Comparative Biochemistry and Physiology, Part C 168 (2015) 28–38

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Biochemical and pharmacological characterization of three toxic phospholipase A_2s from *Daboia russelii* snake venom

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ARTICLE INFO

Article history:
Received 3 September 2014
Received in revised form 19 November 2014
Accepted 26 November 2014
Available online 3 December 2014

Keywords:
Pre/post synaptic neurotoxin
Daboia russelii venom
Venom PLA2

ABSTRACT

Three isoenzymes of phospholipase A_2 (PLA_2), VRV-PL-IIIc, VRV-PL-VII, and VRV-PL-IX were isolated from *Daboia russelii* snake venom. The venom, upon gel filtration on Sephadex G-75 column, resolved into six peaks (DRG75 I–VI). The VRV-PL-IIIc was purified by subjecting DRG75II to homogeneity by rechromatography in the presence of 8 M urea on Sephadex G-75 column. The other two isoenzymes VRV-PL-VII and VRV-PL-IX were purified by subjecting DRG75III to ion exchange chromatography on CM-Sephadex C-25 column. Mol wt. for the three PLA_2s, VRV-PL-IIIc, VRV-PL-VII, and VRV-PL-IX are 13.003 kDa, 13.100 kDa and 12.531 kDa respectively. The VRV-PL-IIIc is not lethal to mice up to 14 mg/kg body weight but it affects blood sinusoids and causes necrosis of the hepatocytes in liver. It causes hemorrhage in kidney and shrinkage of renal corpuscles and renal tubules. The LD_{50}s for VRV-PL-VII and VRV-PL-IX are 7 and 7.5 mg/kg body weight respectively. They induced neurotoxic symptoms similar to VRV-PL-V. All the three PLA2s are anticoagulant and induced varying degree of edema in the foot pads of mice. VRV-PL-V and VRV-PL-VII are shown to act as pre and post synaptic toxins, while VRV-PL-IX acts as presynaptic toxin. This is evident from experiments conducted on cultured hippocampal neurons by patch clamp electrophysiology.

1. Introduction

Phospholipase A_2s (PLA_2) (EC 3.1.1.4) are esterolytic enzymes, which hydrolyse glycerophospholipids at sn-2 position. The PLA_2 enzymes are found as both intracellular and extracellular forms. They have crucial roles in various physiological and pathological processes (Dennis et al., 1991). But snake venom PLA_2 enzymes exhibit wide variety of pharmacological effects such as pre/post-synaptic neurotoxicity, myotoxicity, cardiotoxicity, pro/antiplatelet activity, edema inducing activity etc. (Ownby et al., 1976). Snake venom is also a source of multimolecular forms of PLA_2. The venom of snakes such as *Naja naja*, *Daboia russelii*, and *Trimersurus flavoviridis* have been reported to contain multiple forms of PLA_2 (Vishwanath et al., 1987; Jayanthi and Gowda, 1988; Bhat and Gowda, 1991). Earlier from our laboratory VRV PL-V (Jayanthi and Gowda, 1988) and VRV PL-VIIIa (Kasturi and Gowda, 1989) two toxic PLA_2s were purified and characterized from *D. russelii pulchella* (southern, India). Primary sequence of VRV PL-VIIIa (Gowda et al., 1994) and VRV PL-V has been reported (Vishwanath et al., 1988; Satish, 2004) purified and characterized a multitoxic PLA_2, VRV PL-VI, from the *D. russelii* venom (Northern, India) and has been shown to target pituitary gland. Another PLA2 RVVPF3 was purified and characterized from eastern region *D. russelii* venom (Chakraborty et al., 2002) exhibiting potent hemolytic activity.

The PLA_2s may associate with other proteins/peptides to form a complex and induce potent toxicity, for "Reprotoxin" a protein complex from *D. russelii* venom (Western, India) made up of a PLA_2, a protease and a trypsin inhibitor reported (Kumar et al., 2008). The hemorrhagic complex (MCHR) was isolated and characterized from eastern region *D. russelii* venom (Uma, 1998). It is made up of a PLA_2 and two non enzymatic peptides. Apart from this, there is a variation in the composition of acidic and basic PLA_2 isoforms in the venoms of Russell's viper from different regions (Prasad et al., 1999). Acidic PLA_2s are prominent in northern region *D. russelii* venom (Vishwanath et al., 1988) and of moderate proportion in the eastern region of India. Southern and western regions contain only basic PLA_2s and lack acidic PLA_2s.

The neurotoxic effect of the snake venom is the prominent contributor to the lethal toxicity. Neurotoxicity is brought about either by presynaptic or postsynaptic blockade of the neurotransmission.

Abbreviations: PLA_2, phospholipase A_2; NMDA, N-methyl D-aspartic acid; PC, phosphatidylcholine; TTX, tetrodotoxin; mEPSC, miniature excitatory postsynaptic currents; GABA, gamma amino butyric acid; HEPES, 4-(-2-hydroxyethyl)-1-piperanineethanesulfonic acid; EGTA, ethylene glycol tetra acetic acid; VRV-PL, Vipera russelii venom-phospholipase.

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http://dx.doi.org/10.1016/j.cbpc.2014.11.005
1532-0456/

β bungarotoxin (Bon and Saliou, 1983), Crotoxin (Bon et al., 1988), Taipoxin (Fohlman et al., 1976) and Textilotoxin (Pearson et al., 1993) are among the well studied presynaptic neurotoxic PLA_2 complexes that have been reported from snake venoms. On the other hand only a few postsynaptic neurotoxic PLA_2s are reported from snake venoms. For example Deepa machaiah and Gowda T V (Machiah and Gowda, 2006) have reported the isolation and purification of a postsynaptic PLA2 NN-XIa from *N. naja* venom (Eastern region, India). Bon and Saliou (1983) reported isolation of Ceruleotoxin from the venom of *Bungarus cerulus* which blocks post synaptic nerve terminal, But crotoxin is a complex toxin which exhibits both pre and post synaptic neurotoxicity in experimental animals (Hendon and Fraenkel-Conrat, 1971; Eterovic et al., 1975; Fraenkel-conrat, 1983).

Several classical methods can explain the effect of toxins on the central nervous system. There are mechanisms underlying the selective inhibition of mEPSCs (miniature excitatory postsynaptic currents) of NMDA (N-methyl D aspartic acid) receptors in neurons. Some reports have revealed the involvement of presynaptic NMDA receptors in physiological functions. Alex et al. (2006) reported the isolation of Conantokin G (Con G) from the venom of Conus geographus and its action on NMDA receptor mediated spontaneous EPSCs in cultured cortical neurons. Although two prominent neurotoxic PLA_2 have been reported and characterized from *D. russelii* venom, the pharmacological activities of these two PLA_2 do not completely recapitulate all the toxic effects of the venom such as effects on liver and kidneys. Therefore we set forth to resolve the remaining toxic components of the venom. In the present paper we report the isolation and characterization of three phospholipase A_2 from *D. russelii* venom (Western region). The PLA_2, VRV PL-IIIc induces kidney and liver necrosis. The other three PLA2s VRV-PL-VII and VRV-PL-IX including VRV-PL-V inhibited NMDA and nonNMDA mediated spontaneous excitatory neurotransmission in cultured hippocampal neurons.

2. Materials and methods

2.1. Materials and reagents

Sephadex G-75 and low-range molecular weight markers were purchased from Sigma Chemicals (St. Louis, MO, USA). [^{14}C] Oleic acid was from Perkin Elmer Life Sciences, Inc., USA. Fatty acid-free bovine serum albumin (BSA) was obtained from PAA Laboratories GmbH, Austria. Scintillation cocktail was obtained from Packard Biosciences BV (The Netherlands). All the other chemicals and reagents were of analytical grade purchased from SRL Chemicals, India. Lyophilized *D. russelii* snake venom from western India was purchased from the Haffkine Research Institute, Mumbai, India.

2.2. Animals

Adult Swiss Wister male mice weighing approximately 21 g (30–35 days old) were obtained from the central animal facility, University of Mysore. Animal care and handling were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The Institutional Animal Ethics Committee (IAEC) of the University of Mysore approved the protocols for the animal experiments.

2.3. Gel filtration chromatography and protein estimation

The sephadex G-75 column (1 × 145 cm) equilibrated with 0.05 M phosphate buffer (pH 7.0) was loaded with 100 mg of *D. russelii* venom in 0.5 ml equilibration buffer. Elution was carried out with pre-equilibrated buffer at a flow rate of 15 ml/h and 1.5 ml fractions were collected. Protein elution was monitored at 280 nm using a UV-VIS1601 Shimadzu spectrophotometer. The fractionation showed six discrete peaks (DRG-75 I–VI). Fractions of each peak were pooled separately, and were lyophilized and stored at 4 °C. Protein content for each peak was estimated by the method of Lowry (Lowry et al., 1951). Bovine serum albumin was used as a protein standard.

2.4. Purification of venom PLA2s

2.4.1. VRV PL-IIIc

VRV PL-IIIc was purified according to the method of Kumar J R (Kumar et al., 2008). The Sephadex G-75 column (1 × 145 cm) was first equilibrated with 8 M Urea. The three milligrams of DRG-75-II was dissolved in 0.5 ml of 8 M urea and loaded on to the column. Elution was carried out in the same buffer as described earlier.

2.4.2. VRV PL-VII and VRV PL-IX

The DRG-75-III peak showing PLA_2 activity was loaded (8 mg) on to a CM-Sephadex C-25 cation exchange column (1.5 × 40 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). PLA_2s were resolved by a step-wise gradient elution of phosphate buffers of various molarities and pH. The fractions of 1.5 ml was collected at the rate of 15 ml/h. Protein elution was monitored at 280 nm using a UV–VIS1601 Shimadzu spectrophotometer. Resolved protein peaks were desalted, lyophilized and stored at 4 °C in a refrigerator.

2.4.3. VRV PL-V

The most toxic PLA_2 found in *D. russelii russelii* venom, VRV PL-V was purified according to the method described by Shelke (2000). Briefly, whole venom of *D. russelii* snake venom from southern region India was fractionated on sephadex G-75 column followed by cation exchange chromatography on CM Sephadex C-25 column.

2.5. Phospholipase A_2 assay and positional specificity

Phospholipase A_2 activity was determined using egg phosphatidyl-choline (PC) as substrate according to the method of Bhat and Gowda (1989). The reaction mixture (1 ml) contained 1 μmol of PC in 0.05 M Tris–HCl buffer, pH 7.5, 0.2 ml of diethyl ether, and 40 μmol of Ca^{2+}, mixed well before adding 5 μg of PLA_2 peaks and incubated at 37 °C for 60 min with intermittent mixing. The free fatty acid released was extracted as cobalt soap and then the cobalt was complexed with α-nitroso-β-naphthol and estimated by colorimetry method. The phospholipase A_2 activity is expressed as nanomoles of free fatty acid released per minute. Positional specificity of PLA_2 was determined with [^{14}C] oleate-labeled, autoclaved *Escherichia coli (E. coli)* cells as substrate according to the method of Vishwanath et al. (1987).

2.6. Determination of molecular weights of VRV PL-VII and VRV PL-IX by MS-MALDI

The molecular mass of VRV PL-VII and VRV PL-IX was determined by mass spectrometry in Kratos PC-Kompact MALDI-4 in the positive ionization mode (Linear high, Power: 45).

2.7. Determination of LD_{50}

Groups of 10 mice, each mouse weighing 20–24 g, were injected intraperitonially (i.p.) with VRV PL-VII and VRV PL-IX, separately in 250 μl saline at doses of 1.0–10 mg/kg body weight. In case of VRV PL-IIIc dose in between 1 and 14 mg/kg body weight was injected (i.p). The survival time of each animal was recorded for 24 h. The LD_{50} dose was calculated according to the mathematical scheme of Meier and Theakston (Meier and Theakston, 1986). Animals were also observed constantly for the appearance of signs/symptoms of toxicity.