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Differential mode of attack on membrane phospholipids by an acidic phospholipase A_2 (RVVA-PLA_2-I) from *Daboia russelli* venom

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ABSTRACT

An acidic phospholipase A_2 (RVVA-PLA_2-I) purified from *Daboia russelli* venom demonstrated dose-dependent catalytic, mitochondrial and erythrocyte membrane damaging activities. RVVA-PLA_2-I was non-lethal to mice at the tested dose, however, it affected the different organs of mice particularly the liver and cardiac tissues as deduced from the enzymatic activities measured in mice serum after injection of this PLA_2 enzyme. RVVA-PLA_2-I preferentially hydrolyzed phospholipids (phosphatidylcholine) of erythrocyte membrane compared to the liver mitochondrial membrane. Interestingly, RVVA-PLA_2-I failed to hydrolyze membrane phospholipids of HT-29 (colon adenocarcinoma) cells, which contain an abundance of phosphatidylcholine in its outer membrane, within 24 h of incubation. The gas-chromatographic (GC) analysis of saturated/unsaturated fatty acids' release patterns from intact mitochondrial and erythrocyte membranes after the addition of RVVA-PLA_2-I showed a distinctly different result. The results are certainly a reflection of differences in the outer membrane phospholipid composition of tested membranes owing to which they are hydrolyzed by the venom PLA_2s to a different extent. The chemical modification of essential amino acids present in the active site, neutralization study with polyvalent antivenom and heat-inactivation of RVVA-PLA_2-I suggested the correlation between catalytic and membrane damaging activities of this PLA_2 enzyme. Our study advocates that the presence of a large number of PLA_2-sensitive phospholipid domains/composition, rather than only the phosphatidylcholine (PC) content of that particular membrane may determine the extent of membrane damage by a particular venom PLA_2 enzyme.

1. Introduction

Russell's viper (*Daboia russelli*) is regarded as one of the most medically important venomous snakes in many South-east Asian countries, including India [1]. Depending on the zoogeographic origins of Russell's vipers, their venom composition may vary, as a result of which envenomation by this snake displays an intriguing variation in the clinical manifestation [2,3].

Russell's viper venom is known to contain different isoforms of phospholipase A_2 (EC 3.1.1.4) that hydrolyzes glycerophospholipids at the *sn*-2 position of the glycerol backbone releasing lysophospholipids and fatty acids [4–6]. PLA_2s from venoms, in addition to digestion of pray, exhibit wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrosis, anticoagulation, hypotension, haemolysis, haemorrhage and oedema inducing activities [7]. Different isoforms of PLA_2, which may be acidic, basic or, neutral in nature on the basis of their elution profile from ion-exchange columns, can exist in a single venom [3], and each PLA_2 may exert different pathophysiological effects by a wide range of mechanisms in snakebite victims [2,3,6]. Therefore, it remains a challenging task for the scientists to elucidate the structure–function relationships of this class of protein.

Most of the toxic effects of snake venom PLA_2 are dependent on the hydrolysis of cellular or sub-cellular membrane phospholipids, and/or generation of phospholipid hydrolyzed breakdown products which are themselves lytic and can cause considerable membrane damage [7–9]. Kinetic studies of PLA_2 in the scooting mode establish that these enzymes bind to the intact membrane surface as a prelude to loading of the active site with a single phospholipid molecule, more specifically they bind to the phosphatidylcholine (PC) present in the outer leaflet of the membrane for the lipolysis reaction [5,10]. It is now becoming apparent that the different isoforms of venom PLA_2 can display dramatically different affinities for biomembranes, composed of different phospholipid polar head groups and fatty acyl

Abbreviations: pBPB, p-bromophenacyl bromide; DTT, Dithiothreitol; FA, Fatty acid; IAA, Iodoacetamide; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; RVV, Russell's viper venom; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone; TPCK, Tosyl phenylalanyl chloromethyl ketone

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chains, resulting in their differential membrane damaging activity [5,8,11]. This specificity of phospholipases has been extensively used to explore the physical structure of phospholipids in biological membranes [12]. It is exemplary to mention that only a few reports on membrane hydrolyzing property of PLA_2s from RVV are available [13,14]. Therefore, more precise studies are necessary to advance our understanding of the mechanism(s) of membrane damage and subsequent toxicity of PLA_2 enzymes from RVV.

Perusal of literature has showed that limited attempts have been made to explore the biochemical and pharmacological properties of acidic PLA_2 from snake venom. Recently, we reported the purification and biochemical characterization of an acidic, strong anticoagulant PLA_2 (RVVA-PLA_2-I) from Russell's viper venom [15]. In this study, we are reporting the membrane damaging activity of RVVA-PLA_2-I from *D. russelli* venom. Further, our study provides an insight into the membrane damaging activity of this acidic PLA_2, and suggests that it has a distinct preference for hydrolyzing specific phospholipid domain(s) in intact mitochondrial and erythrocyte membranes, whereas its effect on HT-29 adenocarcinoma cells are minimal, which may be due to the absence of RVVA-PLA_2-I-sensitive phospholipid domains in HT-29 cells.

2. Materials and methods

2.1. Materials

D. russelli venom was purchased from Calcutta Snake Park, Kolkata. CM Sephadex C-50, DEAE Sephadex A-50 and Sephadex G-50 (fine grade) were obtained from Pharmacia Fine Chemicals, Sweden. All other reagents of analytical grade were purchased from Sigma, India. The human colon adenocarcinoma (HT-29) cell was procured from National Centre for Cell Sciences (NCCS), Pune, India. The "In Vitro Toxicology Assay Kit (XTT based)" was purchased from Sigma-Aldrich, India. Polyvalent antivenom was purchased from Bharat Serum and Vaccines Limited, Ambernath. The kits for estimation of total protein, triglycerides, total cholesterol, urea, uric acid, serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) were procured from Coral Clinical Systems (Goa, India). The kits for the measurement of serum levels of glucose, alkaline phosphatase (ALP), and creatine phosphokinase (CPK-MB) were obtained from Erba Diagnostics (Mumbai, India) whereas the kit for estimation of serum lactate dehydrogenase (LDH) was obtained from Fisher scientific (Mumbai, India). The animal food was obtained from Pranav Agrotech, Delhi, India. The RVVA-PLA_2-I, an acidic PLA_2, was purified from crude RVV as described previously [15].

2.2. Methods

2.2.1. Phospholipase A_2 activity

For the screening purpose, PLA_2 activity of crude RVV and RVVA-PLA_2-I was determined as described by Doley and Mukherjee [16] using egg yolk phospholipids as substrate. One unit of PLA_2 activity was defined as the amount of protein which produces a decrease in 0.01 absorbance in 10 min at 740 nm. To determine the substrate specificity and phospholipid head-group preference of RVVA-PLA_2-I different commercially available phospholipids such as PC, PS and PE at a final concentration 1 mM were incubated with 100 nM enzyme at 37 °C for desired time periods and PLA_2 activity was assayed by titrametric method using palmitic acid as fatty acid standard [5].

2.2.2. Membrane damaging activity

Isolation of mitochondria from fresh chicken liver at 4 °C was described previously [5]. For the assay of RVVA-PLA_2-I induced mitochondrial swelling and membrane damage, mitochondrial suspension containing 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissues) in 2.0 ml of assay buffer (20 mM Tris–HCl, pH 7.4, containing 0.3 M sucrose) were incubated with 100 nM of RVVA-PLA_2-I, either in the presence or absence of 2 mM Ca^{2+}, at 37 °C for the desired time period. Mitochondrial swelling was followed spectrophotometrically by the decrease in absorbance at 520 nm for 30 min [5]. One unit of swelling is defined as a decrease in 0.01 absorbance per min of mitochondrial suspension at 520 nm by added RVVA-PLA_2-I (100 nM) as compared to control (mitochondrial suspension without RVVA-PLA_2-I) under the experimental condition. The basic test system without added RVVA-PLA_2-I was served as a control. Qualitative and quantitative analyses of liberated fatty acids and lysophospholipids from the mitochondrial membranes due to the action of RVVA-PLA_2-I were performed by gas-chromatography as described below. A control was also set up where the mitochondria were treated under the identical condition except the addition of RVVA-PLA_2-I.

For the estimation of RVVA-PLA_2-I induced haemolysis and erythrocyte phospholipid hydrolysis, goat blood was collected in 3.8% tri-sodium citrate and erythrocytes were separated by centrifuging the citrated blood at $1000\times g$ for 15 min, washed twice with isotonic K-phosphate buffer, pH 7.4 and suspended in the same buffer at a concentration of 5% (v/v). RVVA-PLA_2-I at a final concentration of 100 nM was added to 3.0 ml of the above said erythrocyte suspension and the haemolysis and erythrocyte phospholipid hydrolysis by RVVA-PLA_2-I were measured as described previously [5,8]. For determining the role of exogenously added phospholipids in haemolytic activity (indirect haemolytic activity), either PC (final concentration of 1 mM) or egg yolk phospholipids (0.1% v/v) were added to the erythrocyte suspension prior to the addition of RVVA-PLA_2-I. The reaction was initiated by the addition of PLA_2 followed by measuring the haemolysis as stated above [5]. For each of the experiment isotonic saline instead of RVVA-PLA_2-I was used as a control.

Erythrocytes or mitochondria, after treatment with RVVA-PLA_2-I, were centrifuged at $10{,}000\times g$ and 1.0 ml of supernatant was used for the extraction and quantification of total lipid and fatty acids from the reaction mixture [5]. For the estimation of phospholipids/lysophospholipids released from the membrane, the total lipid extracted from the supernatant was digested with concentrated HNO_3 in a Kjeldahl flask until a white precipitate was formed and then the liberated Pi was estimated calorimetrically as described by Doley et al. [5].

To study the effect of heating on catalytic and membrane damaging activities, RVVA-PLA_2-I solution (100 nM) was heated at 75 °C for different time periods (5–60 min), cooled immediately in ice-bath and then the required volume was withdrawn for the spectroflurometric study, assay of catalytic and membrane damaging activities of this enzyme.

2.2.3. Gas-chromatography analysis of liberated fatty acids from membranes

The total lipid released from the membranes after the treatment of RVVA-PLA_2-I was extracted and from this total lipid, liberated fatty acids were extracted and methylated as described by Mukherjee et al. [11]. The dry sample (fatty acid methyl esters) was dissolved in a minimum quantity of chloroform and analysed on a GC–MS (Varian 3800, Saturn 2000) system. The samples (1.0 μl) were injected using a split ratio of 100:1 into a fused silica GC column CP-Sil 8 CB low bleed (30 m × 0.25 mm × 0.25 μm) coupled with a CP-Sil 5 CB low bleed/MS (30 m × 0.25 mm × 0.25 μm) column with helium as the carrier gas. The system was equipped with flame ionization detector. The initial oven temperature was 120 °C and a temperature programme of 8 °C per min began at injection and continued to a final oven temperature of 270 °C, which was hold isothermal for 3 min. The injector port and detector temperature were set at 250 °C. The mass spectrometric data were acquired in electron ionization mode (70 eV). The unknown methylated fatty acids were identified by matching both retention time and MS of the unknown compound with those of authentic standards (Saturn 2000 MS library search). The fatty acids were quantitated by measuring and

comparing the GC peak area for released (unknown) fatty acids with the GC chromatogram of standard (known) fatty acids.

2.2.4. Modification and neutralization of RVVA-PLA$_2$-I with inhibitors and commercial antivenom

For RVVA-PLA$_2$-I-inhibition study, different inhibitors such as TPCK, TLCK, PMSF, p-BPB, EDTA, DTT, IAA, and commercial polyvalent antivenom raised in horse against crude RVV were pre-incubated with a constant amount of RVVA-PLA$_2$-I (100 nM) at 37 °C for 30 min [5,15]. The mixture was then assayed for the catalytic and membrane damaging activities of the RVVA-PLA$_2$-I in the corresponding assay system.

2.2.5. Enzyme immunoassay to determine the binding of RVVA-PLA$_2$-I with membranes

Supernatant obtained from the incubation of intact mitochondria or erythrocytes with purified RVVA-PLA$_2$-I (native, heated or chemically modified) at 4 °C for 30 min was tested for free (unbound) toxin concentration by ELISA using horse polyclonal antibodies and rabbit anti-horse IgG peroxidase conjugate [5]. A standard curve of PLA$_2$ was plotted by adding graded concentration of RVVA-PLA$_2$-I (30–200 nM per well) in the wells of the ELISA plate and the concentration of unbound RVVA-PLA$_2$-I was determined from this curve by using an ELISA plate reader (Thermo Electron Corporation, Multiskan ascent, Type 354). Each experiment was repeated thrice to assure the reproducibility.

2.2.6. Measurement of interaction of RVVA-PLA$_2$-I with PC

The dose-dependent interaction of PC with RVVA-PLA$_2$-I was measured by using a fluorescence spectrometer [15]. Briefly, PC was suspended in 20 mM Tris–HCl, pH 8.0 buffer at a final concentration of 0.1–1.0 mM and sonicated for about 5 min at 4 °C with a Labsonic® M (Sartorius) sonicator. To this, 100 nM of RVVA-PLA$_2$-I (dissolved in 20 mM Tris–HCl, pH 8.0) was mixed and fluorescence spectra were obtained at an excitation wavelength of 280 nm, excitation and emission slits of 5 nm (at room temperature ~23 °C). Wavelength shifts were measured by taking the midpoint at two thirds the height of the spectrum. The maximum fluorescence of free protein (I_0) was also measured.

2.2.7. Cytotoxicity assay on tumour cells

Cytotoxicity was assessed on human adenocarcinoma tumour cell line (HT29) as described by us with the following modifications [8]. Briefly, human colon adenocarcinoma (HT-29) cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (50U ml^{-1}), streptomycin (50 mg ml^{-1}) at 5% CO_2 in a humidified incubator at 37 °C. For cytotoxicity assays, 1×10^4 cells were seeded in 96-well plate and left overnight for attachment. Then crude RVV (10 μg ml^{-1}) or RVVA-PLA$_2$-I (10 μg ml^{-1} equivalent to 172 nM), diluted in DMEM media were added to the cells and incubated for either 4 h or 24 h. Cell viability was measured by using in vitro Toxicology Assay Kit (XTT based) according to the manufacturer's protocol and the percentage viability was calculated as the ratio of treated cells to the control (cells without addition of CRVV/RVVA-PLA$_2$-I) cells. Cells were also observed under the inverted phase contrast microscope for any visible morphological changes.

2.2.8. Determination of lethality and toxicity on mice

All the BALB/c mice, weighed between 20 and 30 g, were born in the laboratory breeding colony of the Central Facility of Animal House, Defence Research Laboratory, Tezpur, Assam and were pathogen free. General conditions of captivity were maintained in simulated atmospheric conditions of North East India (temperature 33–36 °C, relative humidity ≥ 75%).In captivity, seasonal variations of physiological functions are entrained by altering six-month periods of summer like long photoperiod (14 h of light/day). The general conditions of captivity were applied as described above and animals were maintained in social groups before and after experimenting. All experimenting protocols using animals were performed according to the "Principles of Laboratory Animal care" (NIH publication 85-23, revised 1985) and approved by the Institutional Animal Ethical Committee.

The acute toxicity was determined as per protocol of OECD/OCED guidelines 425 [17]. For toxicity assessment of RVVA-PLA$_2$-I-in rodents, RVVA-PLA$_2$-I (0.2 mg kg^{-1} body weight) dissolved in 0.1 ml of PBS was injected intraperitoneally into a group of six BALB/c mice. The mice were assigned as a control group (Group I, n = 6) and one RVVA-PLA$_2$-I treated group (Group II, n = 6). The animals were observed at regular intervals for up to 48 h after the injection for any behavioural changes viz., body weight, food and water intake, faecal and urination, grip strength, ear twist, rectal temperature etc., effect on circulatory system, and/or death. Bleeding time along with clotting time was also recorded before sacrificing the animals. Control animals (placebo) were injected with 0.1 ml of PBS only.

From mice sacrificed after 48 h of injection, blood was collected immediately by venipuncture and the serum used for the assay of different parameters viz. total protein, glucose, cholesterol, triglycerides, uric acid and urea levels as well as different enzymatic activities viz. LDH, ALP (alkaline phosphatase), CPK (creatine phosphokinase), SGOT and SGPT by using commercial kits following the instructions of manufacturers. To study the effect of RVVA-PLA$_2$-I on blood cells, RBC and WBC counts were done by an automatic cell counter (Automated haematology cell counter — MS 4(s), Melet Schloesing Laboratories).

2.2.9. Statistical analysis

The statistical analysis of the data was done by Student's *t* test using the software SigmaPlot 11.0 for windows (version 7.0). The value of $p \leq 0.05$ was considered as significant.

3. Results

3.1. Effect of RVVA-PLA$_2$-I on membrane phospholipid hydrolysis

With an increase in incubation time of mitochondria with RVVA-PLA$_2$-I, a significant damage to mitochondria was observed which was further pronounced in the presence of Ca^{2+} (Table 1). A deeper insight into the mode of attack of RVVA-PLA$_2$-I on the mitochondrial membrane was revealed by GC-analysis of liberated fatty acids which showed that straight chain saturated fatty acids such as $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, and $C_{19:0}$ were the most prominent fatty acids released from the membrane within the initial 30 min of attack by RVVA-PLA$_2$-I (Fig. 1). Further, with an increase in the incubation time of mitochondria with the RVVA-PLA$_2$-I, a corresponding increase in membrane phospholipid hydrolysis was observed (Fig. 1).

The RVVA-PLA$_2$-I at a concentration of 100 nM did not exert any appreciable haemolytic activity directly on washed erythrocytes; however, exogenous addition of egg yolk phospholipids (which is a source of PC) to the erythrocyte suspension enhanced the haemolytic activity of RVVA-PLA$_2$-I to a significant extent (Table 2). Either with an increase in the concentration of RVVA-PLA$_2$-I (data not shown) or an increase in the incubation time of PLA$_2$ with the erythrocytes (Table 2), a concomitant enhancement in the release of fatty acids and measured Pi from the erythrocyte membranes was observed. It is worthy to mention that during the initial 30 min of attack, haemolysis could not be detected although RVVA-PLA$_2$-I was able to release the FAs and lysophospholipids from the intact erythrocyte membranes. In contrast, the addition of egg-yolk phospholipids to the erythrocyte suspension resulted in the initiation of haemolysis within 30 min of incubation, and after 120 min, about 47.6% of total erythrocytes were haemolysed (Table 2).

The GC analysis of erythrocyte membrane phospholipid hydrolysis by RVVA-PLA$_2$-I had shown a specific preference for releasing the unsaturated straight chain fatty acids such as fatty acids for example,

Table 1
RVVA-PLA_2-I induced swelling and phospholipid hydrolysis of intact mitochondrial membrane either in the presence or in the absence of 2 mM Ca^{2+}. About 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissue) from chicken liver was incubated with 100 nM of RVVA-PLA_2-I at 37 °C for different time periods. The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	Mitochondrial swelling (U/min)[a]		Phospholipid hydrolysis				Ratio of saturated/unsaturated FA
			μg FA released		μg of measured Pi		
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
0 (control)	0	0	0	0	0	0	0
15	550 ± 2.5	700 ± 1.1	132.9 ± 0.9	159.5 ± 0.5	15.7 ± 2.6	17.0 ± 1.5	5.1 ± 0.5
30	900 ± 1.2	1150 ± 2.5	799.4 ± 1.2	1019.1 ± 1.3	77.8 ± 1.9	85.0 ± 1.2	7.1 ± 0.2
60	1350 ± 1.5	1650 ± 0.5	1503.8 ± 1.6	1954.9 ± 1.8	126.5 ± 1.3	163.4 ± 2.2	8.9 ± 0.8

[a] Mitochondrial swelling was measured spectrophotometrically and one unit of swelling is defined as a decrease in 0.01 absorbance/min of mitochondrial suspension at 520 nm by 100 nM of RVVA-PLA_2-I.

$C_{18:02\text{-}OH}$ and $C_{19:02\text{-}OH}$ from the intact erythrocyte membrane after 30 min of incubation. This result corroborates well with our previous report showing preferential release of unsaturated fatty acids from the erythrocyte membrane during the initial stage of attack by a PLA_2 (NK-PLA_2-I) from *N. kaouthia* venom [18]. However, after 120 min of incubation of erythrocytes with RVVA-PLA_2-I, additional saturated FAs of chain lengths $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{19:0}$, $C_{20:0}$, and $C_{22:0}$, and unsaturated fatty acid such as $C_{18:03}$ were detected. Nevertheless, a few saturated and unsaturated fatty acids ($>C_{20}$) liberated from the membrane due to action of RVVA-PLA_2-I could not be identified (Fig. 2). The TLC analysis of phospholipids and lysophospholipids released from the erythrocyte or mitochondrial membrane after the treatment with RVVA-PLA_2-I demonstrated the release of most of the FAs from the membrane PC pools (Supplementary Fig. S1).

A comparison of ratios of saturated/unsaturated fatty acids released from the intact mitochondrial membrane (Table 1) after the addition of RVVA-PLA_2-I suggested that the enzyme has a distinct preference for hydrolyzing phospholipids containing saturated fatty acids at *sn*-2 position in mitochondrial membrane. But in sharp contrast, during the initial phase of attack, this acidic PLA_2 prefers hydrolysis of unsaturated fatty acids in the erythrocyte membrane (Table 2). Therefore, the ratio of saturated/unsaturated fatty acids released from the erythrocyte membrane was less than 1.0 for up to 30 min of incubation with RVVA-PLA_2-I. Increase in incubation time beyond 30 min resulted in a change in RVVA-PLA_2-I induced fatty acids' release pattern from the erythrocyte membrane owing to a greater hydrolysis of phospholipids containing saturated fatty acids compared to unsaturated fatty acids at sn-2 position (Table 2).

3.2. Effect of inhibitors and antivenom on catalytic and membrane damaging activities

Different irreversible modifiers of serine residue viz. TPCK (inhibitor of chymotrypsin like serine protease), TLCK (inhibitor of trypsin-like serine protease) and PMSF (inhibitor of serine proteases) at 2.0 mM concentration did not affect the catalytic and membrane damaging

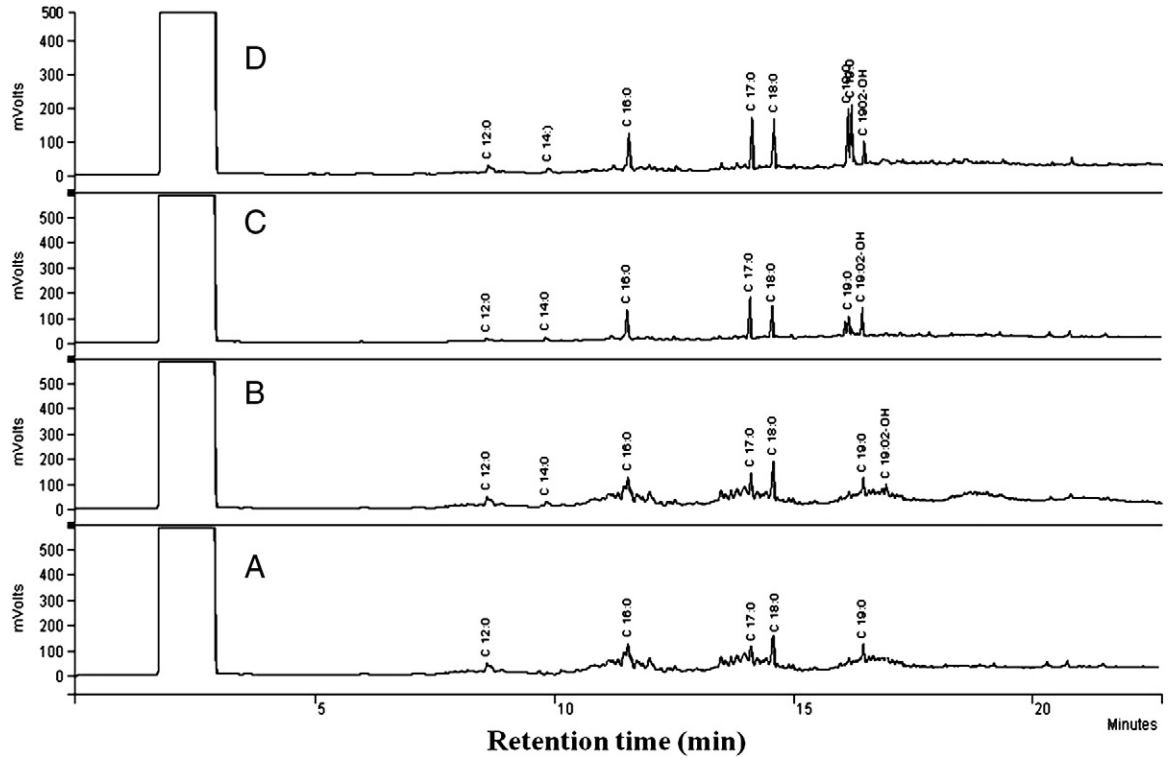

Fig 1. Kinetics of mitochondrial membrane phospholipid hydrolysis by RVVA-PLA_2-I. About 100 mg equivalents of liver mitochondria (mitochondria obtained from 100 mg wet weight of tissue) were incubated with 100 nM of RVVA-PLA_2-I (in the presence of 2 mM Ca^{2+}) at 37 °C for different time periods. The liberated fatty acids were analysed by GC–MS as described in the text. A) control, B) 15 min, C) 30 min, and D) 60 min after incubation of mitochondrial membranes with RVVA-PLA_2-I.

Table 2
RVVA-PLA_2-I induced haemolysis and phospholipid hydrolysis of goat washed erythrocytes. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVA-PLA_2-I at 37 °C and haemolysis (direct and indirect) and erythrocyte phospholipid hydrolysis were determined. Indirect phospholipid hydrolysis was achieved in the presence of egg yolk phospholipids (which is a source of PC). The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	% haemolysis		Phospholipid hydrolysis		Ratio of saturated/unsaturated FA
	Direct	Indirect	μg FA released	μg of measured Pi	
Control	0	0	0	0	0
15 min	0	0	202.5 ± 2.1	28.4 ± 0.9	0.68 ± 0.3
30 min	0	34.5 ± 0.45	1015.3 ± 2.5	163.2 ± 1.2	0.86 ± 0.4
60 min	0.6 ± 0.03	42.1 ± 0.45	2045.1 ± 1.4	253.2 ± 1.7	1.64 ± 0.8
120 min	1.9 ± 0.09	47.6 ± 0.12	2531.9 ± 1.9	302.3 ± 1.1	1.91 ± 0.1

activities of RVVA-PLA_2-I. Therefore, based on our experimental result we propose that serine residue perhaps does not have a contribution to the above mentioned activities of RVVA-PLA_2-I (Table 3). However, the modification of enzyme with pBPB, an inhibitor of histidine residue, drastically reduced the catalytic as well as the membrane damaging activities of RVVA-PLA_2-I indicating a correlation between catalytic and membrane damaging activities of this enzyme (Table 3). Furthermore, as shown in Table 3, EDTA significantly inhibited the catalytic and membrane phospholipid hydrolyzing activity of RVVA-PLA_2-I probably by chelating the Ca^{2+} ion required for the enzymatic activity [15]. The observed significant reduction (37–44%) in catalytic and membrane damaging activities of RVVA-PLA_2-I post-treatment with DTT under the experimental condition was due to partial reduction of disulfide bonds present in this PLA_2 molecule. Presence of 7–8 intra-molecular disulfide bridges has been reported in PLA_2 molecules from snake venom [5–8]. Furthermore, IAA (a sulfhydryl-reactive alkylating reagent used to modify the cysteine residue) at the tested concentrations inhibited the catalytic and membrane damaging activities of RVVA-PLA_2-I almost to the same extent which may be due to the modification of cysteine residues of this PLA_2 responsible for the intramolecular disulfide bond formation (Table 3).

3.3. Binding with phospholipids

The spectroflurometric study revealed the concentration dependent binding of PC with RVVA-PLA_2-I (Supplementary Fig. S2). Binding efficiency of the heat-inactivated RVVA-PLA_2-I had drastically reduced (69%) compared to the PC binding potency of native RVVA-PLA_2-I (Table 3 and Supplementary Fig. S3).In the present study, RVVA-PLA_2-I showed equal binding affinity for both mitochondrial and erythrocyte membranes (data not shown). The membrane binding property of heat-inactivated RVVA-PLA_2-I, as evaluated by the ELISA experiment, revealed the binding of about 65 ± 2% (mean ± S.D., n = 3) of heated RVVA-PLA_2-I (heated for 60 min at 75 °C) to intact erythrocyte or mitochondrial membranes as compared to 100% binding of native (unheated) RVVA-PLA_2-I. This suggested the partial loss of membrane binding property of heat-inactivated enzyme. In contrast, histidine-modified RVVA-PLA_2-I was captured by intact erythrocytes and mitochondria to the

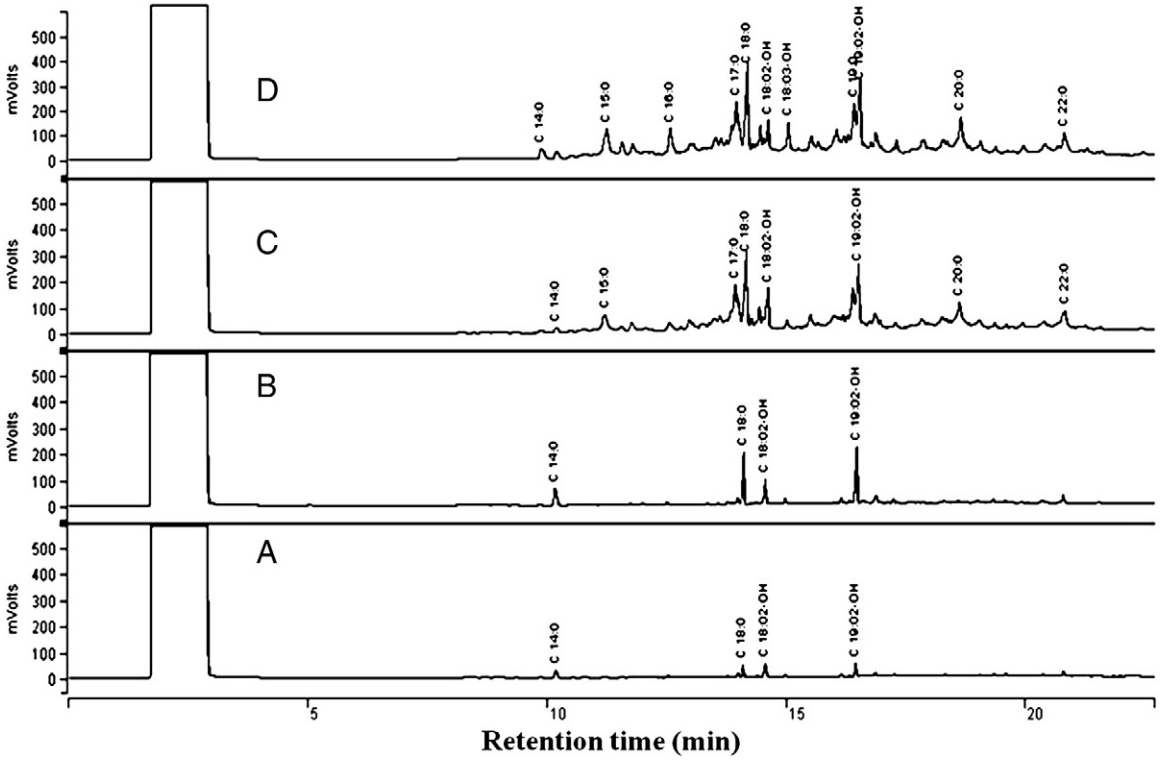

Fig 2. Kinetics of erythrocyte membrane phospholipid hydrolysis by RVVA-PLA_2-I. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVA-PLA_2-I (in the presence of 2 mM Ca^{2+}) at 37 °C for different time periods. The liberated fatty acids were analysed by GC–MS as described in the text. A) control, B) 30 min, C) 60 min, and D) 120 min after incubation of erythrocyte membranes with RVVA-PLA_2-I.

Table 3
Effects of heating, antivenom and inhibitors (at a final concentration of 2 mM) on catalytic and membrane phospholipid hydrolytic activities of RVVA-PLA_2-I (100 nM). Values are mean ± S. D. of triplicate determinations. MM: mitochondrial membrane, EM: erythrocyte membrane.

	% residual activity			Binding with PC (%)
	PLA_2	MM hydrolysis	EM hydrolysis	
Control	100	100	100	100
Chemicals/inhibitors	97.8 ± 1.9	96.0 ± 1.8	96.5 ± 4.7	–
TPCK	94.9 ± 1.7	97.2 ± 1.9	100	–
TLCK	9.7 ± 0.5	12.1 ± 0.8	18.5 ± 0.9	–
pBPB	97.6 ± 1.8	97.7 ± 1.8	95.2 ± 1.7	97.0 ± 1.3
PMSF	63.0 ± 3.1	55.5 ± 1.7	61.5 ± 1.7	–
DTT	56.7 ± 2.8	60.1 ± 2.0	56.6 ± 2.8	–
IAA	20.6 ± 1.5	23.2 ± 1.8	25.9 ± 2.1	–
EDTA				
Antigen: antivenom (w:w)				
1:100	90.1 ± 1.5	89.9 ± 0.5	85.8 ± 1.3	–
1:200	84.4 ± 1.2	76.8 ± 1.8	66.7 ± 2.3	–
1:500	75.4 ± 0.8	55.3 ± 1.7	51.6 ± 2.6	–
Heating at 75 °C				
10 min	97.0 ± 1.8	97.5 ± 0.8	95.9 ± 1.7	97.5 ± 1.8
20 min	94.5 ± 1.7	95.1 ± 1.7	91.8 ± 1.6	91.4 ± 1.6
30 min	90.5 ± 1.5	89.0 ± 0.5	88.5 ± 2.4	86.6 ± 2.3
45 min	85.4 ± 2.3	79.4 ± 1.9	74.4 ± 0.7	75.1 ± 0.8
60 min	70.8 ± 1.5	69.5 ± 1.4	67.2 ± 1.3	69.0 ± 1.4

same extent ($p > 0.05$) as that of the native RVA-PLA_2-I, implying that the modification of histidine residue(s) of RVVA-PLA_2-I did not affect its membrane binding property (Table 3).

3.4. Cytotoxicity assay

The cytotoxicity assay showed that the RVVA-PLA_2-I did not have any cytotoxic effect on HT-29 cells at the tested doses. RVVA-PLA_2-I at a dose of 10 μg ml^{-1} (final concentration of 172 nM) was not detrimental to HT-29 cells after 24 h of treatment (data not shown). In contrast, crude RVV at the same dose (10 μg ml^{-1}) killed more than 18% of cells after 4 h of treatment and almost all the HT-29 cells were dead after 24 h of treatment (data not shown). The inverted phase contrast microscopic observation of RVVA-PLA_2-I treated HT-29 cells did not reveal any gross morphological changes after 24 h of treatment (data not shown). Analysis of cell membrane phospholipid hydrolysis revealed that the RVVA-PLA_2-I failed to hydrolyze the membrane phospholipids of HT-29 cells after 4 h of treatment and about 350 ± 19 μg (mean ± S.D., n = 3) of FAs was released after 24 h of treatment. This suggests that the amount of phospholipids hydrolyzed by RVVA-PLA_2-I in treated HT-29 cells was significantly less ($p < 0.001$) as compared to the phospholipids hydrolyzed in the mitochondrial and erythrocyte membranes.

3.5. In-vivo toxicity assay

Mortality or behavioural changes were not observed in mice after 48 h i.p. injection of RVVA-PLA_2-I at a dose of 0.2 mg kg^{-1} body weight. However, RVVA-PLA_2-I prolonged the bleeding time and the coagulation time of PPP in RVVA-PLA_2-I treated mice as compared to the control mice (data not shown). After the treatment of mice with RVVA-PLA_2-I, no changes were observed in WBC count. Nevertheless, there was a significant decrease ($p < 0.01$) in the RBC count and the Hb content in the blood of treated mice as compared to the control mice (data not shown) suggesting RVVA-PLA_2-I caused lysis of erythrocytes in in-vivo condition.

Table 4 shows the effect of RVVA-PLA_2-I on serum parameters of treated mice. Administration of RVVA-PLA_2-I resulted in a significant increase ($p < 0.05$) in serum enzymes such as ALP, CPK-MB, SGOT, SGPT, and triglycerides. On the other hand, the serum level of LDH was significantly decreased in RVVA-PLA_2-I treated mice as compared to control. However, there was no change in the cholesterol and glucose levels in the serum of RVVA-PLA_2-I treated mice as compared to control mice (Table 4).

4. Discussion

4.1. Correlation between catalytic activity and membrane damaging activity of RVVA-PLA_2-I

Lysis of artificial membranes by PLA_2s from various snake venoms e.g., *Bothrops pirajai* and *Crotalus durissus terrificus* has been shown to be independent of their enzymatic activity, since both native and catalytically inactivated enzymes are fully able to disrupt membranes [19]. Conversely, we have presented contradictory evidences showing that mitochondrial and erythrocyte membrane hydrolyzing properties of Russell's viper acidic PLA_2 (RVVA-PLA_2-I) are dependent on its catalytic activity which is similar to membrane damaging activity of PLA_2 enzymes (NK-PLA_2-I and NK-PLA_2-II) isolated from *Naja kaouthia* venom [5]. The current investigation shows that the different membranes studied in the present investigation have different structural organisations as reflected by their distinctly different sensitivities towards RVVA-PLA_2-I. Nonetheless, this membrane damaging activity is dependent on Ca^{2+} ion because in the presence of the metal chelator EDTA, RVVA-PLA_2-I lost its membrane damaging as well as its catalytic activity in a parallel manner. The degree of membrane phospholipid hydrolysis increases with the increase in the pre-incubation time of the membranes

Table 4
Effect of RVVA-PLA_2-I on different parameters of serum of albino mice. RVVA-PLA_2-I (0.2 mg kg^{-1} body weight) dissolved in 0.1 ml of PBS was injected intraperitoneally into a group of six BALB/c mice (n = 6). Mice were sacrificed after 48 h of injection, blood was collected immediately by venipuncture and the serum was used for the assay of different parameters. Values are mean ± S.D. of six determinations.

	Total protein (g/L)	Glucose (g/L)	CPK-MB (U/L)	LDH (U/L)	ALP (U/L)	SGOT (U/L)	SGPT (U/L)	Cholesterol (g/L)	Triglycerides (g/L)
Control	270.0 ± 0.7	2.11 ± 1.2	27.5 ± 0.65	1281.5 ± 0.9	27.5 ± 0.8	144.0 ± 2.2	50.0 ± 0.91	0.7 ± 0.4	0.2 ± 0.04
Treated	180.5 ± 0.5[a]	2.53 ± 1.5	303.0 ± 1.6[a]	1054.5 ± 1.1[a]	79.3 ± 1.2[a]	191.7 ± 2.6[a]	135.0 ± 1.4[a]	0.8 ± 0.3	1.0 ± 0.05[a]

Significance of difference.
[a] $p < 0.001$.

with RVVA-PLA_2-I, documenting that the membrane damage is partially dependent on the catalytic activity of RVVA-PLA_2-I.

The indirect haemolysis exhibited by RVVA-PLA_2-I was due to the formation of phospholipid hydrolysis products like lysophospholipids and FAs from egg-yolk phospholipids/PC and these products caused further damage to the membrane [20]. The same hypothesis can be put forward to explain the enhanced susceptibility of the erythrocytes in the presence of egg yolk phospholipids/PC and Ca^{2+}, because amongst the tested phospholipids, PC is the most favoured substrate for RVVA-PLA_2-I for binding followed by hydrolysis [15]. The spectroflurometric study has also shown that the presence of Ca^{2+} enhanced the binding of RVVA-PLA_2-I with PC [15], confirming this is a Ca^{2+}-dependent PLA_2. Furthermore, inhibitor studies with specific amino acid modifying reagents, neutralization studies with polyvalent antivenom, and the heat-inactivation data in the present study have suggested a correlation between the catalytic and membrane phospholipid hydrolysis properties of RVVA-PLA_2-I. However, there is enough controversy regarding the role of enzymatic activity in the pharmacological effects of snake venom PLA_2-enzymes and it may be suggested that both enzymatic and non-enzymatic processes have contributed in the pharmacological process [5–8,15].

The heat-inactivation study also leads us to conclude that the binding of RVVA-PLA_2-I to membrane PC followed by its hydrolysis is an essential step for inducing membrane damage [5,8]; because, a parallel inhibition of membrane PC binding, catalytic activity as well as membrane damage in case of heat-inactivated RVVA-PLA_2-I was observed owing to the fact that heat treatment partially distorted PLA_2 structure (Supplementary Fig. S3). This partial unfolding of RVVA-PLA_2-I could not be regained after cooling (Saikia, D. and Mukherjee, A. K., unpublished observation) resulting in a reduction in PC binding property and activity of heat-denatured RVVA-PLA_2-I.

4.2. Differential hydrolysis of mitochondrial and erythrocyte membrane phospholipids: does it reflect the differences in PLA_2-sensitive phospholipid composition of biomembranes?

Membrane surface properties, including membrane fluidity, curvature, surface charge, and membrane-induced structural changes in the enzyme, determine the strength of interaction, cooperatively of membrane binding, and the extent of PLA_2 activation [5,8]. Snake venom PLA_2 induced injury to mitochondria brings about a change in the mitochondrial volume [5,21]. The preferential release of C_{16} to C_{18} FAs from the mitochondrial and erythrocyte membranes during the initial phase of attack by RVVA-PLA_2-I is correlated with its preference for short chain fatty acids at the *sn-2* position. It is presumed that even if PLA_2 binds uniformly across the solid and fluid domains of membrane enriched in the long and short chain fatty acids respectively, enzyme shows a preference for short chain fatty acids, which are in fluid phase [22]. This is due to the lower surface area density of the lipid head groups in short chain fatty acids that are present in fluid phase [23].

Our previous study has shown that RVVA-PLA_2-I preferentially hydrolyzes PC over PS and PE [15]. It is very interesting to observe that RVVA-PLA_2-I preferentially hydrolyzes the phospholipids of erythrocyte membrane compared to mitochondrial membrane even though the latter possesses much greater percentage of PC on its outer leaflet (40.9%) than the outer leaflet of the former membrane (19%) [24]. Therefore, our result suggests that existence of significantly greater number of RVVA-PLA_2-I sensitive regions in the erythrocyte membrane as compared to the mitochondrial membrane might have a relevance to the higher degree of phospholipid hydrolysis of the former membrane [5,8]. These specific domains or venom PLA_2 susceptible region(s) may result from the presence of different fatty acids, more particularly the short-chain fatty acids in these regions of the membrane. Therefore, the presence of a large number of venom PLA_2-sensitive phospholipid compositions, rather than only PC content of that particular membrane, may determine the extent of membrane damage induced by a particular PLA_2 [8].

Furthermore, RVVA-PLA_2-I could hydrolyze the erythrocyte membrane phospholipids within 15 min of incubation which is in sharp contrast to our previous observation where two phospholipase A_2 enzymes viz. NK-PLA_2-I and NK-PLA_2-II from *N. kaouthia* venom showed a lag phase for binding with the erythrocyte membrane and subsequent membrane hydrolysis [5]. Besides, *N. kaouthia* PLA_2 enzymes preferentially hydrolyze the membrane phospholipids of the mitochondria as compared to that of the erythrocyte membranes [5]. It is interesting to note that all these three PLA_2s from snake venom have shown a preference for hydrolyzing PC over PS or PE [5,13]; however, NK-PLA_2-I and NK-PLA_2-II from *N. kaouthia* venom are basic in nature [5] whereas RVVA-PLA_2-I from *D. russelli* venom is an acidic PLA_2 enzyme. Therefore, the differences in overall net charge in a venom PLA_2 molecule may attribute to differential binding and subsequent hydrolysis of phospholipids of a particular membrane [12].

Evidence has been accumulating regarding the presence of nanometer range small-scale structures and lipid domains in the lipid bilayer and such organizational heterogeneity of lipid microdomains may have structural and functional significance [25–27]. The phospholipid-binding domains with secondary binding sites for specific proteins are well known, and this binding is often tightly regulated [28]. The differences in the membrane phospholipid hydrolysis by a venom PLA_2 can be supported by our previous observation that the phospholipid constituents of microsomal membranes are less hydrolyzed as compared to those of lysosomal membranes by the action of *Vipera russelli* venom basic PLA_2 [13,14]. Further, it has been suggested that PLA_2 may be particularly active at domain interfaces which are the sites of structural defects and hence good points of attack for these enzymes [5]. The mechanism by which snake venom phospholipase A_2 sensitive phospholipid domains is formed in the mitochondrial or erythrocyte membrane is not very clear. Although no adequate description of the nature of such membrane lipid domains in terms of their abundance, composition or dynamics has been provided; however, it may be assumed that the physicochemical properties of individual phospholipids, circumstantial effects of membrane proteins, phospholipid/cholesterol ratio, and vitamin E content of the membrane contribute significantly to the formation of such domains [5,8,14,27].

The difference in the release of saturated and unsaturated fatty acids from the membranes may be explained by the fact that in the case of mitochondrial membrane, RVVA-PLA_2-I preferentially binds to one of the PLA_2-sensitive domains of the membrane resulting in quantitative increase in the release of fatty acids from that membrane with respect to time [5]. In a contrast, RVVA-PLA_2-I attacks different phospholipid domains/region(s) of erythrocyte membrane and therefore, this kinetics of erythrocyte membrane phospholipid hydrolysis favours the release of quantitatively as well as qualitatively different FAs with respect to time. The ratio of saturated/unsaturated fatty acids released from the erythrocyte membrane may lead us to assume that during the initial phase of attack, RVVA-PLA_2-I hydrolyzes a particular domain of erythrocyte membrane consisting of mostly the unsaturated fatty acids. Slowly with an increase in time, the reaction products build up which may help in PLA_2 binding and in attacking another domain of the same membrane enriched in more saturated fatty acids rather than unsaturated fatty acids. Interestingly, this result contradicts with the finding of Shukla and Hanahan [12] showing the acidic PLA_2 purified from the venom of *Agkistrodon halys blomhoffi* hydrolyzing only one domain of PC in intact erythrocytes. These differences in erythrocyte membrane phospholipid hydrolysis pattern of acidic PLA_2s from two different snake venoms lead us to conclude that not only the overall acidic charge, but also the charge at a particular region, known as interfacial binding surface (IBS) of PLA_2 may determine its competence for binding with different domains of a membrane [29]. This reinforces the presence of venom PLA_2-specific sensitive regions in a particular membrane. It may be presumed that

by virtue of possessing arrays of phospholipase A_2 isoenzymes, injected venom can induce greater damage to membranes and subsequent toxicity to the cells.

4.3. Why does RVVA-PLA_2-I fail to hydrolyze HT-29 cell membrane phospholipids?

The RVVA-PLA_2-I at the tested dose (10 μg ml^{-1}) did not show any cytotoxicity against HT29 colon adenocarcinoma cells even though PC is the most abundant phospholipids present in the outer cell membrane of HT-29 cells [30]. Lomonte et al. [31] have shown the cytotoxic effect of Myotoxin II from *Bothrops asper* on HT-29 cells at a dose of 50–100 μg ml^{-1} (micromolar range) whereas we have examined the cytotoxicity at a dose of 10 μg ml^{-1} (nanomolar concentration) to avoid any non-specific binding of PLA_2 to cultured cells [5]. Furthermore, considering the proportion of RVVA-PLA_2-I present in RVV, we did not perform cytotoxicity at a very high dose of this PLA_2. Amongst the tested membranes, the least hydrolysis of HT-29 cells can again be explained on the basis that it is not the overall quantity of PC in a membrane but either the availability of PC in a PLA_2-sensitive membrane and/or physicochemical properties of a membrane are the most important criteria in order to elicit the RVVA-PLA_2-I-induced membrane damage [8]. It has been observed that some of the components of the membrane such as cholesterol/phospholipid ratio and vitamin E (α-tocopherol) content may influence its fluidity which in turn modulates the activity of venom PLA_2 enzymes [14,32].

4.4. The RVVA-PLA_2-I is non-lethal but affects the liver and cardiac tissues of experimental animals

RVVA-PLA_2-I (at a dose of 0.2 mg kg^{-1} i.p.) does not show any mortality to the mice even after 48 h of injection suggesting that it is devoid of any lethal effects in mice. The RVVA-PLA_2-I represents 0.1% (w/w) of total RVV protein [13] and on average an adult Russell's viper may inject 225–250 mg of venom (total amount in a bite) to its victim (personal communication from Mr. D. Mitra, in-charge, Calcutta Snake Park, Kolkata). Therefore, a maximum of 0.2 to 0.25 mg of RVVA-PLA_2-I would be injected in a victim by the bite of an adult Russell's viper and this dose corresponds to the nanomolar concentration of RVVA-PLA_2-I in the blood of an adult victim. Since RVVA-PLA_2-I was found to be non-toxic to mice at a much higher dose than this, therefore, it may be suggested that the PLA_2 under study does not contribute to lethality of RVV. It is noteworthy to mention that non-toxic PLA_2 enzymes from snake venom demonstrating phospholipid hydrolysis activity have been reported and the exact contribution of these PLA_2s in snake venom is poorly understood [16,33]. However, evidence has been presented from our laboratory to show that non-covalent interaction of relatively non-toxic PLA_2s from *N. kaouthia* venom with that of weak neurotoxin like molecules (kaouthitoxins) from the same venom resulted in marked synergism to potentiate the cytotoxicity of PLA_2–kaouthiotoxin complex without altering the biological properties of PLA_2 enzymes [19]. The proteome analysis of *D. russelli siamensis* venom revealed the presence of complex of PLA_2 with other unidentified venom proteins [34]. Presence of such PLA_2-interacting component(s) from *D. russelli russelli* venom remains to be elucidated.

Increase in the level of most of the tested enzymes in the serum of RVVA-PLA_2-I treated mice suggests that it might be affecting different organs, particularly the liver and cardiac tissues of mice. Every living cell contains its complement of enzymes, most of which are intracellular and are released into the circulation only after breakdown of the cell or impairment of its membrane post-envenomation [2]. Elevated CPK-MB level suggested damage to the skeletal muscle and is indicative of myonecrosis, whereas increased SGOT, SGPT and ALP levels in the serum of treated mice indicated damage to the liver tissues reinforcing RVVA-PLA_2-I targets cardiac and hepatic tissues.

5. Conclusion

In conclusion, RVVA-PLA_2-I preferentially hydrolyzed phospholipids of erythrocyte membranes compared to liver mitochondrial membranes. Interestingly, this PLA_2 could not hydrolyze HT-29 colon adenocarcinoma cell membrane phospholipids after 24 h of treatment suggesting a differential mode of attack on membrane phospholipids by RVVA-PLA_2-I. The GC analysis of saturated/unsaturated fatty acid release pattern from intact mitochondrial and erythrocyte membranes after the addition of RVVA-PLA_2-I suggested the existence of a significantly greater number of RVVA-PLA_2-I sensitive domains in the erythrocyte membrane as compared to the mitochondrial membrane. Although, the exact nature of the membrane domain(s) responsible for binding with this acidic PLA_2 from Russell's viper could not be identified; however, our study has provided enough evidences in support of membrane domain hypothesis. Further studies to identify the nature of these membrane domains are in progress.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.08.005.

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