µ 4.6/250 column (Pharmacia Biotech). The mobile phases were 0.1% TFA in $H_2O$ (A) and 0.1% TFA in acetonitrile (B). Fractions of 0.5 ml were eluted with a gradient from 25% to 35% of B in 40 ml. CA was used as a mixture of the isoforms present in the preparation, eluted between 29.3 and 30% of B. Only the isoform $CB_2$ (34.1–34.3% of B) was employed in the experiments.

### 2.3. Iodination procedure

To avoid using of a mixture of CB isoforms or concomitant labeling of CA, isolated $CB_2$ was firstly radio iodinated using the chloramine-T method [19–21]. Then, the Ctx complex was reformed by incubating $^{125}ICB_2$ with the corresponding amount of CA in the native complex, for 15 min at room temperature. $CA-^{125}ICB_2$ was loaded onto a $0.8 \times 18$ cm Sephadex G-25 column, previously calibrated with the unlabeled $CA–CB_2$ complex. Fractions of 0.75 ml were eluted with 0.1% BSA in PBS and the radioactivity present in aliquots of 5 µl each was counted in a Gamma Counter. Specific activities ranged from 3700 to 4200 cpm/fmol of $^{125}ICB_2$.

### 2.4. Synaptosomes

Synaptosomes were prepared as described [22]. Briefly, the cerebral cortex of Wistar female rats was dissected and immersed in 0.32 mM sucrose, 1 mM EDTA, 0.25 mM DTT, pH 7.4 at 10% w/v. The tissue was homogenized and centrifuged at 1,000g for 10 min. Two ml of the supernatant were loaded onto the top of a discontinuous Percoll gradient (23%, 15%, 10% and 3%) prepared in the sucrose solution. After centrifugation at $32,500 \times g$ for 5 min, the synaptosomal fraction was collected at the interface between 15 and 23% of Percoll and diluted 4-fold in buffer A (25 mM HEPES, 10 mM glucose, 140 mM choline, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 0.2% BSA, pH 7.4). Synaptosomes were pelleted by centrifugation at $15,000 \times g$ for 15 min and resuspended in about 8 ml of buffer A. This step was repeated until complete removal of the Percoll in the sample was achieved. The final protein concentration was determined using bovine serum albumin as a standard [23].

### 2.5. General conditions for the binding assays

The binding experiments were performed based on Degn et al. [21]. Triplicates were used throughout and incubations were performed in a rocking water bath at 37 °C for 1 h, unless otherwise stated. After the incubation period, the samples were filtered through a GFB disc, previously soaked in buffer A containing 20 mg/ml of BSA. The radioactivity on the filters was counted for 1 min in a Gamma Counter, following two washings with 5 ml of buffer B (5 mM HEPES, 10 mM glucose, 140 mM choline, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 7.0% BSA, pH 7.4). Control samples were prepared with $CA-^{125}ICB_2$ alone (total binding) and in the presence of a 1000-fold excess of $CA–CB_2$ (nonspecific binding). Values for nonspecific binding to the discs, obtained by filtering equivalent concentrations of $CA-^{125}ICB_2$ alone, were

Table 1
Incubation conditions, potency ($IC_{50}$) and equilibrium dissociation constants ($K_d$) in binding assays of Ctx ($CA-^{125}ICB_2$) to rat brain synaptosomes (S), in the presence of CNF or $CA-CB_2$ as competitors

| Condition | Reactions and incubation times | | $IC_{50}$ nM | $K_d$ nM |
|---|---|---|---|---|
| A | $CA-^{125}ICB_2 + S + CA\text{-}CB_2$ ⇌ (2h) | $S-^{125}ICB_2 + S\text{-}CB_2 + CA$ | 8 | $1.2 \pm 0.3$ |
| B | $CA-^{125}ICB_2 + S + CNF$ ⇌ (2h) | $S-^{125}ICB_2 + CNF\text{-}^{125}ICB_2 + CA$ | 100 | $3.2 \pm 1.2$ |
| C | $CA-^{125}ICB_2 + S$ ⇌ (1h) $S-^{125}ICB_2 + CA$ $\xrightarrow{+CNF}$ (1h) | $CNF\text{-}^{125}ICB_2 + S-^{125}ICB_2 + CA$ | $1200^a$ | n.d. |
| D | $CA-^{125}ICB_2 + CNF$ ⇌ (1h) $CNF\text{-}^{125}ICB_2 + CA$ $\xrightarrow{+S}$ (1h) | $CNF\text{-}^{125}ICB_2 + S-^{125}ICB_2 + CA$ | 255 | n.d. |

n.d.=not determined.
$^a$ This value was obtained by extrapolation of the corresponding curve (C) in Fig. 3.

subtracted from the corresponding test samples. Data analyses were performed using the GraphPad Prism version 4 software.

### 2.6. Competition experiments

Varying concentrations of CA, $CB_2$ or $CA-CB_2$ in the range $10^{-11}$ to $10^{-6}$ M were used in the presence of a fixed concentration of $10^{-10}$ M of $CA-^{125}ICB_2$. For CNF, concentrations between $10^{-12}$ and $10^{-6}$ M were employed, under the following incubation conditions (Table 1): B-[$CA-^{125}ICB_2 + CNF + S$] for 2 h; C-[$CA-^{125}ICB_2 + S$] (1 h) plus CNF (1 h); D-[$CA-^{125}ICB_2 + CNF$] (1 h) plus S (1 h). A control sample (A in Table 1) was prepared by incubating [$CA-^{125}ICB_2 + CA-CB_2 + S$] for 2 h. Thirty μg of synaptosomes were used per test sample.

Data were analyzed using non-linear regression of the sigmoidal dose–response curve, according to the following equation

$$Y = (\text{Bottom}) + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(X - \text{LogIC50}) \cdot \text{HillSlope}}}$$

The potency of the competitors was expressed as the concentration of inhibitor that competes for half the specific binding ($IC_{50}$).

### 2.7. Saturation binding experiments

Specific binding at equilibrium was measured at concentrations of $CA-^{125}ICB_2$ in the range $10^{-11}$ to $10^{-9}$, in the presence of a 1000-fold excess of $CA-CB_2$ or CNF and 1 μg of synaptosomes. The equilibrium dissociation constants ($K_d$) were determined after fitting the data to a rectangular hyperbola using non-linear regression.

## 3. Results

The results for the biological system used in the present work, that is, $CA-^{125}ICB_2$ as the toxin and rat brain synaptosomes as target receptors, were firstly validated by comparison with saturation and competition binding data of unlabeled Ctx and its subunits, already reported for analogous systems. Typical association curves were obtained (data not shown), in accordance to those previously reported for whole labeled $^{125}ICtx$ and guinea pig synaptosomal membrane fragments [21] or presynaptic membranes from *Torpedo marmorata* electric organ [24]. The inhibition of $CA-^{125}ICB_2$ binding to rat brain synaptosomes by unlabeled $CA-CB_2$ and its isolated subunits, CA or $CB_2$, also followed the same pattern already published for the other two neuronal membranes

[21,24]. $CA-CB_2$ and CA were able to completely inhibit the specific binding of $CA-^{125}ICB_2$ to rat brain synaptosomes in a dose-dependent manner, with $IC_{50}$ of 16 nM and 97 nM, respectively (Fig. 1). The competition profile of $CB_2$ was concentration-dependent, as it acted as an inhibitor at intermediate concentrations only ($10^{-8}$ to $10^{-6}$). After that, a tendency to an increase in the $CA-^{125}ICB_2$ specific binding was noticed, following a polynomial pattern (Fig. 1). This paradoxical effect of the subunit CB has been also verified before [21,24,25].

Once the biological system was validated, CNF was included in the experiments. The homogeneity of the CNF preparation to be used was checked by SDS-PAGE (Fig. 2). Two components of molecular masses 23 and 18 kDa, respectively, were detected. Further treatment with PNGase F clearly demonstrated that the 18-kDa component corresponded to the deglycosylated form of the CNF monomer. CNF was,
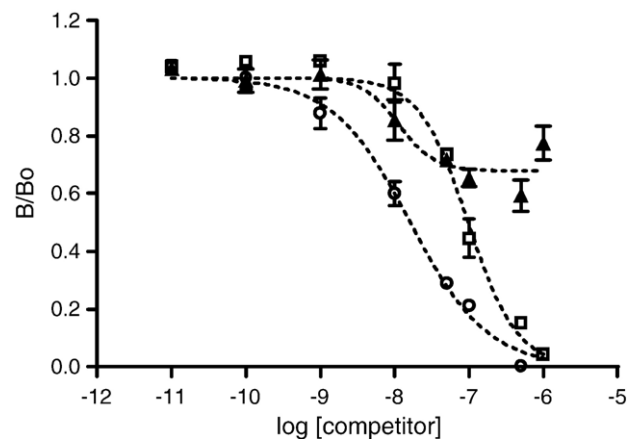


Fig. 1. Inhibition of the specific binding of $CA-^{125}ICB_2$ to synaptosomes by the unlabeled complex and its isolated components. $CA-^{125}ICB_2$ was incubated with synaptosomes in the presence of increasing concentrations of $CA-CB_2$, $CB_2$ or CA. $B_0$ is the concentration of $CA-^{125}ICB_2$ bound in the absence of any competitor, whereas B is the concentration of $CA-^{125}ICB_2$ bound in the presence of a competitor at a given concentration. Symbols: (○) $CA-CB_2$, (□) CA and (▲) $CB_2$. Values are expressed as mean±S.D. of triplicates.
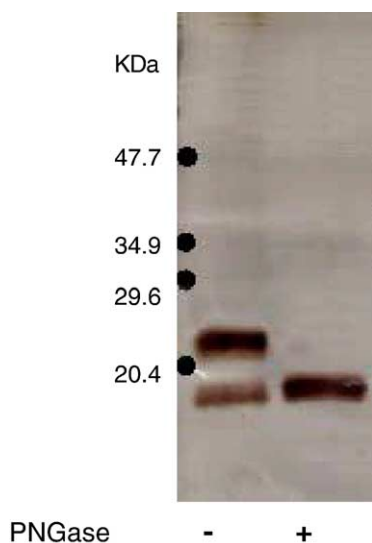
Fig. 2. SDS-PAGE (12.5%) of CNF (2.5 μg per well), before and after deglycosylation with PNGase F. The gel was silver impregnated. The molecular masses of standard proteins (prestained broad range markers, Bio-Rad Laboratories, Inc.) are indicated on the left side.

then, used in the experiments as the natural mixture of glycosylated and deglycosylated monomers.

Competition binding assays with synaptosomes were performed with a fixed concentration of $CA-{}^{125}ICB_2$ and increasing concentrations of CNF over six orders of magnitude. The time scale of addition of reagents was varied as detailed in Table 1 (B to D). Incubation periods of 2 h for one-step and 1 h each for two-step reactions were based on previous kinetic experiments, where equilibrium plateaus were reached after 40 min of reaction, in typical hyperbolic curves for every case. Competition by unlabeled $CA-CB_2$ was run in parallel to be taken as reference (Table 1A). Under all experimental conditions, a dose-dependent inhibition was obtained for CNF (Fig. 3). When an excess of CNF was simultaneously incubated with $CA-{}^{125}ICB_2$ and synaptosomes (Table 1B) the $IC_{50}$ (100 nM) was very close to that determined before for CA (97 nM, Fig. 1). Pre-incubation of $CA-{}^{125}ICB_2$ and CNF followed by the addition of synaptosomes (Table 1D) reduced the initial ratio $B/B_o$ to 60% (inset of Fig. 3). An $IC_{50}$ of 25 nM was found. When the labeled toxin was already bound to the synaptosomes (Table 1C), the displacement by CNF reached a maximum of 45%, even with the highest dose of CNF tested (1 μM) (Fig. 3). This percentage remained unchanged even after prolonged incubation times up to 240 min (data not shown).

Saturation curves for the binding of labeled Ctx to synaptosomes, in the presence of $CA-CB_2$ or CNF as competitors, are shown in Fig. 4. Equilibrium dissociation constants ($K_d$) of $1.2\pm0.3$ nM and $3.2\pm1.2$ nM were determined for $CA-CB_2$ and CNF, respectively.

## 4. Discussion

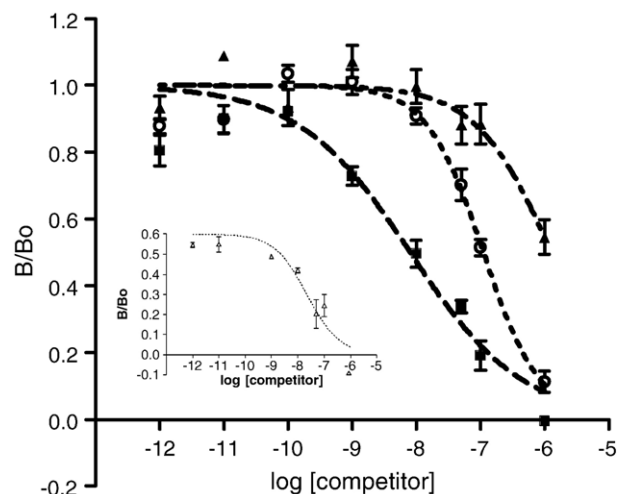The strategy of using a Ctx complex made of one single isoform of CB and total CA ($CA-CB_2$) was employed based



Fig. 3. Inhibition of the specific binding of $CA-{}^{125}ICB_2$ ($10^{-10}$ M) to synaptosomes (S) by CNF. $B_0$ is the concentration of bound $CA-{}^{125}ICB_2$ in the absence of any competitor, whereas B is the concentration of bound $CA-{}^{125}ICB_2$ in the presence of a given concentration of a competitor. Unlabeled $CA-CB_2$ was used as control. Order of addition of reagents and incubation times (in brackets) were as follows: (■) A. [$CA-{}^{125}ICB_{2+}$ S+ $CA-CB_2$] (2 h)-reference curve; (○) B. [$CA-{}^{125}ICB_{2+}$ S+ CNF] (2 h); (▲) C. [$CA-{}^{125}ICB_{2+}$ S] (1 h) plus CNF (1 h). Inset: D. (△) [$CA-{}^{125}ICB_2+$ CNF] (1 h) plus S (1 h). Values are expressed as mean±S.D. of triplicates.

on the fact that CNF binds preferentially to $CB_2$ [8]. Nevertheless, our results should agree with those performed with mixtures of CB isoforms, considering that $CB_2$ is the major isoform in every venom of *C. d. terrificus* analyzed in our laboratory, so far.

Although the activity of the Ctx complex was not directly assayed after radio labeling, it was shown before that Ctx's structure and function were not greatly modified by the iodination method used in the present work [21,24]. Besides, the ready replacement of $CA-{}^{125}ICB_2$ by unlabeled Ctx
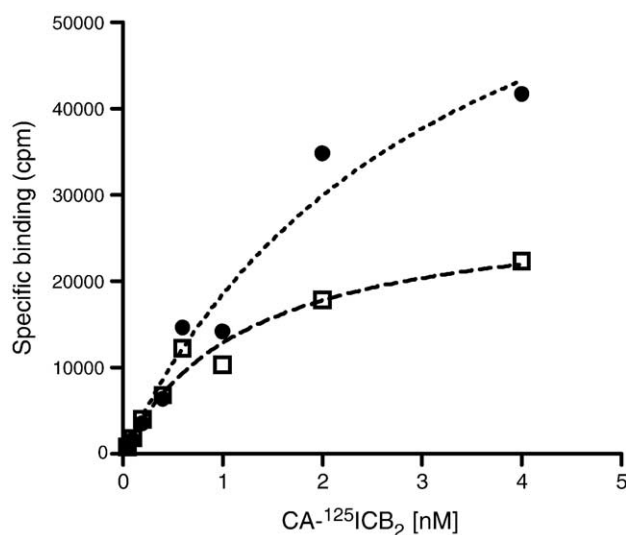


Fig. 4. Saturation binding curves of $CA-{}^{125}ICB_2$ to rat brain synaptosomes, in the presence of $CA-CB_2$ (□) and CNF (●) as competitors. The values are mean of triplicates. Data fitting to a rectangular hyperbole gave equilibrium dissociation constants ($K_d$) of $1.2\pm0.3$ nM and $3.2\pm1.2$ nM for $CA-CB_2$ and CNF, respectively.

offered indirect evidence that the procedure did not compromise the binding properties of the toxin.

The presence of calcium ions in binding studies of toxins with $PLA_2$ activity has been a matter of discussion in the literature. The kinetic parameters for Ctx binding to guinea pig synaptosomes were almost unaffected by the presence of calcium ions [21]. For *Torpedo* membranes, instead, concentrations between 1 and 10 mM $CaCl_2$ have been considered ideal for the specific binding of Ctx [26]. However, an increase in the non-specific binding of Ctx was unexpectedly observed after 10 min. This effect, absent when the calcium ions were omitted, was attributed to the hydrolysis of the membranes due to the addition of a large amount of $PLA_2$ activity [26]. For rat brain synaptosomes (present study), the non-specific binding of $CA-^{125}ICB_2$ was stable up to 90 min in the presence of 1.8 mM $CaCl_2$ (data not shown) and this concentration was maintained throughout.

In an extensive study on the inhibition of Ctx binding to guinea pig synaptosomal membrane fragments [21], a series of competitors have been classified as strong, moderate, weak, very weak and non-inhibitors, based on their strength of inhibition. Native Ctx was among the strongest inhibitors tested, with an $IC_{50}$ around 10 nM [21,24]. A close value was found here for rat brain synaptosomes ($IC_{50} = 16$ nM) demonstrating an agreement in the results with both biological preparations. As for CA, its potency seemed more dependent on the biological membrane. CA was as potent as Ctx for guinea pig synaptosomal membrane fragments [21] but its $IC_{50}$ increased ten times for *Torpedo* membranes [24] and for rat brain synaptosomes (present work). So, based on these $IC_{50}$ values (around 100 nM), CA should not be considered as a strong but a moderate inhibitor for the latter two target membranes.

CNF was the first member of the γ-type PLIs to be purified and characterized as an oligomer of polypeptide subunits of a single type, in its native form [8]. Later on, when a γ-PLI from *Laticauda fasciata* snakes was described as composed of two different types of polypeptide subunits, the homomeric nature of CNF was questioned [27]. At that time, the conclusion was that a SDS-PAGE band of 20 kDa, that appeared in the CNF preparation originally reported [7], was most probably the second subunit of CNF. Presently, the SDS-PAGE profiles obtained before and after deglycosylation of CNF (Fig. 2), leave no doubt about the homomeric nature of CNF. The positioning of CNF in the subclass II of γ-type PLIs can, thus, be no longer contested. Nevertheless, the oligomeric aggregates of CNF are formed by a mixture of glycosylated and deglycosylated monomers, the ratio of which will vary among specimens of *C. d. terrificus* snakes (unpublished results).

A direct competition between the target receptor and CNF for $^{125}ICB_2$ could be followed with the simultaneous incubation of CNF, $CA-^{125}ICB_2$ and synaptosomes (Table 1B, Fig. 3). Comparison of the $IC_{50}$ value for $CA-CB_2$ and CNF indicates that $CA-CB_2$ is about ten times more potent as a competitor than CNF, under our experimental conditions (Table 1A and B). This finding would be expected, though, taking into account the chaperone role of CA in the homologous competition. An additional consideration is that, when CNF is the competitor, part of $^{125}ICB_2$ is sequestered from $CA-^{125}ICB_2$ to give rise to the $CNF-^{125}ICB_2$ complex (Table 1B). The formation of this new complex, with CNF replacing CA in Ctx [8], was more evident when CNF was pre-incubated with $CA-^{125}ICB_2$, before the addition of synaptosomes (Table 1D). Under this assay condition, the initial ratio $B/B_o$ was reduced to 60%, due to the formation of that new stable complex in the first incubation step. The real amount of $CA-^{125}ICB_2$ available to synaptosomes in the second incubation period was, then, greatly reduced (Table 1D, inset of Fig. 3).

Comparable $IC_{50}$ values were found for CNF and CA as competitors (100 and 97 nM, respectively). CA alone, obviously, does not play its chaperone role. CNF can be considered, then, as potent a competitor as CA for the Ctx binding to rat brain synaptosomes. (Figs. 1 and 3).

Although unlabeled Ctx was taken as a reference for the assays with CNF, the molecular models being dealt with are quite different. The reference model comprises two ligands (labeled and unlabeled Ctx) competing for the same receptor site (S) on membranes, that is, two ligands versus one receptor. Two new complexes, $S-^{125}ICB_2$ and $S-CB_2$, will be formed. As of CNF, it will not be competing for the receptor site (S) but for the ligand (CB) instead, through two different pathways. Firstly, the formation of the stable $CNF-^{125}ICB_2$ complex (Table 1B) will sequester part of $^{125}ICB_2$, reducing the amounts of the labeled ligand available to binding to the synaptosomes. Secondly, CNF will act as an alternative receptor for $^{125}ICB_2$ already bound to the synaptosomes. These characteristics make the competition by CNF a unique and complex model.

In the absence of a more adequate mathematical treatment to describe this peculiar competition, the equation of the law of mass action, commonly employed for analysis of radioligand binding experiments, was applied to our data. The equilibrium dissociation constant ($K_d$) of $S-^{125}ICB_2$ was determined as $1.2 \pm 0.3$ nM in the presence of $CA-CB_2$ as competitor (Fig. 4, Table 1A). This value is very close to that
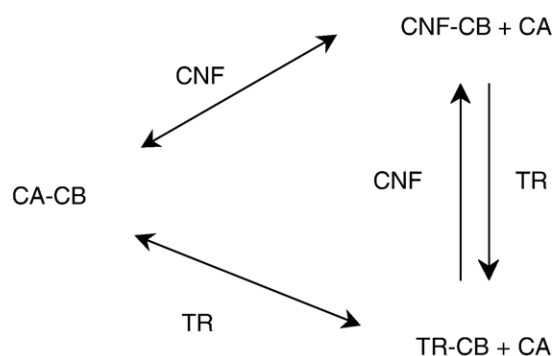


Fig. 5. Schematic representation of the interactions between Ctx, its target receptor (TR) on pre-synaptic membranes and CNF [8], updated with the inclusion of a new equilibrium step between $CNF-CB$ and $TR-CB$.

reported for Ctx and guinea pig synaptosomes ($K_d$=2 nM) [21]. When CA−CB$_2$ was replaced by CNF, a $K_d$ of 3.2±1.2 nM was found. This increased value of $K_d$ does not mean that CNF is a better competitor than Ctx because the mechanisms of competition in each case are different, as already discussed. This $K_d$ reflects an apparent increase in the dissociation of S-$^{125}$ICB$_2$ due to a decrease in the real concentration of $^{125}$ICB$_2$ available for the membrane receptor associated to a competition of CNF for $^{125}$ICB$_2$ bound to the synaptosomes.

The paradoxical concentration-dependent effect of CB for guinea pig synaptosomal fragment [21] and for *Torpedo* membranes [24] was confirmed for rat brain synaptosomes (Fig. 1). The potentiation effect for concentrations of CB$_2$ above $10^{-6}$ M could be possibly explained by its tendency to form aggregates due to its highly basic character [28], its binding to nonspecific sites on the membranes or both [25]. In either case, the final result will be a decrease in the concentration of free CB competing for the membrane receptor and the induction of an increase in the "specific binding" of labeled Ctx, as already suggested [24].

In conclusion, our results demonstrate that CNF is able to totally inhibit the pharmacological action of Ctx and that this action can be partially extended to the toxin already bound to synaptosomes. The schematic model for the interaction of Ctx with its target receptor and CNF proposed before [8] can thus be updated with the inclusion of an additional equilibrium step between CNF−CB and TR−CB, representing the competition between TR and CNF for CB (Fig. 5). CNF, in its natural form or modified, may constitute a useful tool to investigate the mechanism of action and intoxication by Ctx and other PLA$_2$-toxins on targets in the nervous system, as well as in the development of a future therapeutics for snake bites.

## Acknowledgements

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