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Biochemical Pharmacology 63 (2002) 755–761

Biochemical Pharmacology

Inhibition of crotoxin phospholipase A_2 activity by manoalide associated with inactivation of crotoxin toxicity and dissociation of the heterodimeric neurotoxic complex

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Received 25 April 2001; accepted 30 October 2001

Abstract

Crotoxin (CACB complex) is a convulsant heterodimeric neurotoxic phospholipase A_2 (PLA_2). The role of phospholipid hydrolysis in its epileptogenic properties remains unresolved. We, thus, studied the effect of manoalide (MLD), a PLA_2 inhibitor, on the toxin catalytic activity and its central and peripheral toxicity. Incubation of crotoxin with MLD fully and irreversibly inactivated its enzymatic activity. Interestingly, crotoxin also lost its central neurotoxicity after intracerebroventricular injection and peripheral toxicity after intravenous administration. MLD-treated crotoxin prevented the high affinity binding of [^{125}I]-radiolabeled crotoxin on rat cortex synaptic plasma membranes. Further analysis of MLD-treated crotoxin by non-denaturing PAGE and surface plasmon resonance indicated that the crotoxin complex was dissociated after MLD treatment. Although the loss of MLD-treated crotoxin peripheral neurotoxicity could not be attributed to this dissociation, the presence of free CA subunit might explain the observed competition in binding experiments. In conclusion, the dissociation of the crotoxin complex by MLD, as demonstrated in this study, did not permit to specify the role of the enzymatic activity in crotoxin epileptogenic properties. Other approaches would be required to resolve this question.

Keywords: $sPLA_2$ neurotoxin; Crotoxin; Manoalide; Inactivation; Surface plasmon resonance

1. Introduction

The neurotoxicity and convulsant properties of toxic venom low molecular mass (ca. 14 kDa) secretory phospholipases A_2 ($sPLA_2$s) after their intracerebroventricular injection (i.c.v.) have been acknowledged for more than 20 years but usually with poor details concerning namely their possible sites of action or the type of seizures they induce. This is for instance the case for crotoxin (CTX), the heterodimeric β-neurotoxin from the venom of the South American rattlesnake *Crotalus durissus terrificus* [1]. CTX is made of the non-covalent association of a toxic $sPLA_2$ subunit, CB, and of a non-catalytic and non-toxic subunit, CA that enhances the pharmacological action of CB by targeting it onto its receptor [2]. We recently reassessed CTX neurotoxic characteristics and showed that after the i.c.v. injection of a small dose (7 pmol) intoxication leads to violent tonic-clonic seizures, similar to what is observed in brainstem seizures, and then death [3]. Comparing different enzymes, we have also previously shown that these $sPLA_2$ exerted different epileptogenic properties but the key of this diversity has not been understood as yet [3].

It is generally accepted that both phospholipid hydrolysis and binding to membrane acceptors may well participate in the (peripheral) neurotoxicity of this class of toxic enzymes. However, the exact role of the catalytic activity has been a matter of debate for years and no clear picture of its involvement in central toxicity is available as yet [4]. Previously published studies are of little help because of the use of unsuitable substrates in PLA_2 assays as recently

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Abbreviations: MLD, manoalide; CTX, crotoxin; $sPLA_2$, secretory phospholipase A_2; SPM, synaptosomal plasma membrane; SPR, surface plasmon resonance.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00896-6

emphasized [5], or because of the lack of data on the binding properties of the native or chemically-inhibited enzymes that were tested [6,7]. Indeed, chemical inhibition of $sPLA_2$s may induce unwanted structural modifications (e.g. [8–10]) that may hinder binding to membranes although this possibility is usually not acknowledged, probably leading to confusing conclusions. We recently provided some data supporting the implication of phospholipid hydrolysis in the central toxicity of CB, the $sPLA_2$ subunit of CTX. Its irreversible inactivation by alkylation with *p*-bromophenacyl bromide (pBP), a commonly used PLA_2 inhibitor [11], led to a complete suppression of both its central toxicity and epileptogenic properties [3]. Similarly, pBP treatment of the toxic $sPLA_2$ isoform from *Naja m. mossambica* venom abolished its central toxicity [12].

In order to examine the role of the catalytic activity of CTX in its central effects, we were thus interested in inhibiting irreversibly its hydrolytic action while ideally not affecting its binding properties in the central nervous system (CNS). While isolated CB, after dimerization, can be inhibited by pBP, the CTX complex is not sensitive to this inhibitor [11] and one has to look for another compound. MLD, a non-steroidal sesterterpenoid isolated from the marine sponge *Luffariella variabilis*, proved to irreversibly inhibit various venom (e.g. [13,14]) and mammalian $sPLA_2$s [15]. The mechanism of inhibition by MLD has been extensively studied in the case of $sPLA_2$ from bee and *Naja n. naja* venoms (e.g. [14,16,17]). Bianco *et al.* brought arguments in favor of the absence of conformational modifications of the monomeric cobra $sPLA_2$ after MLD inactivation [18]. On the other hand, the effects of MLD have not yet been examined in the case of an heterodimeric $sPLA_2$ such as CTX, made of the non-covalent association of subunits. Similarly, no studies have dealt with the potential changes in $sPLA_2$ binding properties after MLD treatment. Indeed, despite the possible importance of phospholipid hydrolysis and the subsequent release of lipid mediators in the physiological action of $sPLA_2$ neurotoxins, these toxins may also act centrally through selective binding on a neuronal acceptor (receptor) whose nature, diversity and roles remain to be fully determined (as reviewed by [19]). Although some reports suggest a role for central receptors in the neurotoxicity and the lethality of $sPLA_2$ neurotoxins [20–22], their real importance for the epileptogenic properties of these toxins does not appear clearly [3]. In the case of CTX, binding sites have been described on membrane preparations from guinea-pig [23,24] and porcine brains [25] as well as on intracellular proteins [26]. It was thus interesting to confirm the presence of such binding sites for CTX in rat brain, the rodent species in which we previously studied its convulsant properties [3].

In this communication, we show that MLD fully inhibits the catalytic activity of CTX and prevents seizures and death normally occurring after the i.c.v. injection of a toxic dose (7 pmol) in rats. We also show that although MLD dissociates the CTX complex, the loss of the peripheral neurotoxicity of CTX, as assessed in mice, cannot be attributed to this mechanism suggesting that the MLD treatment of CTX impairs its toxicity through $sPLA_2$ inhibition or the chemical modification of important pharmacological sites. Nevertheless, dissociation precludes from gaining insights into the binding properties of MLD-inhibited CTX on the high-affinity binding sites present in rat brain and into the respective roles of binding and phospholipid hydrolysis in the epileptogenic properties of CTX.

2. Materials and methods

2.1. Materials

CTX and isolated subunits were purified from *C. durissus terrificus* venom as previously described [27]. [^{125}I]CTX was prepared using the chloramine T method as already reported [28] and radioactive sodium iodide Na[^{125}I] (carrier-free) was from Amersham Pharmacia Biotech. Two batches were obtained with specific radioactivities of 1090 and 1660 Ci mmol^{-1}. The 9-anthryldiazomethane (ADAM) was from Serva Feinbiochemica. Purified fatty acids and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma. All the other chemicals were of the best analytical grade.

2.2. Animal experiments

The investigations with animals were conducted in compliance with the French legislation on ''protection of animals used for experimental and other scientific purposes'' and in accordance with the Directives of the European community on this subject. Experimental protocols were submitted to and approved by our institutional animal use and care committee. Every attention was taken to avoid unjustified discomfort to the animals. Rats were Wistar strain males (Iffa-Credo, 260–280 g) and mice were Swiss strain males (Elevage Janvier, 30–40 g).

2.3. Chemical inhibition of CTX by MLD

The non-steroidal sesterterpenoid manoalide is a tricyclic molecule which structure has been known for years [17]. MLD can be found under two different forms (open or closed) depending on various factors, in particular the pH [17,29]. MLD (BIOMOL Research Laboratories, Inc., TEBU) was first dissolved in methanol to achieve a 24 mM stock solution that was stored at -20°. MLD was added at a molar excess of 368 for one to CTX in Tris–HCl 0.1 M, pH 8, then incubated at 40°. Methanol did not exceed 6% (v/v) in the final solution. First experiments on the PLA_2 activity of CTX, determined with a synthetic

substrate, indicated that the inactivation was very fast (80 and 98% inactivation after 2 and 10 min of incubation, respectively). Latter MLD treatments of CTX were conducted for 60 min. The reaction medium was then dialyzed, for at least 1 hr, against cold normal saline or distilled water using a Fastdialyser® system equipped with a Cellu.Sep T1 dialysis membrane with a molecular mass cutoff of 3500 Da (Interbiotech, Interchim). As a control, CTX was also submitted to the same protocol in the absence of MLD and the resulting material, hereafter referred as CTX (sham), was tested in comparison with MLD-treated CTX.

2.4. PLA_2 activity assay

PLA_2 activity was first determined with the fluorimetric assay described by Radvanyi *et al.* [30] using 1-palmitoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphomethanol (β-py-C_{10}-HPM, Molecular Probes, Interchim) as a fluoro-substrate. The residual activity of CTX following MLD treatment is expressed as a percentage of the mean activity of native CTX, i.e. 13.0 ± 1.4 μmol min^{-1} mg^{-1} CTX (mean ± SEM, $n = 21$, six separate experiments). PLA_2 activity was also measured with a natural substrate by quantifying after HPLC separation the release of free fatty acids (FFAs) from mouse brain mini-slices [3,31]. Briefly, the native and MLD-treated CTX were added to the brain mini-slices at a concentration of 100 nM and incubated at 37° for 1 hr, before FFAs extraction. The basal content of FFAs (at 0 time) was always deduced.

2.5. Toxicity assay

Injection of CTX and electrocorticographic (ECoG) recordings were performed as previously described [3]. MLD-treated CTX and CTX (sham) samples were tested for central neurotoxicity in rats after the i.c.v. injection of 7 pmol. The lethal potency of MLD-treated CTX and CTX (sham) was also tested in mice following the i.v. injection of toxin diluted in Tris–HCl 0.1 M, pH 8. Further controls were added by testing by the same route aliquots of CTX that underwent each step of the inhibition procedure separately (addition of methanol, heating and dialysis).

2.6. Binding experiments

Synaptosomes were prepared from rat cerebral cortex. A modified form of the P2 preparation of synaptosomes was used as previously described by McMahon *et al.* [32] with modifications. Briefly, the tissue was homogenized in 20 mL g^{-1} of tissue of ice-cold homogenization buffer (320 mM sucrose; 1 mM Na_2EDTA, 2 H_2O and 10 mM Tris–HCl, pH 7.4) with a Teflon–glass homogenizer before undergoing a two-step centrifugation procedure. Synaptosomal plasma membranes (SPM) were prepared from the pellet of synaptosomes osmotically lysed by resuspension four times in 10 vol of Tris–HCl 10 mM, pH 7.4 buffer and centrifugation at 52,000 *g maximum* for 10 min at 4°. The final washed pellet was resuspended in 2 mL of buffer. The protein content was determined by a modification of the Folin-Lowry protein assay, as previously described [3], using BSA as a standard. The suspension was then snap-frozen in liquid nitrogen and kept at −80° until use. The day of the experiment, the thawed suspension was diluted in the binding incubation buffer to reach the final protein concentration of 2 mg mL^{-1}.

Binding experiments were performed at 20° on SPM (370 μg of SPM proteins per mL) as previously described [28]. Briefly, saturation curves for [^{125}I]CTX binding to SPM were determined by the incubation of membranes with [^{125}I]CTX (0–500 nM) in binding buffer for 1 hr. Non-saturable binding to membranes was determined in parallel incubations containing a 200-fold excess of unlabeled toxin. In competition studies, SPM were incubated with 1 nM [^{125}I]CTX, in the presence or absence of unlabeled competitor for 1 hr. The data were analyzed using the non-linear curve-fitting program GRAFIT (Erithacus Software Ltd.).

2.7. Gel electrophoresis of CTX and MLD-treated CTX

Native CTX, MLD-treated CTX and CTX (sham), dialyzed against cold distilled water, were analyzed by PAGE at pH 6.5 in 20% precast polyacrylamide Phast® gels in the absence of detergents and reducing agents, using a Phastsystem® apparatus (Amersham Pharmacia Biotech.). Protein bands were stained with a Silver staining kit (Amersham Pharmacia Biotech.).

2.8. Surface plasmon resonance (SPR) studies of the interaction of CTX and MLD-treated CTX with the monoclonal antibody A73.13

SPR studies were performed at 25°, using a Biacore® 2000 system (Biacore AB). The running and dilution buffer in all experiments was 10 mM Hepes, 150 mM NaCl, 0.005% surfactant P20, pH 7.4. MAb A73.13 [33] was covalently coupled via primary amino groups on CM5 sensor chip surface according to the manufacturer's instructions. The SPR signals for immobilized MAb A73.13 on two different flow cells were found to be 3,500 and 12,900 resonance units (RU), where 1 RU corresponds to an immobilized protein concentration of 1 pg mm^{-2}. Different concentrations of native, MLD-treated CTX or isolated CA subunit (0–20 μg mL^{-1}) were injected with a flow rate of 10 μL min^{-1} on the assay as well as control (blank immobilization) flow cells and their binding was monitored. Between each injection, surfaces were regenerated with 5 μL of Gly/HCl 0.05 M pH 2.0. After subtraction of the signals recorded in the control flow cell, data were analyzed using the BIAevaluation 3.1 software (Biacore).

3. Results

3.1. Inactivation of CTX by MLD

The treatment of CTX with MLD resulted in the complete inhibition of its catalytic activity as assessed using the synthetic substrate β-py-C_{10}-HPM (residual activity $0.3 \pm 0.1\%$; mean ± SEM, $n = 18$, four separate experiments) as well as using mouse brain mini-slices. Indeed, no differences in FFAs could be found between spontaneous hydrolysis (CTX replaced by saline) and the one catalyzed by MLD-treated CTX. As of CTX (sham), there was no significant change in the hydrolytic potency of the toxin (Fig. 1).

3.2. Inhibition of the toxicity of MLD-treated CTX

Results of the toxicity assessment are presented in Table 1. Following the i.c.v. injection of 7 pmol, a lethal and epileptogenic dose, MLD-treated CTX did not exert any apparent neurotoxic effects as shown by the absence of changes in behavior or ECoG of the rats. They survived 48 hr before being euthanasied. Conversely, CTX (sham) exerted the neurotoxic properties of native CTX, consisting in delayed, short-lasting tonic-clonic episodes of convulsions and death in a few hours.

Considering the peripheral toxicity of the toxin, the i.v. injection of about two LD_{50} of native CTX in mice (400 pmol) induced their quick death within a few hours. Conversely, the i.v. administration of a larger dose of MLD-treated CTX (2000 pmol or ca. 10 LD_{50}) to other mice did not induce toxic symptoms to any of them. This loss of toxicity is linked with the chemical modifications induced by MLD on CTX since the same procedure in the absence of MLD did not induce any significant loss of lethality after injection of 400 pmol of toxin. On the other hand, the i.v. injection of isolated native CB at a dose that would be reached after the injection of the highest dose of CTX used, if a complete dissociation occurred, i.e. 2000 pmol, provoked the death of the mice intoxicated within 17 hr with obvious clinical symptoms appearing within the first hour.

Table 1
Effect of MLD treatment on the central and peripheral toxicity of CTX and comparison with the isolated subunit CB[a]

Nature of the toxin	Route/dose (pmol)	Group size	Clinical signs	Death
MLD-treated CTX	i.c.v./7	3	No	No
Native CTX	i.c.v./7	2	Yes	Yes
MLD-treated CTX	i.v./2000	3	No	No
CTX (sham)	i.v./400	9	Yes	Yes
Native CTX	i.v./400	4	Yes	Yes
Native CB	i.v./2000	6	Yes	Yes

[a] Clinical signs after i.c.v. injection refer to the symptoms previously described [3] and to convulsive seizures. After intravenous (i.v.) injection, major symptoms are rapid prostration and labored respiration. The time frame chosen for the results in the "death" column was 24 hr. In each experimental groups, a given response was always observed in 100% of the group and therefore, does not require statistical comparison.

3.3. Inhibition of [^{125}I]CTX specific binding by CTX and MLD-inhibited CTX

[^{125}I]CTX bound to rat cortex SPM in a saturable manner. The maximal number of binding sites was estimated to be ca. 2 pmol mg^{-1} of proteins (data not shown).

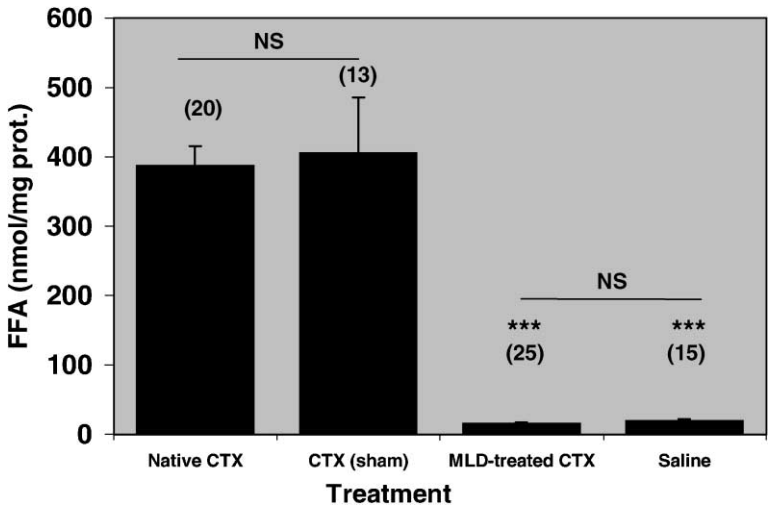

Fig. 1. Inhibition by MLD of the CTX-induced release of free fatty acids from mouse brain mini-slices. PLA_2 activity was measured by quantifying after HPLC separation the release of FFAs from mouse brain mini-slices induced by the action of 100 nM of the indicated treatment for 1 hr at 37°. The basal content of FFAs (at time 0) was always deduced. The results are presented as the mean ± SEM of n values (number between parenthesis) obtained in at least five separate experiments except for the spontaneous hydrolysis (saline, 3). The four groups are compared using Kruskal–Wallis non-parametric test ($P < 0.0001$) followed by pairwise comparisons using Mann–Whitney test and the Bonferroni procedure (number of pairwise comparisons $k = 6$). Level of significance was set at 5%. $^{***}P < 0.00017$ ($\alpha = 0.001$) (difference to native CTX). NS means not significantly different.

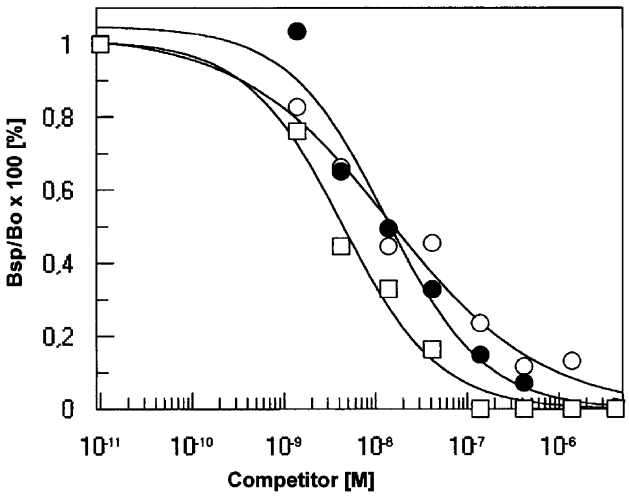

Fig. 2. Inhibition of [^{125}I]CTX specific binding on rat brain synaptic membranes by native, MLD-treated CTX or isolated CA subunit. SPM were prepared from synaptosomes obtained from rat cerebral cortex as described in Section 2. [^{125}I]CTX (1 nM) was incubated with SPM (370 μg mL^{-1} of protein) in the presence of increasing concentrations of CTX (○), MLD-treated CTX (●) or CA (□). Non-specific binding (Bns) was obtained in experiments where unlabeled CTX was present in 1000-fold molar excess over [^{125}I]CTX. Specific binding (Bsp), defined as Bt-Bns, is shown relative to the maximal specific binding determined in the absence of any competing ligand (B_0). Values are means of at least two experiments.

The high affinity and specific binding of [^{125}I]CTX to rat cortex SPM was analyzed in the presence of CTX, MLD-treated CTX or the isolated CA subunit. Competition experiments confirmed the specific nature of [^{125}I]CTX binding to rat SPM as shown by its complete inhibition by CTX at a half effective concentration (IC_{50}) of 17 nM. Interestingly a complete inhibition was also obtained with (1) MLD-treated CTX that further proved to be as effective as CTX with the same IC_{50} and with (2) isolated CA crotoxin subunit (Fig. 2).

3.4. Dissociation of CTX following MLD treatment

PAGE of MLD-treated CTX in non-denaturing conditions indicated dissociation of the MLD-treated CTX complex (Fig. 3, third lane from the left). This was not observed with native CTX and CTX (sham) (Fig. 3).

The monoclonal antibody (MAb) A73.13 directed against the non-catalytic subunit CA of CTX recognizes an epitopic region of CA (A-I) and is able to bind to the CTX complex but not to CB [33]. Biacore technique was used to determine the kinetic parameters of MAb A73.13 binding to MLD-treated CTX and compare them with those obtained with CTX or the isolated CA subunit. For these experiments, MAb A73.13 was immobilized onto the sensor chip surface and various concentrations of native CTX, MLD-treated CTX or CA were injected. Interestingly, the binding kinetic parameters (k_{on}, k_{off} and K_d^{app}) obtained for MLD-treated CTX are closer to the values determined for isolated CA subunit than to those

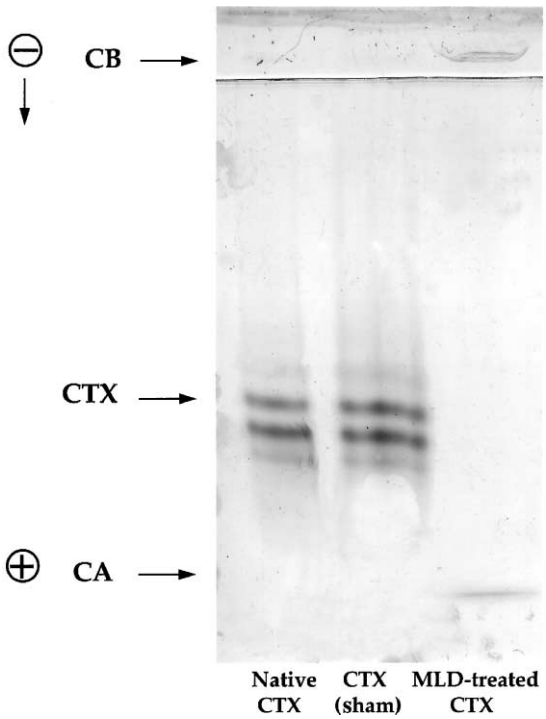

Fig. 3. PAGE analysis of CTX and MLD-treated CTX. Native CTX, MLD-treated CTX and CTX (sham), following dialysis against cold-distilled water, were analyzed by PAGE at pH 6.5 in 20% precast polyacrylamide Phast® gels in the absence of detergents and reducing agents using a Phastsystem® apparatus. Aliquots of 0.4 μg of the proteins were applied on the gel and stained with silver nitrate. Arrows indicate the relative migration of CTX and of its isolated subunits CA and CB. The very basic CB remains in the stacking gel under our PAGE conditions whereas the very acidic subunit CA migrates very quickly. Silver staining of these isolated bands is faint under our conditions. The three bands visible in lanes 1 and 2 are the three isoforms present in the CTX mixture.

Table 2
Kinetic parameters of the interaction of CTX, MLD-treated CTX and of the isolated subunit CA with immobilized monoclonal anti-CA antibody A73.13[a]

	k_{off} (10^{-3} s^{-1})	k_{on} (10^4 M^{-1} s^{-1})	K_d^{app} (nM)
CTX	0.23 ± 0.09	2.9 ± 0.2	8 ± 1
MLD-treated CTX	3.20 ± 0.20	10.0 ± 2.0	32 ± 8
CA	4.10 ± 0.04	7.0 ± 1.0	58 ± 10

[a] Surface plasmon resonance studies were performed at 25° using a Biacore® 2000 system. The apparent dissociation constant K_d^{app} is the ratio of the dissociation rate constant k_{off} to the association rate constant k_{on}. Kinetic parameters are presented as mean ± SE of three independent experiments.

found for CTX (Table 2), confirming dissociation of CTX after MLD treatment.

4. Discussion

This investigation shows that MLD irreversibly and completely inactivates the PLA_2 activity of CTX. This was demonstrated using β-py-C_{10}-HPM, an anionic substrate for which CTX has a preference compared to

zwitterionic substrates like β-py-C_{10}-HPC [30], and confirmed with phospholipids in their natural membrane environment, the brain mini-slices. The nature of the biochemical reaction between CTX and MLD remains to be established and may at least be as complex as what has been described with monochain $sPLA_2$s and involve the chemical modification of some residues, e.g. lysine residues [14,16,17,34]. At variance with pBP that has been shown to react specifically with the histidine 48 residue of the phospholipase subunit CB, when it is isolated but not when it is associated with CA in the CTX complex [11], MLD seems to be able to react with the CTX complex. Further to the irreversible inactivation of CTX enzymatic activity, MLD treatment induces the loss of CTX central toxicity and epileptogenic properties as suggested by the lack of clinical and ECoG signs after the i.c.v. injection of a dose known to induce severe seizures and death with native CTX [3]. The concomitant loss of both properties as well as the fact that CTX (sham) retained both its toxicity and its enzymatic activity suggest that the two properties are associated in accordance with data previously obtained with pBP-alkylated CB [3]. However, the loss of toxicity could be explained by other events including modifications of lysine residues, that have been reported to be important for the central neurotoxicity of some $sPLA_2$ neurotoxins [9], or structural modifications, as suggested in the case of alkylation by pBP of the mammalian pancreatic enzyme [8] and of the toxic $sPLA_2$ notexin [10]. Owing to the non-covalent association of CA and CB, dissociation is facilitated and MLD, like some natural inhibitors of CTX [35], may have thus destabilized the CTX complex in addition to having inhibited the catalytically active subunit CB. CB is at least 20 times less toxic than CTX by the i.c.v. route [3] and dissociation may account for the loss of central neurotoxicity observed in our paradigm. Indeed, PAGE clearly shows that MLD treatment dissociates CTX and SPR confirmed this result. However, injection by i.v. route of high doses of MLD-treated CTX, comparable to CB toxic and lethal doses, demonstrated the MLD-induced loss of peripheral toxicity, in accordance with previous data [13]. Again, it cannot however be inferred from this result that enzymatic activity of CB is essential to its peripheral neurotoxicity as other biochemical modifications may have been induced, such as modifications of binding properties.

In this report, we have brought evidence that high-affinity binding sites exist in rat cortex SPM. The IC_{50} value for homologous competition (17 nM) and the estimated number of binding sites were both comparable to results previously reported for guinea-pig brain [23,24]. Because of the MLD-induced dissociation of CTX, it has not been possible to gain insight into the modification of binding properties of CTX. During dissociation, free CA subunit is generated and this may explain the competitive inhibition of [^{125}I]CTX binding on rat brain SPM that we observed with MLD-treated CTX. Indeed, our results show that isolated CA subunit is able to competitively displace crotoxin from rat brain SPM with similar parameters as MLD-treated CTX confirming results previously reported in the case of synaptosomal membranes from *Torpedo* electric organ [28] or guinea-pig brain preparations [24].

In conclusion, previous experiments suggested that both a significant hydrolytic activity on brain membranes under *in vivo* conditions [3,12] and affinity for certain subtypes of brain acceptors [22] were probably important for neurotoxicity and potentially the type of seizures without clearly determining the superiority of one or the other. MLD proved here to be a very potent inhibitor of the catalytic activity of CTX. It also suppressed, or highly reduced, the central and peripheral neurotoxicity of CTX and induced its dissociation.

Inactivation of crotoxin PLA_2 activity without dissociating the complex and/or inducing structural alterations would be required to definitively specify without ambiguity the role of enzymatic activity in its epileptogenic action. Inhibitors of common use such as MLD or pBP do not fulfill these requirements and therefore, other approaches may be required.

Acknowledgments

This work was partly supported by grants from the French Ministry of defense (DSP/DGA Grant no. 14/96).

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