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Neurotoxicity of coral snake phospholipases A2 in cultured rat hippocampal neurons

Nathalia Delazeri de Carvalho[a], Raphael CaioTamborelli Garcia[d], Adilson Kleber Ferreira[b], Daniel Rodrigo Batista[e], Antonio Carlos Cassola[e], Durvanei Maria[b], Ivo Lebrun[b], Sylvia Mendes Carneiro[c], Solange Castro Afeche[a], Tania Marcourakis[d], Maria Regina Lopes Sandoval[a,*]

[a]Laboratory of Pharmacology, Butantan Institute, Av. Dr. Vital Brasil 1500, São Paulo, SP 05503 900, Brazil
[b]Laboratory of Biochemistry and Biophysics, Butantan Institute, São Paulo, SP 05503 900, Brazil
[c]Laboratory of Cellular Biology, Butantan Institute, São Paulo, SP 05503 900, Brazil
[d]Department of Clinical and Toxicological Analysis, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP 05508 000, Brazil
[e]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

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ABSTRACT

The neurotoxicity of two secreted Phospholipases A2 from Brazilian coral snake venom in rat primary hippocampal cell culture was investigated. Following exposure to Mlx-8 or Mlx-9 toxins, an increase in free cytosolic Ca^{2+} and a reduction in mitochondrial transmembrane potential ($\Delta\Psi m$) became evident and occurred prior to the morphological changes and cytotoxicity. Exposure of hippocampal neurons to Mlx-8 or Mlx-9 caused a decrease in the cell viability as assessed by MTT and LDH assays. Inspection using fluorescent images and ultrastructural analysis by scanning and transmission electron microscopy showed that multiphase injury is characterized by overlapping cell death phenotypes. Shrinkage, membrane blebbing, chromatin condensation, nucleosomal DNA fragmentation and the formation of apoptotic bodies were observed. The most striking alteration observed in the electron microscopy was the fragmentation and rarefaction of the neuron processes network. Degenerated terminal synapses, cell debris and apoptotic bodies were observed among the fragmented fibers. Numerous large vacuoles as well as swollen mitochondria and dilated Golgi were noted. Necrotic signs such as a large amount of cellular debris and membrane fragmentation were observed mainly when the cells were exposed to highest concentration of the PLA2-neurotoxins. PLA2s exposed cultures showed cytoplasmic vacuoles filled with cell debris, clusters of mitochondria presented mitophagy-like structures that are in accordance to patterns of programmed cell death by autophagy. Finally, we demonstrated that the sPLA2s, Mlx-8 and Mlx-9, isolated from the *Micrurus lemniscatus* snake venom induce a hybrid cell death with apoptotic, autophagic and necrotic features. Furthermore, this study suggests that the augment in free cytosolic

*Corresponding author.
E-mail addresses: mrlsando@usp.br, mrlsando@butantan.gov.br (M.R.L. Sandoval).

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Ca^{2+} and mitochondrial dysfunction are involved in the neurotoxicity of Elapid coral snake venom sPLA2s.

1. Introduction

In the Americas, the *Elapidae* family is represented by coral snakes that comprise 120 species and subspecies belonging to the genera *Micruroides*, *Leptomicrurus* and *Micrurus* (McDowell, 1987; Roze, 1996). *Micrurus* is the most representative genus as far as abundance and diversity are concerned, with a great number of species found in South America and Southern United States. However, the biochemistry and pharmacology of components from coral snake venoms have not been thoroughly studied so far.

Recently, the Brazilian Ministry of Health has considered ofidism as a neglectful disease. Accidents involving *Micrurus ssp*. are considered a medical emergency due to the high risk of death which is usually due to respiratory failure. However, there are rare reports in literature aimed at understanding the mechanisms involved in the degeneration processes induced by pre and postsynaptic neurotoxins present in coral snake venoms.

On the other side, venoms belonging to *Elapidae* family from the Old World have been extensively studied as sources of presynaptic phospholipases A2 (PLA2s) and postsynaptic neurotoxins in peripheral and central nervous system (Clapp et al., 1995; Da Silva Junior et al., 1991; Dorandeu et al., 2002; Khow et al., 2003; Kost et al., 2005). Snake presynaptic neurotoxins endowed with PLA2 activity, which are prominent components of elapid venoms, play an important role in envenomation of the prey by causing a persistent blockade of the neuromuscular transmission (Kini, 1997; Schiavo et al., 2000). Death usually follows due to respiratory paralysis with little or no damage to other organs (Connolly et al., 1995; Prasarnpun et al., 2005). The toxicity of PLA2s from *Micrurus* venoms in the peripheral nervous system showed that a presynaptic PLA2 isolated from *Micrurus dumerilli* venom also evoked neuromuscular blockade in vertebrate nerve–muscle preparations and triphasic changes in spontaneous neurotransmitter release from motor neuron terminals (Belo et al., 2005).

Neurotoxic PLA2s have been isolated mainly from the venom of the two families of venomous snakes Elapidae and Viperidae (Lambeau and Lazdunski, 1999). Secreted PLA2s (sPLA2s) are small proteins of 13–19 kDa that comprise 17 groups (Ho et al., 2001; Rouault et al., 2003), however, neurotoxic sPLA2 have been found only in groups IA, IIA and IIIA (Kini, 1997; Pungercar and Krizaj, 2007). Those from elapids venoms belong to the group IA and no mammalian equivalent has been described so far (Lambeau and Lazdunski, 1999). Secreted PLA2s are enzymes that catalyze the hydrolysis of the sn-2 ester bond in 1, 2-diacyl-sn-3-phosphoglycerides of glycerophospholipids to produce free fatty acids and lysophospholipids.

Concerning the mechanisms of action of the sPLA2s-neurotoxins, recent studies have made major contributions to understand their molecular mechanisms. Thus, data from literature has claimed that snake presynaptic PLA2 neurotoxins block nerve terminals by binding to neuronal membranes receptors (Lambeau and Lazdunski, 1999) 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 later stage, other changes in nerve terminals take place such as increased plasma membrane permeability to ions and internalization of the toxins, markedly impairing the functional and structural integrity of nerve terminals (Pungercar and Krizaj, 2007; Rossetto et al., 2006; Rosso et al., 1996).

Due to the complexity of the anatomically fine structure of the neuromuscular junction (NMJ) and to the inherent limited possibility of experimental approach at the molecular level of this tissue preparation, further progress has been achieved through the utilization of primary neuronal cultures from the central nervous system (Herkert et al., 2001; Paoli et al., 2009; Rigoni et al., 2004) and NSC34 cell line, a very similar model to actual motoneuron (Pungercar and Krizaj, 2007). Despite the target of neurotoxins sPLA2 is NMJ, it is well established that they are highly toxic when injected into the central nervous system (CNS) (Gandolfo et al., 1996; Kolko et al., 1999) or added to neuronal cell cultures (Bazan et al., 1995; Bazan, 1998; Kolko et al., 1999). Therefore, data obtained with cultured CNS neurons were relevant and it is accepted that replication would not be complete but it has contributed to the knowledge of the basic molecular events (Paoli et al., 2009; Pungercar and Krizaj, 2007).

Considering neuronal injury, β-bungarotoxin (β-BuTX), the most investigated presynaptic PLA2 neurotoxin isolated from the elapid snake *Bungarus ssp*. venom, induces widespread neuronal cell death throughout the mammalian CNS (Francis et al., 1997). Moreover, intracerebroventricular (i.c.v.) injection of *Naja mocambique*-PLA2 provoked extensive cortical and subcortical injury to forebrain neurons and fiber pathway lesions (Brazil, 1972). This neuronal injury was also observed after i.c.v. injection of other PLA2 neurotoxins as paradoxin (Masroori et al., 2010), crotoxin (Masroori et al., 2010), *β-bungarotoxin* (Shakhman et al., 2003) and *N. mocambique* PLA2 (Clapp et al., 1995). In addition, we previously investigated the neurotoxicity of four sPLA2s toxins (Mlx-8, 9, 11 and 12) isolated from the venom of the elapid snake *Micrurus lemniscatus* after microinjection into the brain (Oliveira et al., 2008). This study showed the presence of isolated and clustered spikes on EEG records, behavioral alterations characterized mainly by forelimb clonus, compulsive scratching and severe neuronal damage. Thus, the present work was designed to investigate in detail the neurotoxic effects of two sPLA2 toxins (Mlx-8 and Mlx-9) isolated from *M. lemniscatus* venom on cultured primary hippocampal neurons. The integrity of mitochondria through the transmembrane potential