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Research report

Phospholipases A2 isolated from *Micrurus lemniscatus* coral snake venom: Behavioral, electroencephalographic, and neuropathological aspects

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Abstract

The present study evaluated four phospholipase A2 (PLA2) (Mlx-8, Mlx-9, Mlx-11 and Mlx-12) isolated from *Micrurus lemniscatus* snake venom (Elapidae). The effects of intrahippocampal administration of these toxins have been determined on behavior, electroencephalography, and neuronal degeneration in rats. These four PLA2 toxins induced motor and EEG alterations in a dose-dependent manner. Behavioral convulsions were characterized by clonic movements and were often accompanied by EEG alterations. Mlx toxins were convulsive but weakly epileptogenic, since low rates of seizure discharges were observed in EEG records. Neuronal injury seemed to depend on the dose of the toxin used. The highest doses of toxins caused severe intoxication and death of some animals. The injury of hippocampal cells was characterized by massive neuronal loss and hippocampal gliosis. A high occurrence of compulsive scratching was observed. Moreover, the onset of seizures induced by Mlx toxins was markedly delayed. The similarities between the effects of Mlx PLA2s and those isolated from other Elapidae snakes venoms suggest that their toxicity are probably due to their specific binding to neuronal membranes and to the catalysis of phospholipid hydrolysis, producing lysophospholipids and fatty acids. These compounds likely disturb the membrane conformation, causing a marked increase in the release of neurotransmitters and concurrent inhibition of vesicle fission and recycling. These toxins can be a useful tool to investigate properties of endogenous secretory PLA2s and therefore may be important both to study mechanisms involved in neurotransmitter release at nerve terminals and to explain the convulsive properties of PLA2s toxins.

Keywords: Elapidae venom PLA2; *Micrurus lemniscatus* toxins; Hippocampal injury; Seizure; EEG; Neurotoxicity

1. Introduction

Brazilian snakes of the genus *Micrurus* (coral snakes, family Elapidae) provoke severe envenomation in humans, characterized by neurotoxicity of rapid evolution, which may be fatal. Some toxic components have been isolated from coral snake venoms such as phospholipases A2 (PLA2) [2,35,40,43,48] and pre- and postsynaptic neurotoxins [1,2,13,16,20,52–56]. Concerning the mechanisms of toxicity of PLA2 isolated from *Micrurus* venoms at the peripheral nervous system, Dal Belo et al. [13] showed that a presynaptic PLA2 isolated from *Micrurus dumerilli* venom evoked neuromuscular blockade in vertebrate nerve-muscle preparations and triphasic changes in spontaneous neurotransmitter release from motor neuron terminals. The facilitatory phase of the response was accompanied by an increase in the quantal content, whereas in the depression phase it was reduced. The blockade is most likely mediated by activation of Na^+ channels, complemented by blockade of K^+ channels, mainly presynaptically.

Therefore, few investigations have dealt with isolated toxins from *Micrurus* venoms and their effects on central nervous system (CNS). Venoms of Old World elapids have been extensively studied as sources of presynaptic PLA2s and postsynaptic neurotoxins at peripheral and central nervous system [7,14,17–19,30,32]. Several low-molecular-weight secretory PLA2s have been characterized from venoms of snakes, insects, and mollusks (vPLA2s) and were classified into groups I, II, III and IX [51]. Some of these PLA2s are potent neurotoxins

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acting at presynaptic level, and they present high affinity for neuronal N-type receptors, as described by Lambeau et al. [30]. The N-type receptors are present in high levels in rat brain membranes and contain at least two binding sites of high affinity for PLA2-OS2 toxin purified from the venom of Taipan snake *Oxyuranus scutellatus scutellatus*. They bind a variety of neurotoxic vPLA2s with high affinity, but have low or very low affinities for non-toxic vPLA2s such as OS1 toxin, suggesting their involvement in the neurotoxic effects of various vPLA2s [30,37]. The role of catalytic activity of PLA2s to induce toxic effects is unclear, because not all vPLA2s display toxic effects, although both toxic and non-toxic vPLA2s have similar catalytic activities [24].

The neurotoxicity and/or convulsant properties observed after intracerebral injection of major neurotoxic PLA2s such as crotoxin [8,14,15,36], paradoxin [14], bee venom PLA2 (BVP), OS2 toxin [17] and *Naja mocambique* PLA2 [32] have been demonstrated for several years. Dorandeu et al. [14] characterized central neurotoxicity of paradoxin and crotoxin in rodents, with a special emphasis on their potential epileptogenic properties and neuropathologic alterations. Gandolfo et al. [17] described the mainly behavioral effects including compulsive scratching, convulsions and epileptic EEG alterations. The involvement of secretory PLA2-group I/B in seizures was investigated by [28]. They found that kainic acid as well as electroshock induce sPLA2-IB and COX-2 gene expression various time points after injection of the convulsant drugs. sPLA2-IB could be important in COX-2-induced cascade reactions leading to neuronal cell death.

Concerning the effects of secretory PLA2 in the CNS Kolko et al. [27] showed that sPLA2-OS2 toxin induces a neuronal death in primary cortical neurons that is partially mediated through neuronal signaling cascade initiated with binding of sPLA2 to a membrane receptor with activation of cytosolic PLA2, arachidonic acid release, production of PAF, and induction of COX-2. In regard to neuronal injury, β-bungarotoxin (β-BuTX), the most investigated presynaptic PLA2 neurotoxin isolated from an elapid venom, induces widespread neuronal cell death throughout the mammalian CNS [19,47]. Moreover, intracerebroventricular injection of *Naja mocambique*-PLA2 provoked extensive cortical and subcortical injury to forebrain neurons and fiber pathway lesions [11].

The roles of endogenous sPLA2 in the nervous system are poorly understood. It is know that some brain pathologies such as ischemia, brain trauma and epilepsy lead to enhanced secretory PLA2 activity [4]. These PLA2s are known to be stored in synaptic vesicles and released upon neurotransmitter stimulation or depolarization [23,33] and potentiates glutamatergic excitotoxicity [25,26]. Moreover, glutamate ionotropic receptors antagonists inhibit sPLA2-induced neuronal cell death [26].

As mentioned above, neurotoxic PLA2s from Old World elapids have been investigated, but the central neurotoxicity induced by venoms of *Micrurus* snakes from Americas has not been studied. Thus, the aim of this work was to investigate in detail the central effects of four neurotoxic PLA2s isolated from the venom of Brazilian coral snake *Micrurus lemniscatus*. We determined the acute effects of intrahippocampal administration of these toxins on the electroencephalogram (EEG) and behavioral modifications. We also analyzed their ability to induce neuronal injury in the hippocampus.

2. Materials and methods

2.1. Venom

Freeze-dried fresh *M. lemniscatus* venom was obtained from Laboratory of Venomous Animals, Federal University of Bahia, Brazil.

2.2. RP-HPLC purification of M. lemniscatus venom

M. lemniscatus venom (10 mg) was diluted in 5 ml of Milli-Q water. After filtration in a 0.45-μm filter (Millipore), 400-μl samples (800 μg) were applied to a C_8 reversed-phase column (Shim-Pack; 4.6 mm × 250 mm, 5 μm particle) coupled to a HP 1100 series HPLC system. The elution was carried out under a flow rate of 1 ml/min, and monitored at 214 nm. A linear gradient of trifluoroacetic acid (TFA) (solvent A) (0.1% TFA in water) and acetonitrile (solvent B) (90% acetonitrile + 10% solvent A), from 10% to 35% of solvent B in 50 min, was used to elute proteins. Peaks were manually collected according to the optical density (Fig. 1).

2.3. Mass spectrometry

Previously lyophilized samples were dissolved in 50% ACN, containing 0.1% formic acid and deposited into the MSQ Surveyor (Thermo Finningan, MA, USA) autosampler microplate. Typically, 20 μl aliquots of the samples are injected under a 50 μl/min constant flow of 50% ACN, containing 0.1% formic acid, under positive ionization mode and were scanned from 80 to 1800 *m/z* in 1 s. Instrument control, data acquisition and processing were performed by XCalibur software package (Thermo Finningan, MA, USA).

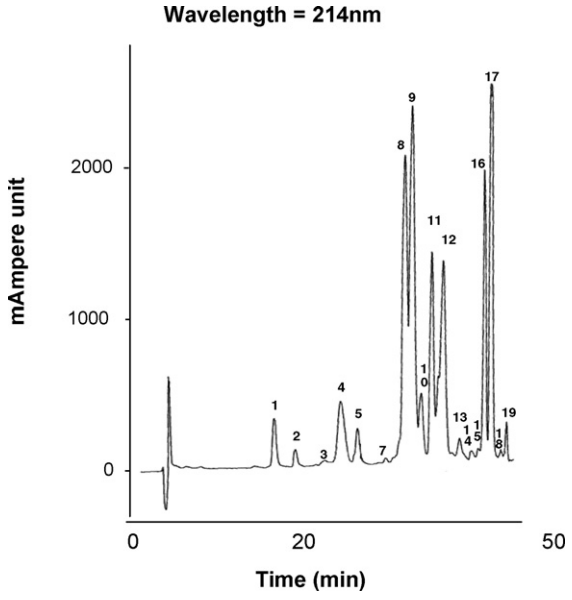

Fig. 1. Analytical RP-HPLC profile of *Micrurus lemniscatus* venom on a C_8 column eluted with trifluoroacetic acid (0.1%) (solvent A) and acetonitrile (90% + 10% A) (solvent B) and a solvent gradient developed from 10% to 35% of acetonitrile within 50 min, monitored at 214 nm and 0.05 aufs (absorbance unit full scale). Peak 8, 9, 11 and 12 (Ml-08, Ml-09, Ml-11 and Ml-12) were studied in the present work. They showed PLA2 activity and neurotoxic effects.

2.4. Phospholipase A2 activity

PLA2 activity was determined colorimetrically [31], as modified by Santoro et al. [46] using SoftMax Pro 4.8 software for Spectra Max 190 spectrophotometer (Molecular Devices). Samples of 1.5 μl of *M. lemniscatus* crude venom and its fractions (Ml-8, Ml-9, Ml-11 and Ml-12 toxins, 0.25 μg/μl) were added to 150 μl of substrate reagent (100 mM NaCl, 10 mM $CaCl_2$, 7 mM Triton X-100, 0.265% soybean lecithin, and 99 μM phenol red, pH 7.6) in a microplate maintained at 37 °C. The decrease in absorbance ($\Delta A_{558\,nm}$) was recorded for 2 min to calculate the maximum velocity, V_{max}, of reaction (U/min). The definition of 1 unit of PLA2 activity was taken as the amount of enzyme in assay producing a decrease of 1.0 absorbance units per min. Data were expressed as specific PLA2 activity (V_{max}/mg).

2.5. Animals

Male Swiss mice weighing 23–25 g were maintained for 7 days before the toxicity assay in the laboratory. They were grouped 16 per plastic cage (40 cm × 34 cm × 17 cm) in a room with constant temperature (22 ± 1 °C) on a 12 h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided *ad libitum*. Male Wistar rats weighing 250–260 g were maintained for 7 days before the stereotaxic surgery in the laboratory. They were grouped 5 per plastic cage (40 cm × 34 cm × 17 cm) in a room with constant temperature (22 ± 1 °C) on a 12 h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided *ad libitum*. Animals were maintained in accordance with the guidelines of the Department of Pathology of the School of Veterinary Medicine, São Paulo University, based on the guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

2.5.1. Toxicity assay

Purified toxins Mlx-08, Mlx-09, Mlx-11 and Mlx-12 were injected intravenously (tail vein) in mice. The toxicity of the toxins was determined by testing four doses (1, 10, 20 or 40 μg/mouse) in four mice per dose. The animals were observed until 48 h after the toxin injection.

2.5.2. Surgery

Rats were anesthetized with a mixture of pentobarbital (1 g) and chloral hydrate (4 g) in 100 ml of 0.9% NaCl (3 ml/kg, i.p.) and inserted in a stereotaxic frame. A stainless steel guide cannula was implanted into one side of the dorsal hippocampus (AP −4.3, L 3.5, and V 3.0 mm, according to Paxinos and Watson [38] and fixed with dental acrylate. In the contralateral hippocampus (AP −4.3, L 3.5, and V 3.5 mm), bipolar twisted electrodes were implanted to record hippocampus EEG. Electrodes were anchored to the skull with dental acrylate. For recording of surface EEG, jeweler screws were inserted into the skull over the left and right occipital cortex, which functioned as electrodes. An additional screw placed in the frontal sinus served as a reference (indifferent) electrode. After surgery, the animals were housed individually and allowed to recover for 3–4 days.

2.5.3. Experimental design

On the experimental day, the rats were transferred to an acrylic cage (30 cm × 30 cm × 39 cm). After 15 min of acclimatization, the EEG was recorded for 15 min. Then a volume of 1 μl (speed of injection: 1 μl/5 min) was injected into the hippocampus with either the vehicle (0.1 M phosphate buffer; $n = 5$) or one of the toxins (Ml-8, Ml-9, Ml-11 or Ml-12 toxin). The following doses of toxins were injected: toxin Ml-8, 1.0 ($n = 5$) 1.4 ($n = 8$) or 2.1 μg/μl ($n = 6$); toxin Ml-9, 0.57 ($n = 5$); 1.15 ($n = 4$) or 4.5 ($n = 6$) μg/μl; toxin Ml-11, 1.0 ($n = 5$) or 2.0 μg ($n = 5$); toxin Ml-12, 1.43 ($n = 5$) or 2.86 μg/μl ($n = 5$). Brain injections were made with a metal injector that protruded 0.5 mm beyond the tip of the guide cannula.

EEG and behavior were recorded continuously for periods ranging from 7 to 9 h after injection. Additional EEG recordings were taken at 24 and 48 h after injection. We also recorded epilepsy-related behavioral signs. The convulsive episodes were classified on an 8-point scale, as suggested by Pinel and Rovner [39]. It was not possible to perform a quantitative analysis of the EEG record for these neurotoxins because the EEG alterations occurred at an irregular way and presented a low frequency. They are not intense and regular as described by classical convulsive drugs. The LD_{50} values for these toxins were not determined, but the percentages of death observed after intrahippocampal injection of different doses of the toxins were calculated.

2.5.4. Histological analysis

Seven days after toxin injection, the animals were deeply anesthetized with CO_2 and fixed by transaortic perfusion with phosphate buffered saline (PBS) followed by 10% formalin solution. The brains were removed, stored in formalin, dehydrated and embedded in paraffin. Coronal sections of 10 μm were cut from a 700 μm brain block including the cannula track. The septum-temporal levels corresponding to bregma −3.90 to 4.60 mm were chosen to determine cell counts. Every seventh slice from either side of the track was mounted on a glass slide and stained with cresyl violet. We magnified the slices 400 times and counted all cells with a clearly visible nucleus using a 100-μm^2 grid in the hippocampus CA1, CA3, and CA4 pyramidal cell layers, and in the granulate cell layer of the dentate gyros. The cells were counted by a blind observer using the method of Bagetta et al. [3].

2.6. Statistical analysis

Cell counts in experimental and control groups were compared by ANOVA, followed by Dunnett test. Data were reported as mean ± standard deviation (S.D.). Values were considered significant when $P \leq 0.05$.

3. Results

3.1. Venom purification and biochemical characterization of the protein toxins

Fig. 1 presents a representative RP-HPLC profile of the fractionation of *M. lemniscatus* venom. The numbered peaks were collected and assayed for PLA2 and neurotoxic activities. The active fractions, namely, 8, 9, 11 and 12 have had their molecular mass profile assessed by ESI-Q-MS and the calculated molecular masses are presented in Table 1. Fig. 2 contains a representative mass spectrum of fraction 9, presenting the ionic envelope and the deconvoluted molecular mass distribution in the inset showing a single component present in the chromatographic peak, no other significant mass envelopes were found. The same pattern was obtained with the other collected peaks.

3.2. Phospholipase A2 activity

Mlx-8, Mlx-9, Mlx-11 and Mlx-12 toxins isolated from *M. lemniscatus* venom presented PLA2 activity, as demonstrated by their high specific phospholipase A2 activity (Table 2).

Table 1
Calculated molecular mass for the most active isolated fractions of the *Micrurus lemniscatus* venom

Peak #	Retention time (min)	Molecular masses (relative intensity)
Mlx-08	35.07	7489.3 (15%); 13531.3 (85%)
Mlx-09	36.04	13568.0 (95%); 13607.3 (5%)
Mlx-11	39.04	7127.7 (40%); 7190.4 (45%); 7213.3 (5%)
Mlx-12	40.40	22307.4 (30%); 22379.9 (70%)

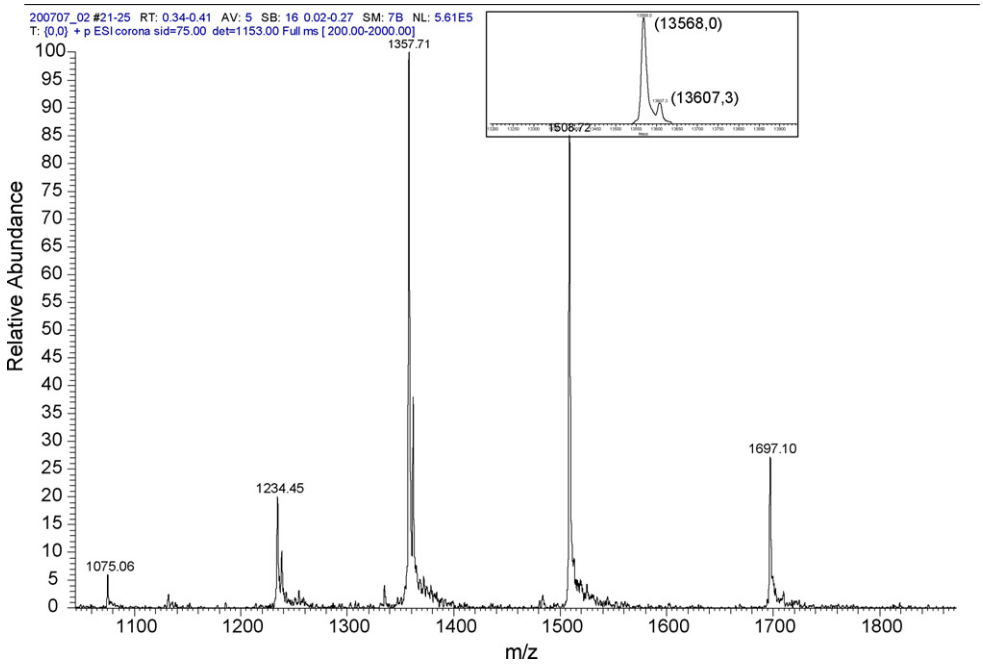

Fig. 2. Representative mass spectrometric analysis of peak 9, presenting the protein ionic envelope. Inset: deconvoluted mass spectrum of sample 9. The calculated molecular masses are typed besides the peaks.

3.3. Toxicity

The i.v. injection of Mlx-8, Mlx-9, Mlx-11 and Mlx-12 toxins in concentration of 1, 10, 20 or 40 μg/mouse by tail vein showed no toxicity. The animals were observed until 48 h after the toxin injection and none of them presented behavioral alterations (Table 2).

The intrahippocampal injection of these toxins in rats induced severe symptoms of intoxication as showed in Table 3. The percentage of death was dose-dependent.

3.4. Intrahippocampal injection of Mlx-8, Mlx-9, Mlx-11 and Mlx-12 toxins isolated from M. lemniscatus venom

3.4.1. Behavioral and electroencephalographic effects

The intrahippocampal administration of Mlx-8 (1.0, 1.4 and 2.1 μg/μl) induced a variety of symptoms, which included wet dog shake, compulsive scratching, vigorous jumping, vocalization, circling behavior, wild running and periods of immobility. The EEG records showed isolated and cluster spikes. The onset of these effects occurred approximately 4–5 h after injection. In addition, more severe symptoms, e.g. wild running, bilateral forelimb clonus with rearing and falling, were also observed, characterizing a limbic convulsion (Table 3). These symptoms were dose-dependent, and seizures were classified at level 5 on an 8-point scale described by Pinel and Rovner [39]. At this time, the EEG record showed isolated and cluster spikes, which evolved to short epileptic discharges in the cortex and hippocampus. Seizures were often accompanied by behavioral convulsions. Convulsions had the peculiarity of being elicited by handling the animal. Besides the behavioral and electroencephalographic effects described above, Mlx-8 (2.1 μg/μl) induced generalized limbic convulsions at level 7 (Pinel and Rovner [39], characterized by bilateral forelimb clonus, followed by tonic extension of limbs. Dyspnea was also observed. Occasionally, convulsive episodes were present 24 h after injection of the highest dose of Mlx-8 (2.1 μg/μl). Loss of balance was still observed 4 days after toxin injection (Table 3). Animals injected with 2.1 μg/μl manifested severe symptoms of intoxication, and 80% of them died at the following day after injection.

Table 2
Lethal toxicity and PLA2 activity of *M. lemniscatus* venom and its purified toxins

Venom/toxins	Lethal toxicity, LD_{50} (μg/mouse)			PLA2 specific activity (U/mg)[a], $X \pm$ S.E.M. ($n = 3$)
	i.p.	i.v.	Reference	
Crude venom	5	–	[6]	167.16 ± 15.88
Mlx-08	–	>40	Present study	263.75 ± 14.84
Mlx-09	–	>40	Present study	251.49 ± 12.68
Mlx-11	–	>40	Present study	168.28 ± 11.53
Mlx-12	–	>40	Present study	176.83 ± 10.87

[a] Concentration of 0.25 μg/μl was used for venom or toxins. Data were determined in the present study.

Table 3
Behavioral, EEG and histopathologic effects of intrahippocampal injection of PLA2s toxins isolated from *M. lemniscatus* venom

Treatment	Dose (μg/rat)	Measure parameters			
		EEG	Behavior	Neuronal lesion	Lethality (%)
Vehicle	–	Normal	Normal	None	
	1.0	Isolated spikes	WDS, compulsive scratching, bilateral forelimb clonus	CA1, CA3, DG	0
Mlx-8	1.4	Isolated and clusters spikes	WDS, compulsive scratching, jumping vocalization, circling behavior, wild running, periods of immobility, bilateral forelimb clonus	CA1, CA3, DG	33.33
	2.1	Isolated and clusters spikes. Short-lasting epileptic discharges	Bilateral forelimb clonus with rearing and falling, tonic extension of the members, breathing difficulties. Convulsion elicited by handling the rat	CA1, CA3	80.0
	0.57	Few isolated spikes	Staring, WDS, wild running	None	0
Mlx-9	1.15	Isolated and cluster spikes. Short-lasting epileptic discharges	WDS, wild running, bilateral forelimb clonus with rearing and falling, generalized clonic convulsions	CA1, CA3, DG	37.50
	4.5	Isolated and cluster spikes. Short-lasting epileptic discharge	Severe intoxication, generalized clonic–tonic convulsions, salivation, dyspnea, comatose and loss of balance	CA1, CA3, CA4, DG	66.66
Mlx-11	1.0	Isolated spikes	WDS, staring and immobility	CA1, CA3, CA4	0
	2.0	Isolated and cluster seizures. Short-lasting epileptic discharges	WDS, compulsive scratching, jumping, vocalization, circling behavior, immobility, breathing difficulties, salivation, barrel rotation, bilateral forelimb clonus with rearing and falling, forelimb tonic extension, wild running	CA1, CA3, CA4, DG	50.0
Mlx-12	1.43	Normal	WDS and scratching	None	0
	2.86	Isolated and cluster seizures. Short-lasting epileptic discharges	WDS, staring, compulsive scratching, vocalization, wild running, circling behavior, barrel circling, breathing difficulties, bilateral forelimb clonus with rearing and falling, generalized tonic–clonic convulsion, seizures elicited by handling the animal	CA1, CA3, CA4	20.0
	4.30	Short-lasting epileptic discharges	Severe intoxication, death	ND	86.0

Qualitative comparison of EEG alterations in hippocampus until 24 h after the injection of Mlx-8, -9, -11 or -12. Abbreviations: WDS, wet dog shake; DG, dentate gyrus; ND, not determined.

The onset of the intoxication symptoms was similar for 1.15 and 4.5 μg/μl Mlx-09. Animals laid on side in a moon-shape posture. Their motor activity was reduced, and crawling and increasing loss of balance were observed. Behavioral alterations initiated 2–4 h after injection of 1.15 or 4.5 μg/μl Mlx-9. The percentage of disturbances in rats and the severity of each symptom increased in a dose-dependent manner. Firstly, the rats showed staring, and thereafter they showed behavioral disturbances, including wet dog shake and wild running. At this time, the EEG records showed isolated and cluster spikes. Approximately 3 h after injection, behavioral disturbances evolved to episodes of limbic convulsion at level 5 [39], characterized by wild running, jumping, bilateral forelimb clonus with rearing and falling. Intense salivary secretion was also observed. Eight hours after toxin injection, one rat presented generalized tonic–clonic convulsions at level 7 [39], followed by death. These behaviors were accompanied by isolated and cluster spikes and short-lasting epileptic discharges (Table 3 and Fig. 3). One animal presented a delayed onset (about 16 h) to show wet dog shake, forelimb clonus, eyes blinking, ear clonus and generalized behavioral seizures (level 7). Cluster and/or isolated spikes were recorded 24 h after toxin administration (Fig. 3). Only isolated spikes were observed 48 h after toxin injection (Fig. 3). Hypersensitivity to touch and sound and aggressive behavior were also noticed in the following days. Vocalization and compulsive scratching were not observed. After intrahippocampal injection of 0.57 μg/μl Mlx-9, rats showed staring, and thereafter behavioral disturbances included wet dog shake and wild running. The EEG analysis of rats showed isolated spikes. More severe signs of intoxication were observed in animals treated with 4.5 μg/μl Mlx-09, which initiated after a long delay, around 16 h, after toxin injection. Behaviors were characterized by wet dog shake and repetitive generalized clonic–tonic convulsion (level 7). Other intoxication signs were

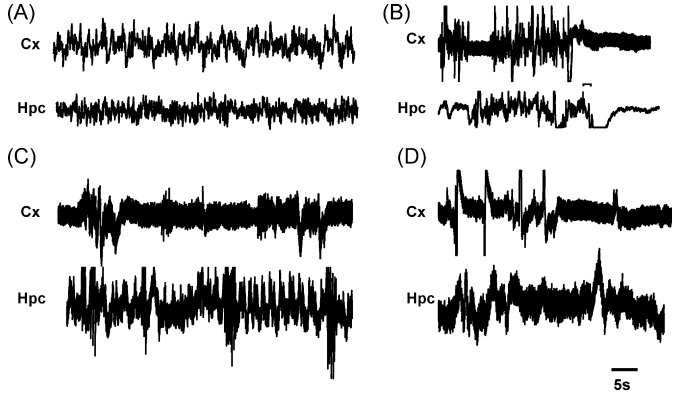

Fig. 3. Electroencephalographic record of an individual rat, illustrating the alterations observed after injecting 1.15 μg/μl Mlx-09 into the dorsal hippocampus. (A) Control record obtained from a rat injected with 1.0 μl of 0.1 M phosphate buffer into the hippocampus; (B) short epileptic discharges observed 6 h after Mlx-9 injection; (C) cluster of spikes observed 24 h after toxin injection and (D) isolated spikes and wet dog shakes (●) observed 24 h after toxin injection. The records were from cortex (Cx) and hippocampus (Hpc).

also observed, such as penile erection, Straub tail, increased salivation and dyspnea. From this period until the sacrifice, animals presented an intense comatose condition and loss of balance. The EEG was normal at the day of toxin injection, but the EEG recorded 24 h after injection showed isolated and cluster spikes and short-lasting epileptic discharges. Isolated spikes were still recorded 48 h after injection (Table 3). Only four animals (33.33%) treated with 4.5 μg/μl Mlx-09 survived until the sacrifice day.

The intrahippocampal administration of 1.0 μg/μl Mlx-11 induced weak symptoms such as wet dog shake, staring and immobility 2–3 h after toxin injection. At this time, EEG records showed isolated spikes (Table 3). Mlx-11 (2.0 μg/μl) induced severe symptoms characterized by repeated wet dog shakes, compulsive scratching, vigorous jumping, vocalization, circling behavior, wild running and periods of immobility, dyspnea, salivation, barrel rotation, and behavioral limbic seizures characterized by bilateral forelimb clonus with rearing and falling, which evolved to forelimb tonic extension (level 7) [39]. Only cluster and isolated spikes were recorded over 8 h of observation (Table 3). Fifty percent of the animals injected with 2.0 μg/μl Mlx-11 died at the injection day. Thus, a quantitative histological analysis was not made for this group. The observation of hippocampal areas of surviving rats revealed neuronal loss in CA1, CA3 and CA4 subfields and dentate gyrus.

Mlx-12 (1.43 μg/μl) only induced wet dog shake and scratching 4–6 h after injection. No EEG alterations were observed. Mlx-12 (2.86 μg/μl) induced frequent wet dog shakes, staring, compulsive scratching, vocalization, wild running, periods of immobility, circling behavior, barrel circling, dyspnea, ear clonus, and Straub tail that initiated approximately 2–3 h after toxin injection (Table 3). These behavioral disturbances gradually evolved to limbic seizures, characterized by bilateral forelimb clonus with rearing and falling (level 5), which in turn evolved to generalized tonic convulsion (level 7) [39]. Similar seizures to that provoked by Mlx-8 were elicited by handling animals. The EEG record showed isolated and cluster spikes (Table 3) that evolved to short epileptic discharges in the cortex and hippocampus. They were accompanied by behavioral disturbances such as myoclonus and wet dog shake (Table 3). Mlx-12 (4.3 μg/μl) induced a severe intoxication such as compulsive scratching, vocalization, dyspnea, salivation, forelimb clonic–tonic extension (level 7) and death of 86% of the animals.

3.4.2. Histological analyses

Analyses of hippocampal sections by light microscopy showed lesions characterized by massive neuronal loss and hippocampal gliosis. Animals that were injected with different doses of four toxins, except the lowest doses of Mlx-9 (0.57 μg/μl) and Mlx-12 (1.43 μg/μl, data not showed), significantly exhibited fewer cells in CA1 and CA3 subfields of the hippocampal formation, at the ipsilateral side of injection, than control animals (Figs. 4 and 5). Mlx-12 (1.43 μg/μl) was not able to induce any histological alteration in hippocampal neurons (Table 3). Compared to the vehicle, all doses of toxins, except the highest dose of Mlx-12 (2.86 μg/μl), significantly decreased the number of cells in dentate gyrus subfields of the hippocampal formation, at the ipsilateral side of injection (Fig. 4). Only

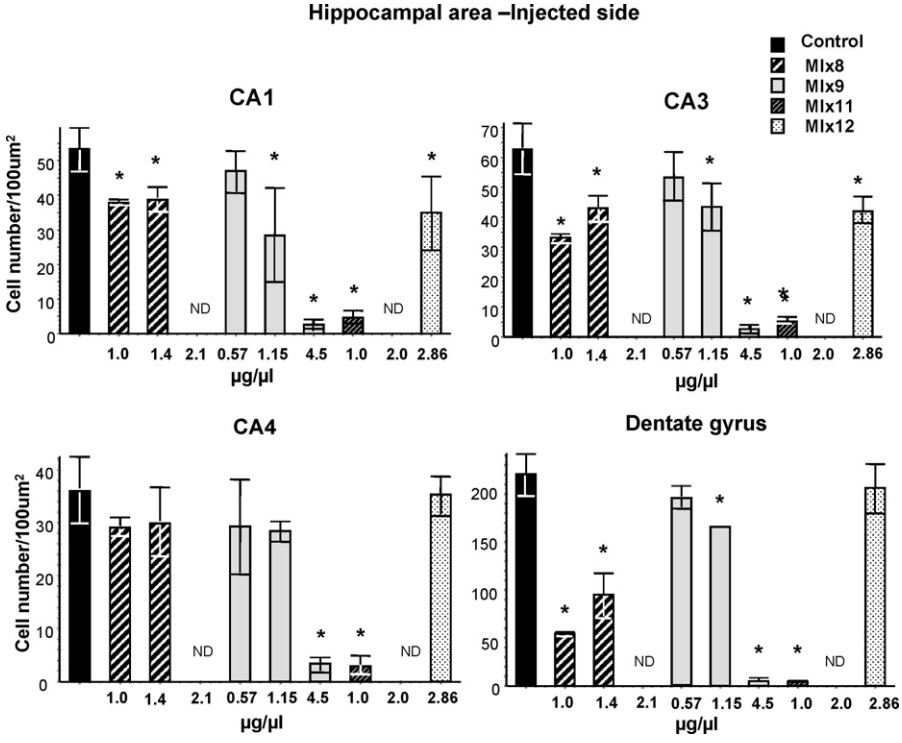

Fig. 4. Hippocampal sections were analyzed by light microscopy. The number of cells/100 μm² in the pyramidal (subfields CA1, CA3 and CA4) and dentate granular layers were quantified 7 days after intrahippocampal injection of vehicle or different doses of Mlx-8, Mlx-9, Mlx-11 and Mlx-12. Data are expressed as means ± S.D. *$P < 0.05$ compared to the control group (analysis of variance by the Dunnett test). ND: not determined. The majority of animals of these groups died before the sacrifice day.

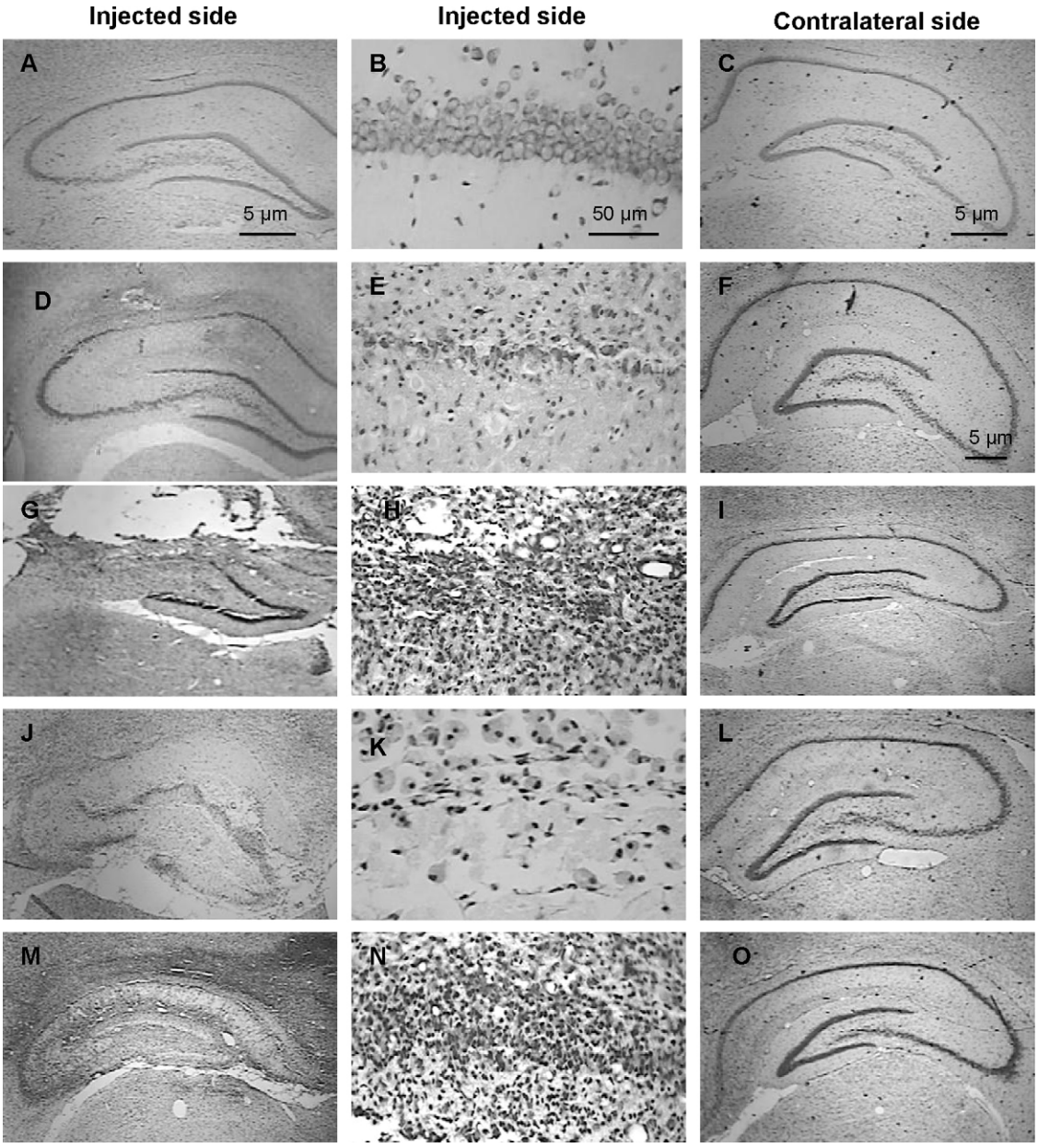

Fig. 5. Photomicrographs of the dorsal hippocampus showing a typical pattern of neurodegeneration 7 days after microinjection of 1.4 μg/μl Mlx-8 (D–F); 1.15 μg/μl Mlx-9 (G–I); 1.0 μg/μl Mlx-11 (J–L) and 2.86 μg/μl Mlx-12 (M–O) toxins isolated from *M. lemniscatus* venom. The control hippocampus and contralateral side of the injection appear to be unaffected (A–C). Cresyl violet stain. The photomicrograph was made 0.2 mm from the injection site. Magnification: A, C, D, F, G, I, J, L, M and O, 40×; B, E, H, K and N, 400×.

the hippocampal injection of 4.5 μg/μl Mlx-09 and 1.0 μg/μl Mlx-11 induced a massive neuronal loss in CA4 subfield of hippocampus, at the ipsilateral side of injection, compared to controls animals (Fig. 4). Mlx toxins were not able to induce neuronal loss at the contralateral side of microinjection.

Animals injected with Mlx-08 2.1 μg/μl manifested severe symptoms of intoxication. The survival animals (20%) showed neuronal loss in the CA1 and CA3 subfields and in the dentate gyrus of hippocampus. Fifty percent of the animals injected with Mlx-11 2.0 μg/μl died at the injection day. The observation of hippocampal areas of surviving rats revealed neuronal loss in CA1, CA3 and CA4 subfields and dentate gyrus. Thus, a quantitative histological analysis was not performed for these groups.

4. Discussion

Mlx-8, Mlx-9, Mlx-11 and Mlx-12 present in *M. lemniscatus* venom are PLA2s, as revealed by their typical molecular mass presented in Table 1 and induce neurotoxicity after microinjection into the hippocampus.

The *M. lemniscatus* crude venom present high toxicity and some works show that the LD_{50} is closed of 5–10 μg/mouse after i.p. injection [6,10]. The neurotoxicity of other elapid crude

venoms have been reported. Lysz and Rosenberg [32] reported that *Naja naja* venom present a LD_{50} of great than 10 μg/mouse (i.p.). Khow et al. [22] reported the LD_{50} of *Bungarus candidus* venom was 3.5 μg/mouse (i.v.).

However, purified PLA2 neurotoxins from elapid venoms have showed weak toxicity when administered by a peripheral route. In order to estimate the LD_{50} in mice various doses of Mlx-08, Mlx-09, Mlx-11 or Mlx-12 toxins were tested by i.v. injection, but we observed that none of them showed toxic effects after peripheral administration (until 40 μg/mouse). Otherwise, the intrahippocampal injection of these toxins induced severe neurotoxic effects and the approximate dose able to induce 20–50% of lethality range between 1.15 and 2.86 μg/rat (Table 3). Nevertheless, the LD_{50} of the toxin Mlx-09 after i.c.v. injection was previously determined. It is 0.088 μg/mouse (personal communication). We have observed that other PLA2 neurotoxins have also determined the LD_{50} after intracerebral administration as PLA2 mocambique toxin, 0.6 μg/mouse [11], paradoxin, 1.22 μg/kg of mouse [14], OS2-PLA2, 100 pmol/mouse inducing 40% of death [17] and crotoxin, 3.3 pmol/mouse [14]. In addition, the finding described by Lysz and Rosenberg [32] corroborate with our results. These authors showed that the approximate LD_{50} value following i.p. injection into mice rose from 10 μg/mouse for the *Naja naja* crude venom to 900 μg/mouse (i.p.) for the final partially purified phospholipase (peak I fraction). On the other hand, the i.c.v. injection of this same fraction in rats present a LD_{50} of 3.8 μg/rat and a CD_{50} (convulsive dose) of 1.7 μg/rat. In order to make sure that the delayed effects of PLA2 were not due to a peripheral action, rats were injected with 330, 660 or 990 μg/rat of the enzyme and none of them present ill-effects [32]. On the other hands, we can find data showing neurotoxins that present high toxicity after intraperitoneal administration. Thus, three beta-bungarotoxins, beta-3, beta-4 and beta-5 bungarotoxins from *Bungarus multicinctus* venom present LD_{50}s of 0.066, 0.072 and 0.13 μg/g of mouse, respectively [29].

Therein, at the present work we studied the effects of intrahippocampal administration of Mlx-8, Mlx-9, Mlx-11 and Mlx-12 toxins and observed these toxins induced severe toxic symptoms and the percentage of death was dose-dependent. Thus, all these toxins induced behavioral, electroencephalographic disturbances and neuropathological injuries when injected into the hippocampus. These four PLA2 toxins induced motor and EEG alterations in a dose-dependent manner. In regard to their features to cause neuronal injury, they also seem to depend on the dose of the toxin used. The highest doses of toxins caused severe intoxication and death of some animals. Thus, the histological analysis was not possible to be performed in these groups (2.1 μg/μl Mlx-8 and 2.0 μg/μl Mlx-11). A high occurrence of compulsive scratching was observed after Mlx-11 and Mlx-12 hippocampal injection and in the i.c.v. injection of paradoxin [14] OS2-PLA2 [17] and crotoxin [14]. Behavioral convulsions induced by Mlx toxins were often accompanied by EEG alterations. Similarly to the kainate model [9] convulsions induced by Mlx-8 and Mlx-12 were also elicited by handling animals. In this way paradoxin i.c.v. injected also induces an hypersensibility to sound or touch [14]. Mlx toxins were convulsive but weakly epileptogenic, since low rates of seizure discharges were observed in EEG records. These effects are quite similar to those induced by other PLA2s, OS2 toxin [17], PLA2 mocambique toxin [11] and paradoxin, an analog of taipoxin, in rodents [14]. Nonetheless, crotoxin, a neurotoxic PLA2, presents high convulsive and epileptogenic properties [14,34]. Dorandeu et al. [14] proposed the classification of neurotoxic sPLA2s into different groups, depending on their epileptogenic properties, based on two criteria: the presence of epileptic paroxysmal discharges recorded in cortex and convulsions. Using this criteria, we could classify Mlx toxins as convulsive, but weakly epileptogenic.

Concerning the works that have been investigated the behavior, EEG and/or neuropathologic effects of various PLA2s toxins, we observed that paradoxin, OS2-PLA2 and Bee-PLA2 toxins present similar and different effects when compared with the date showed in the present work. In this way, paradoxin i.c.v. injected induced myoclonic-like jump, clonic movements of forelegs (pedaling), but generalizing convulsion was not observed [14]. Nevertheless, convulsion was observed in our study. On the other side, the EEG alterations were quite similar, i.e. isolated spikes and polyspikes discharges were present in both studies. Other effects such as grooming, flat posture, decrease of the motor activity, crawling, loss of balance, compulsive scratching, comatose state, pedaling, plioerection, ptosis, hypersensibility to sound or touch were also observed by Dorandeu et al. [14] and in the present study. Regarding the studies developed with OS2-PLA2 and Bee-PLA2 toxins [17], these showed effects closed related with those observed for us such as wet dog shake, pedaling (forelimb clonus), compulsive scratching, convulsion, vocalization, breathing difficulties, spike-wave discharges and fill episodes of seizures. The onset of behavioral alteration was delayed for all these PLA2s toxins. The onset for most of the mainly effects described for Mlx toxins are in the 80–380 min range.

The neuronal lesions induced in the present work by intrahippocampal administration of Mlx toxins, which demonstrate PLA2 activity, were also observed after i.c.v. injection of paradoxin [14], crotoxin [14], β-bungarotoxin [47] and *Naja mocambique* PLA2 [11]. Thus, recent studies have demonstrated that β-bungarotoxin-induced death of cultured neurons was mediated, in part, by excessive generation of nitric oxide triggered by $[Ca^{2+}]i$ overloading. In addition, activation of NMDA receptors and L-type calcium channels is apparently involved in β-bungarotoxin-induced increase in calcium levels [49]. These studies have demonstrated that the mechanisms involved in PLA2 neurotoxicity are not restricted solely to the catalytic activity of PLA2s. Besides, endogenous PLA2s are known to be activated by some brain pathological conditions, such as epilepsy, ischemia and cerebral trauma, and to be involved in neuronal degeneration. As stated by Cole-Edwards and Bazan [12] the excitatory amino acid glutamate activates PLA2 and phospholipase C, generating arachidonic acid, PAF, and DAG, which are implicated in neuronal plasticity.

The appearance of the mainly neurotoxic effects induced by Mlx-8, Mlx-9, Mlx-11 and Mlx-12 were observed 3–5 h after toxin injection. Indeed, the onset of seizure development was markedly delayed for OS2 PLA2 [17], paradoxin [14] and *Naja*

mocambique PLA2 [11]. On the other hand, the seizure-inducing effects of other convulsants such as bicuculin, pilocarpine [50] kainate [5] alpha-dendrotoxin [3] or toxins from *Tityus serrulatus* venom [21,44,45] were evident within 30 min. These drugs are known to act in specific neuronal structures such as ionic channels or receptors.

In fact, the intracerebral injection of PLA2 toxins used in the present work and of other neurotoxic PLA2s markedly delayed the onset of behavioral seizures and electrographic seizures [11,14,17]. These findings show that neurotoxic PLA2 could have similar mechanisms of action. We could postulate that the underlying mechanisms whereby PLA2s delay the onset of neurotoxicity observed in our experiments could be similar to those described elsewhere. Thus, only the catalytic activity of PLA2 toxins such as β-bungarotoxin, paradoxin, and OS2 toxin cannot cause neuronal lesions, since an acidic PLA2 isoenzyme from *Vipera russellii* was virtually inactive to cause neuronal damage. Moreover, Gandolfo et al. [17] showed that OS1 PLA2 – a close structural homologue of OS2 neurotoxic toxin, showing high PLA2 activity – had no neurotoxicity, clearly evidencing that toxicity of PLA2s is not directly related to their enzymatic activity. Thus, the characterization of neuronal (N-type) and muscular (M-type) PLA2 receptors showed that neurotoxicity is probably initiated by the binding of these neurotoxins to specific brain and muscular receptors [30].

In spite of the present study have not investigated the mechanisms of action of the Mlx-PLA2 toxins, recent studies have made major contributions to understand the molecular mechanisms of neurotoxic PLA2s. Thus, data from literature have claimed that snake presynaptic PLA2 neurotoxins block nerve terminals by binding to neuronal membranes and by catalyzing phospholipid hydrolysis, producing lysophospholipids and fatty acids. These compounds change the membrane conformation causing enhanced fusion of synaptic vesicle via hemifusion intermediate, with release of neurotransmitters and concurrent inhibition of vesicle fission and recycling. At a late stage, other changes in nerve terminals take place such as increased plasma membrane permeability to ions and internalization of toxins, markedly impairing the functional and structural integrity of nerve terminals [41,42]. Thus, the neurotoxicity induced by PLA2s studied herein could also be attributed, at least in part, to those mechanisms described above.

In conclusion, we demonstrated for the first time that the venom of South America coral snake *M. lemniscatus* contains neurotoxic PLA2s, similar to those characterized in Old World elapid venoms, which can induce behavioral disturbances, electroencephalographic seizures and neuronal degeneration. The seizures were characterized by clonic movements. Mlx toxins were convulsive but weakly epileptogenic. Moreover, the onset of seizures induced by Mlx toxins was markedly delayed. The injury of hippocampal cells was characterized by massive neuronal loss and hippocampal gliosis. Researches using *in vivo* and *in vitro* approaches are currently in progress in our laboratory to investigate the mediators involved in Mlx neurotoxicity. These toxins can be a useful tool to investigate properties of endogenous secretory PLA2s and therefore may be important both to study mechanisms involved in neurotransmitter release at nerve terminals and to explain the convulsive properties of PLA2s toxins.

Conflict of interests

The authors declare no conflict of interests.

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