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Two Acidic, Anticoagulant PLA$_2$ Isoenzymes Purified from the Venom of Monocled Cobra *Naja kaouthia* Exhibit Different Potency to Inhibit Thrombin and Factor Xa via Phospholipids Independent, Non-Enzymatic Mechanism

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Abstract

Background: The monocled cobra (*Naja kaouthia*) is responsible for snakebite fatality in Indian subcontinent and in southwestern China. Phospholipase A$_2$ (PLA$_2$; EC 3.1.1.4) is one of the toxic components of snake venom. The present study explores the mechanism and rationale(s) for the differences in anticoagulant potency of two acidic PLA$_2$ isoenzymes, Nk-PLA$_2\alpha$ (13463.91 Da) and Nk-PLA$_2\beta$ (13282.38 Da) purified from the venom of *N. kaouthia*.

Principal Findings: By LC-MS/MS analysis, these PLA$_2$s showed highest similarity (23.5% sequence coverage) with PLA$_2$ III isolated from monocled cobra venom. The catalytic activity of Nk-PLA$_2\beta$ exceeds that of Nk-PLA$_2\alpha$. Heparin differentially regulated the catalytic and anticoagulant activities of these Nk-PLA$_2$ isoenzymes. The anticoagulant potency of Nk-PLA$_2\alpha$ was comparable to commercial anticoagulants warfarin, and heparin/antithrombin-III albeit Nk-PLA$_2\beta$ demonstrated highest anticoagulant activity. The anticoagulant action of these PLA$_2$s was partially contributed by a small but specific hydrolysis of plasma phospholipids. The strong anticoagulant effect of Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$ was achieved via preferential, non-enzymatic inhibition of FXa ($Ki = 43$ nM) and thrombin ($Ki = 8.3$ nM), respectively. Kinetics study suggests that the Nk-PLA$_2$ isoenzymes inhibit their "pharmacological target(s)" by uncompetitive mechanism without the requirement of phospholipids/Ca^{2+}. The anticoagulant potency of Nk-PLA$_2\beta$ which is higher than that of Nk-PLA$_2\alpha$ is corroborated by its superior catalytic activity, its higher capacity for binding to phosphatidylcholine, and its greater strength of thrombin inhibition. These PLA$_2$ isoenzymes thus have evolved to affect haemostasis by different mechanisms. The Nk-PLA$_2\beta$ partially inhibited the thrombin-induced aggregation of mammalian platelets suggesting its therapeutic application in the prevention of unwanted clot formation.

Conclusion/Significance: In order to develop peptide-based superior anticoagulant therapeutics, future application of Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$ for the treatment and/or prevention of cardiovascular disorders are proposed.

Introduction

The Indian monocled cobra (*Naja kaouthia*) is one of the major venomous snakes of the Elapidae family. This species of snake is responsible for major snakebite fatality in India, Bangladesh, Nepal, Myanmar, and south-western China [1]. Snakebite remains a neglected tropical disease which requires immediate medical attention. The venom of *N. kaouthia* is enriched with various enzymes and non-enzymatic protein/peptide toxins [1]. Among the snake venom enzymes, PLA$_2$s, because they have a crucial role in inducing various pharmacological effects on snakebite victims, are an interesting group of proteins [2,3].

Snake venom PLA$_2$ enzymes exhibit a wide variety of pharmacological effects such as neurotoxicity, cardiotoxicity, cytotoxicity, membrane damaging activity, myotoxicity, necrotic, anticoagulant, hemolytic, haemorrhage and edema induction by various mechanisms of action [2–11]. The structure-function relationship and the mechanism of action of this group of small proteins are still subtle, complex and thus impose intriguing challenges to the toxinologists [2,12].

The systemic neuromuscular blockade and other clinical signs of *N. kaouthia* envenomation have been described. However, the mechanism(s) of anticoagulant action of snake venom PLA$_2$ enzymes still remains incomprehensible and a detailed molecular

mechanism of anticoagulant action of PLA$_2$ enzymes from *N. kaouthia* venom has never been explored. Our previous studies have shown the biochemical and pharmacological characterization as well as membrane damaging activities of major PLA$_2$ isoenzymes purified from the venom of *N. kaouthia* of different geographical origins [5–7]. These Nk-PLA$_2$s were found to prolong the Ca-clotting time of citrated platelet poor plasma [7]; however, their mechanism of anticoagulant action was not explored. Snake venom PLA$_2$ enzymes may prolong the blood coagulation in victims by different ways; either by enzymatic hydrolysis of plasma phospholipids which is required to initiate the coagulation process and/or binding with essential blood coagulation factors through a non-enzymatic mechanism [2–3,8–9]. Studies on the mechanism of such anticoagulants will advance our understanding of 'vulnerable' sites in the coagulation cascade [12]. This study may further help us to design novel strategies towards developing anticoagulant therapeutics for treating cardiovascular disorders (CVDs) and also new functional diagnostic test kits in the field of haemostasis [2,10,13].

In the present study, we report the purification and the mechanism of anticoagulant action of two major PLA$_2$ enzymes purified from the venom of *N. kaouthia* of eastern India origin.

Materials and Methods

The crude *N. kaouthia* venom sample (pooled) was obtained from authorized, licensed venom dealer Mr. D. Mitra, Calcutta Snake Park, Kolkata [5,6,8]. Lyophilized monovalent antivenom (against *N. kaouthia* venom) was obtained from Vins Bioproducts Limited, India (batch no: 30AS11001; expiry date: 04/2015). All other chemicals used were of analytical grade and were procured from Sigma-Aldrich, USA.

Purification of two PLA$_2$ isoenzymes

All the fractionation steps were carried out at 4°C. Twenty five mg (dried weight) of *N. kaouthia* crude venom, dissolved in 0.5 ml of 20 mM Tris-HCl, pH 7.4, was injected to a FPLC-HiPrep CM FF16/10 column (20 ml) pre-equilibrated with 20 mM Tris-HCl, pH 7.4 (buffer A). The column was coupled to a Fast Protein Liquid Chromatography (FPLC) system (Akta Purifier 10, Wipro GE Healthcare). After washing the column with 30 ml (1.5 CV) of equilibration buffer, the bound proteins were eluted from 0 to 50% linear gradient of buffer B (20 mM Tris-HCl, pH 7.4 containing 1M NaCl) for 100 min at a flow rate of 1.0 ml/min. One ml fraction was collected in each tube. The elution of protein was monitored at 280 nm and the peaks were then screened for protein content [14], PLA$_2$ (see below), and plasma clotting activities (see below).

The cation-exchange unbound fractions showing maximum anticoagulant as well as PLA$_2$ activities were pooled, desalted, lyophilized, dissolved in 0.5 ml of 20 mM Tris-HCl, pH 7.4 and was then applied to an anion exchange FPLC Hiprep DEAE FF16/10 column (20 ml) pre-equilibrated with the above buffer. After washing the column with 30 ml of equilibration buffer (1.5 CV), bound proteins were eluted from 0 to 50% linear gradient of buffer B (20 mM Tris-HCl, pH 7.4 containing 1M NaCl) for 100 min at a flow rate of 1.0 ml/min. One ml fraction was collected in each tube. The elution of protein was monitored at 280 nm, and the peaks were then screened for protein content [14], PLA$_2$, and plasma clotting activities. The purity and molecular mass of 1.0 µg protein of pooled fractions showing high PLA$_2$ and anticoagulant activities were determined by MALDI-TOF-MS (4800 Plus MDS SCIEX, Applied Biosystems) as described previously [3,15].

Protein identification by LC-MS/MS

Twenty five microgram of the lyophilized sample after reduction and alkylation was subjected to in-solution digestion with proteomics grade trypsin (Promega) for overnight at 37°C [15]. The digested peptides were reconstituted in 15 µl of the 0.1% (v/v) formic acid and were subjected to RP-HPLC-MS/MS analysis with collision induced dissociation as the fragmentation method. The data so generated was searched for the identity of tryptic peptides using MASCOT 2.4 search engine on a Proteome discoverer 1.4 platform. The data was searched against Uniprot Swiss-Prot database (non redundant database with reviewed proteins) and Uniprot TrEMBL (database with unreviewed proteins) database against the taxid snake from NCBI. Minimum of two high confident peptides was used as a prerequisite to identify the protein(s) of interest.

PLA$_2$ activity assay

The PLA$_2$ activity was assayed as described previously [5,8] by using egg-yolk phospholipids as substrate. One unit of PLA$_2$ activity has been defined as the amount of protein which produces a decrease in 0.01 absorbance after 10 min at 740 nm. The substrate specificity of purified PLA$_2$ was determined by using different commercially available phospholipids (1 mM) viz. phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE). The enzyme activity was assayed by titrametric method with palmitic acid as a free fatty acid standard [6,8]. The PLA$_2$ activity is expressed as ng of fatty acid released/min/mg of protein.

Anticoagulant activity assay

Platelet poor plasma (PPP) was used for anticoagulant activity assay [3,8]. One unit of anticoagulant activity has been arbitrarily defined as one second increase in clotting of PLA$_2$-treated PPP compared to the clotting time of control PPP [5,8]. To determine the dose-dependent anticoagulant activity different amounts (0.1 to 1.0 µg) of Nk-PLA$_2$ were incubated with PPP (300 µl) for 3 min at 37°C followed by an addition of 40 µl of 250 mM CaCl$_2$ in order to initiate the clot formation. As a positive control, the anticoagulant activity of heparin and warfarin was also determined under identical experimental conditions. The effect of pre-incubation on anticoagulant activity was determined by pre-incubating 300 µl PPP with 0.5 µg PLA$_2$ for 5–30 min at 37°C and then the anticoagulant activity was determined as stated above.

To determine the release of PLA$_2$-induced free fatty acids from PPP, 0.5 µg PLA$_2$ (dissolved in 20 mM Tris-HCl, pH 7.4) was pre-incubated with PPP (300 µl) for 3–30 min at 37°C, and thus the liberated fatty acids were extracted and micro titrated by using 0.01 N NaOH and phenolphthalein red as indicator [8]. A control (PPP treated with buffer) was also run in parallel. The fatty acids liberated from the PPP (treated with PLA$_2$ or buffer) were extracted and quantified as described by Doley et al. [6].

Thrombin and Factor Xa inhibition assay

To observe the effect of purified PLA$_2$ isoenzymes on amidolytic activity of thrombin or FXa, purified PLA$_2$ (1.0 µg in 20 mM Tris-HCl, pH 7.4) was pre-incubated with thrombin (0.03 NIH U/ml) or FXa (0.15 µM) for 30 min at 37°C and then by assaying the activity of these coagulation factors against their chromogenic substrates (0.2 mM) T1637 and F3301, respectively [3,8]. The release of p-nitroaniline (pNA) was monitored for 15 min at intervals of 1 min at 405 nm in a plate reader (Multiskan^{TM} GO, Thermo Scientific, USA). For every experiment, a control was run

in parallel. The activity of thrombin or FXa towards their substrates was considered 100% activity and other values were compared with this.

The dose-dependent (0–0.2 μg) and time-dependent (5–30 min) antithrombin activity of purified PLA$_2$ against fibrinogen (7.5 μM), which is the physiological substrate for thrombin, was also determined as described previously [3]. In another set of experiments, the FXa inhibition assay was performed by pre-incubating 20 nM FXa with 1.0 μg of PLA$_2$ for 60 min at 37°C and then the reaction was initiated by adding prothrombin (1.4 μM) to this mixture. The prothrombin activation was determined by formation of thrombin using its chromogenic substrate T1637 [8]. Thrombin formation was also determined by 15% SDS-PAGE analysis of prothrombin activation products after 1 h incubation of prothrombin (10.0 μg) with 20 nM FXa (pre-treated with PLA$_2$ or buffer) at 37°C, pH 7.4. The formation of thrombin was confirmed by the peptide mass fingerprinting analysis of ~36 kDa protein. For each experiment, a control was also run in parallel where buffer instead of PLA$_2$ was added.

Determination of inhibitory constant (K$_i$) for the inhibition of FXa and thrombin

For determining the inhibitory constant (*K*i value) of Nk-PLA$_2$α on amidolytic activity of FXa, a fixed concentration of FXa (20 nM) was pre-incubated with two different concentrations of Nk-PLA$_2$α (50.0 nM and 100.0 nM) in a 96-well microplate at 37°C for 60 min. A control was run in parallel where FXa was incubated with buffer (20 mM Tris-HCl, pH 7.4) under identical experimental conditions. Then graded concentrations (0.1–0.8 mM) of chromogenic substrate for factor Xa (F3301) were added and the final volume of reaction was adjusted to 100 μl with 20 mM Tris-HCl, pH 7.4. After incubation for 10 min, the release of pNA was determined at 405 nM. For kinetic analysis, the reaction rate (V) was plotted against substrate concentrations (S) at each inhibitor concentration and the data was fitted to a hyperbolic Michaelis-Menten model using GraphPad Prism 5.0 software. The inhibitory constant (*K*i) was determined by using the uncompetitive model (shown below) for enzyme inhibition by using the same software.

$$VApp = \frac{V\max}{1 + \frac{I}{Ki}} \qquad (1)$$

$$KmApp = \frac{Km}{1 + \frac{I}{Ki}} \qquad (2)$$

$$V = \frac{VApp \times S}{KmApp + S} \qquad (3)$$

In the above equations (1) and (2), I indicates the inhibitor concentration; *VApp* and *Vmax* represent maximum velocity in the presence and absence of the inhibitor, respectively. The *KmApp* and *Km* in equation 2 denote the Michaelis constant in presence and absence of inhibitor, respectively.

The *K*i value for inhibition of amidolytic activity of thrombin by Nk-PLA$_2$β was determined by pre-incubating a fixed concentration of thrombin (0.03 NIH U/ml) with two different concentrations of Nk-PLA$_2$β (50.0 nM and 100.0 nM) at 37°C, pH 7.4 for

30 min. In control, thrombin was incubated with buffer (20 mM Tris-HCl, pH 7.4) instead of Nk-PLA$_2$β under identical experimental conditions. The reaction was initiated by adding graded concentrations (0.1–0.8 mM) of chromogenic substrate for thrombin (T1637) in a final reaction volume of 100 μl adjusted with 20 mM Tris-HCl, pH 7.4. After incubation for 10 min at 37°C, the release of pNA was determined at 405 nM in a microplate reader. The kinetics analysis was done by using the GraphPad Prism 5.0 software and the *Ki* value was determined as stated above. The experiments were repeated three times to assure the reproducibility.

Spectrofluorometric assay of interaction of PLA$_2$ with thrombin/FXa and PC

To study the protein-protein interaction, thrombin/FXa was pre-incubated with NK-PLA$_2$ (1:10 molar ratio) for 20 min at room temperature and the fluorescence spectra were acquired as described previously [3,8,10]. As a control, the fluorescence spectra of individual proteins were also determined. For determination of interaction of Nk-PLA$_2$ with PC in presence of 0.5 mM EDTA (to prevent the PC hydrolysis), our previously described procedure was followed [3]. The above experiments were repeated three times to assure the reproducibility.

Platelet aggregation inhibition assay

For preparation of platelet rich plasma, citrated blood (1:9, v/v) was centrifuged at 200× g for 20 min. The *in vitro* platelet aggregation inhibition property was investigated by pre-incubating different concentrations (0.5 to 2.0 μg) of purified PLA$_2$ with thrombin (1 NIH U/ml) for 30 min at 37°C followed by induction of platelet aggregation. A control reaction was also set up where instead of purified Nk-PLA$_2$, thrombin (1 NIH U/ml) was incubated with 1× PBS (20 mM K-phosphate, 150 mM NaCl, pH 7.4) at 37°C. The platelet aggregation was monitored in a Chrono-log dual channel aggregometer for 10 min. The experiment was repeated three times to assure the reproducibility.

Effect of histidine modifier, heparin and commercial antivenom on enzymatic and anticoagulant activities

The PLA$_2$ inhibitor (histidine modifier) p-bromophenacyl bromide (5 mM), heparin (1 IU) or graded amounts of monovalent antivenom (against *N. kaouthia* venom) were incubated with a fixed concentration of PLA$_2$ (1.0 μg/ml) at 37°C for 30 min. The catalytic, anticoagulant, and thrombin/FXa inhibitory activity of PLA$_2$ was then assayed as stated above. The activity of PLA$_2$ in presence of its inhibitor/antivenom was thus compared with control (without inhibitor/antivenom).

Statistical analysis

Results were presented as mean ± standard deviation (S.D.) of three experiments. Statistical analysis of the data was performed using a Student's t test in Sigma Plot 11 for Windows (version 7.0). Values of p≤0.05 were considered significant.

Results

Isolation and purification of two PLA$_2$ isoenzymes from *N. kaouthia* venom

Fractionation of crude *N. kaouthia* venom through Hiprep CM FF 16/10 column separated the proteins into 6 peaks, Nk(H)CEXP1 to Nk(H)CEXP6 (Fig. 1a).The peak 1 [Nk(H)CEXP1] eluted with the equilibration buffer showed a significant PLA$_2$ as well as anticoagulant activity. By re-

fractionation of this peak through Hiprep DEAE FF16/10 column, the proteins were separated into 8 fractions (Fig. 1b). The peak Nk(H)AEXP4 and Nk(H)AEXP5 demonstrated significant PLA$_2$ as well as anticoagulant activity. The proteins of these peaks were found to be homogenous through MALDI-TOF-MS analysis with molecular mass of 13463.91 Da and 13282.38 Da, respectively (Figs. 1c–d). A summary of purification of these purified anticoagulant PLA$_2$s, named Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$ is shown in Table 1.

LC-MS/MS identification of proteins

By LC-MS/MS analysis, both the PLA$_2$s under study showed highest similarity (score 463) with PLA$_2$ (EC 3.1.1.4) III isolated from monocled cobra venom (accession no. 2144440) with 23.5% sequence coverage. Furthermore, the PLA$_2$s under study also demonstrated similarity (18-15% sequence coverage) with PLA$_2$s isolated from *Naja naja* (accession no. 395192), anticoagulant PLA$_2$ from *N. n. sagittifera* (accession no. 66361231), and an acidic PLA$_2$ natratoxin from *N. atra* (accession no. 209573225) venom samples. Besides, both these PLA$_2$s were found to possess identical sites for trypsin cleavage and therefore, they demonstrated similar trypic fragmentation pattern. The NCBI BLASTP search of one of the tryptic peptides GGSGTPVDDLDR (Mr. 1188.5 Da) showed putative conserved domains of PLA$_2$-like superfamily. Another tryptic peptide sequence NMIQCTVPSR (Mr. 1148.5 Da) demonstrated 100% sequence similarity with an acidic PLA$_2$ enzymes natratoxin (accession no. A4S04) isolated from the Chinese cobra *N. atra* venom. Furthermore, another tryptic peptide sequence viz. NLYQFK (Mr. 812.4 Da) was found

to contain the N-terminal sequence of both these PLA$_2$ isoezymes purified from *N. kaouthia* venom.

Substrate specificity, anticoagulant activity and inhibition of platelet aggregation

Both NK-PLA$_2$s showed preferential hydrolysis of PC over PS or PE (data not shown), which is similar to the phospholipid hydrolysis pattern of PLA$_2$s, which we previously isolated from the venom of *N. kaouthia* [5–7]. The specific activity of Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$ towards PC was determined as 2.3×10^5 and 4.5×10^5 units/mg, respectively. Both enzymes displayed optimum activity at the pH range of 8.0–9.0 and at 37–45°C (data not shown). Like other snake venom PLA$_2$ enzymes [6–8], heating Nk-PLA$_2\alpha$ or Nk-PLA$_2\beta$ at 75°C for 30 min did not result in any significant decrease in catalytic activity of heated enzyme as compared to control (unheated) enzyme (data not shown).

A comparison of anticoagulant activity of the purified PLA$_2$s with commercial anticoagulants heparin and warfarin is shown in Fig. 2a. Both these PLA$_2$s increased the Ca-clotting time of PPP in a dose-dependent manner, however to a significantly different extent (Fig. 2a). The anticoagulant activity of Nk-PLA$_2\beta$ was found to be significantly higher ($p<0.05$) compared to Nk-PLA$_2\alpha$, heparin or warfarin under identical experimental conditions. It is worthy of mention that heparin shows anticoagulant effect by binding with antithrombin-III (AT-III) present in PPP, which in turn inhibits thrombin and activated factor X [16]. Pre-incubation of Nk-PLA$_2\alpha$ (0.2 μg) and Nk-PLA$_2\beta$ (0.2 μg) at a ratio of 1:1 (w:w) for 20 min at room temperature prior to the addition of PPP did not allow the plasma to clot within 10 min. Conversely, the
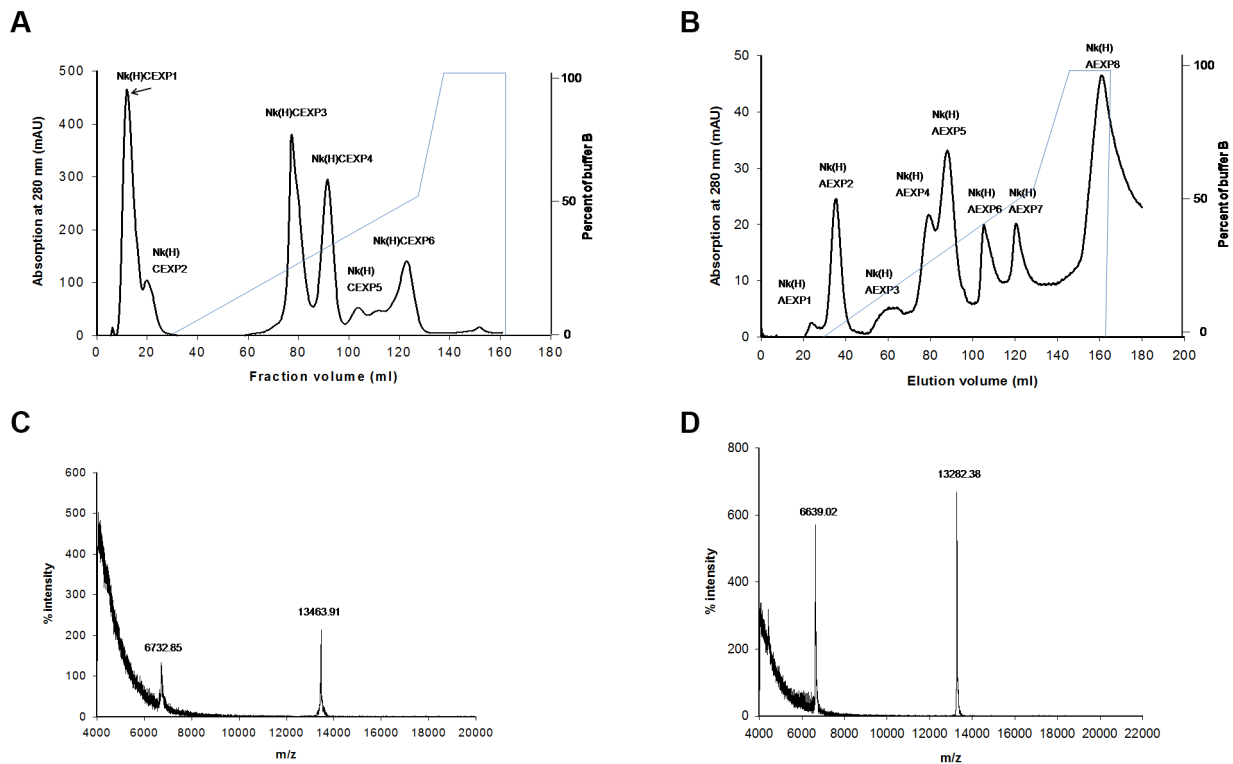

Figure 1. Purification and determination of molecular masses of PLA$_2$ isoenzymes isolated from *N. kaouthia* venom. (a) Fractionation of crude *N. kaouthia* venom (25 mg dry weight) done on a HiPrep CM FF16/10 FPLC cation-exchange column. The fractionation conditions are described in the text. The peak showing major PLA$_2$ and anticoagulant activity is marked with an arrow. (b) Chromatogram resulting from anion-exchange fractionation of cation-exchange unbound peak [Nk(H)CEXP1] by using a Hiprep DEAE FF16/10 FPLC column. (c) and (d) MALDI-TOF mass spectrum of Nk-PLA$_2\alpha$ [peak Nk(H)AEXP4], and Nk-PLA$_2\beta$ [peak Nk(H)AEXP5], respectively.
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Table 1. A summary of the purification of PLA_2 isoenzymes from the venom of *N. kaouthia*.

Fraction	Total protein (mg)	Yield of protein (%)	PLA$_2$ activity		Purification
			Total activity	**Specific activity**	**(fold)**
			(Units)[a]	**(Units/mg)**	
Crude venom	16.1	100	24.2×10^4	1.5×10^4	1.0
Nk(H)CEXP1	1.5	9.3	2.6×10^4	1.7×10^4	1.1
Nk(H)AEXP4	0.1	0.6	0.4×10^4	4.1×10^4	2.7
(NK-PLA$_2\alpha$)					
Nk(H)AEXP5	0.2	1.2	1.1×10^4	5.4×10^4	3.6
(NK-PLA$_2\beta$)					

The PLA$_2$ activity was assayed by egg-yolk phospholipids hydrolysis method using 0.1 M Tris-HCl, pH 8.0 buffer. Data are from a typical experiment.
[a]One unit of PLA$_2$ activity is defined as decrease of 0.01 absorbance at 740 nm per 10 min at 25°C.
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anticoagulant activity of individual Nk-PLA$_2\alpha$ or Nk-PLA$_2\beta$ under identical experimental conditions was found to be much lower (Fig. 2a).

Increasing of the pre-incubation of PPP with Nk-PLA$_2\alpha$ or Nk-PLA$_2\beta$ for 0–5 min before addition of CaCl$_2$ enhanced the anticoagulant potency of both the enzymes (Fig. 2b). However, increasing of the pre-incubation of Nk-PLA$_2\alpha$ or Nk-PLA$_2\beta$ with PPP from 5 to 30 min did not reveal any significant difference ($p > 0.05$) in the release of fatty acids from plasma phospholipids (Fig. 2c). This result indicates that only a minor plasma phospholipids hydrolysis done by Nk-PLA$_2$ isoenzymes is sufficient

to exert their anticoagulant effect [17]. The Nk-PLA$_2\alpha$ did not show inhibition of thrombin-induced platelet aggregation at a dose of 2.0 µg (data not shown). Conversely, Nk-PLA$_2\beta$ demonstrated dose-dependent partial inhibition of thrombin-induced platelet aggregation and saturation was observed at 2.0 µg PLA$_2$ (Fig. 3).

Inhibition of thrombin and FXa

None of the Nk-PLA$_2$s showed amidolytic activity against the chromogenic substrate for FXa or thrombin. The Nk-PLA$_2\alpha$ at a dose of 1.0 µg completely inhibited the amidolytic activity of FXa (0.15 µM); conversely, the Nk-PLA$_2\beta$ under identical experimen-
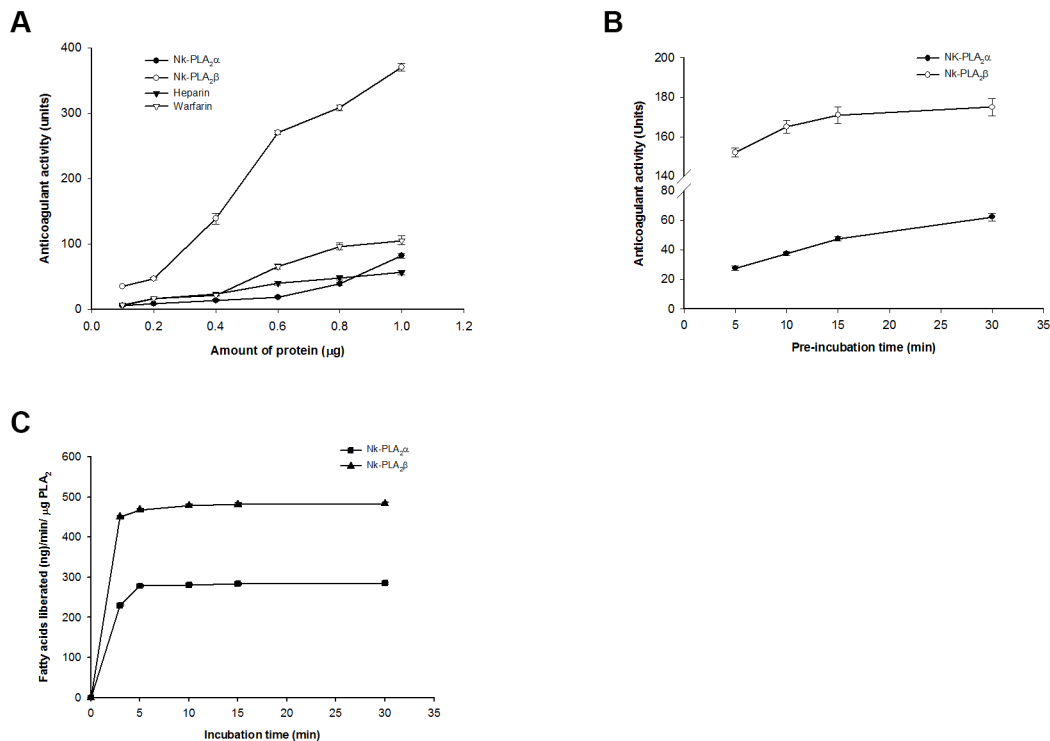

Figure 2. Comparison of anticoagulant activity and plasma phospholipids hydrolytic activity of Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$. (a) A comparison of dose-dependent anticoagulant activity (Ca-clotting time of citrated PPP) of Nk-PLA$_2\alpha$, Nk-PLA$_2\beta$, heparin and warfarin. Unit is defined as 1s increase in clotting of PPP in presence anticoagulants compared to the clotting time of control PPP. (b) Effect of PPP/PLA$_2$ pre-incubation time on anticoagulant activity. (c) Effect of PPP/PLA$_2$ pre-incubation on release of free fatty acids from plasma phospholipids. Values are mean ± SD of triplicate determination.
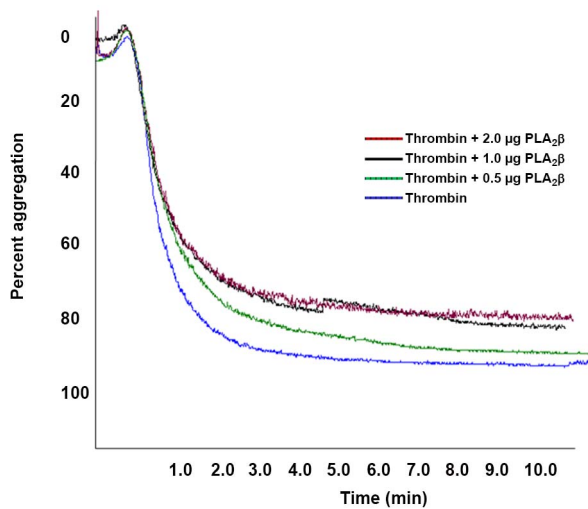doi:10.1371/journal.pone.0101334.g002

Figure 3. Inhibition of thrombin-induced platelet aggregation by different doses of Nk-PLA$_2$β. The platelet aggregation was induced by with thrombin (1.0 NIH U/ml) pre-incubated with Nk-PLA$_2$β (0.5–2.0 μg/ml) or 1× PBS (control) for 30 min at 37°C. The platelet aggregation was monitored in a Chrono-log dual channel aggregometer for 10 min. The data represent a typical experiment; however, the experiment was repeated three times to assure the reproducibility. doi:10.1371/journal.pone.0101334.g003

tal conditions displayed partial inhibition to FXa (Fig. 4a). The Michaelis-Menten plot showed (Fig. 4b) that Nk-PLA$_2$α decreased in a dose-dependent manner, the Km as well as the $Vmax$ values of factor Xa towards its chromogenic substrate (Table 2). The turnover number ($Kcat$ value) was also found to decrease with an increase in inhibitor (Nk-PLA$_2$α) concentration (Table 2). The Ki value for the inhibition of amidolytic activity of factor Xa by Nk-PLA$_2$α was calculated at 42.1 nM. Furthermore, Nk-PLA$_2$α was found to display a greater strength than Nk-PLA$_2$β in inhibiting the prothrombin activation property of FXa (Fig. 5a). The SDS-PAGE analysis (under reduced conditions) of prothrombin degradation products by FXa in presence or absence of Nk-PLA$_2$α or Nk-PLA$_2$β also supports the above observation (Fig. 5b).

The Nk-PLA$_2$ isoenzymes differentially inhibited the amidolytic activity (Fig. 6a) and fibrinogen clotting activity of thrombin (Fig. 6b). The Michaelis-Menten plot for the inhibition of amidolytic activity of thrombin at two different concentrations of

Nk-PLA$_2$β is shown in Fig. 6c. The Nk-PLA$_2$β dose-dependently decreased the Km, $Vmax$, and $Kcat$ values of thrombin towards its chromogenic substrate (Table 2). The K_i value for thrombin inhibition displayed by Nk-PLA$_2$β was determined at 9.4 nM. It was observed that under identical experimental conditions, the inhibition of fibrinogen clotting activity of thrombin done by Nk-PLA$_2$β was more pronounced (p<0.01) compared to the same activity displayed by Nk-PLA$_2$α (Fig. 6b). Increasing of the time (5–30 min) during which Nk-PLA$_2$β was pre-incubated with thrombin (prior to addition of fibrinogen) linearly decreased the fibrinogen clotting activity (or increased the fibrinogen clotting time) of thrombin (Fig. 6d).

Spectrofluorometric analysis of binding of PLA$_2$s with thrombin, FXa or PC

The interaction of FXa with Nk-PLA$_2$α (at 1:10 ratio) resulted in an increase in the emission of fluorescence signal (measured using excitation at 280 nm) of Nk-PLA$_2$α–FXa complex (Fig. 7a). However, the interaction of Nk-PLA$_2$β with FXa decreased the fluorescence signal of Nk-PLA$_2$β-FXa complex compared to the fluorescence signal produced by Nk-PLA$_2$β (Fig. 7a). Moreover, the fluorescence signal at ~345 nm produced by Nk-PLA$_2$β was found to be higher than that of Nk-PLA$_2$α suggesting presence of more tryptophan residue in the former PLA$_2$ enzyme. The interaction of Nk-PLA$_2$α or Nk-PLA$_2$β with thrombin showed that the intrinsic fluorescence of Nk-PLA$_2$β was significantly increased in the presence of thrombin (Fig. 7b); conversely, interaction of Nk-PLA$_2$α with thrombin influenced the fluorescence signal only to a marginal extent (Fig. 7b).

The effect of interaction between Nk-PLA$_2$α/Nk-PLA$_2$β and PC is shown in Fig. 7c. The interaction between Nk-PLA$_2$α and PC in the presence of EDTA (to prevent the PC hydrolysis) resulted in a small decrease in the emission intensity with respect to the emission strength of PC alone. However, interaction effect of Nk-PLA$_2$β with PC was found to be more pronounced because a large decrease in emission intensity was observed (Fig. 7c).

Effect of histidine modifier (PLA$_2$ inhibitor) and antivenom on catalytic and anticoagulant activity

Like other snake venom PLA$_2$ enzymes, the catalytic as well as anticoagulant activity of Nk-PLA$_2$α and Nk-PLA$_2$β was drastically and simultaneously reduced by pBPB, a modifier of histidine (Table 3). The monovalent antivenom was found to inhibit the catalytic and anticoagulant activity of Nk-PLA$_2$α and Nk-PLA$_2$β
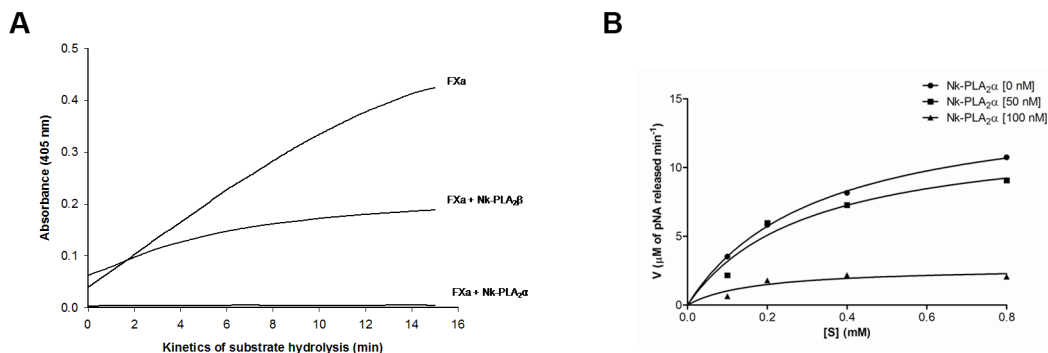

Figure 4. Effects of Nk-PLA$_2$ isoenzymes on inhibition of amidolytic activity of FXa. (a) The Nk-PLA$_2$α (1.0 μg) or Nk-PLA$_2$β (1.0 μg) was preincubated with FXa (0.15 μM) against its chromogenic substrate F3301 (0.2 mM) for 60 min at 37°C, pH 7.4 before the amidolytic activity assay. A control (FXa treated with buffer) was also run in parallel. The values are mean of triplicate determinations. **(b)** Michaelis-Menten plot to determine the inhibitory constant (K$_i$) of Nk-PLA$_2$α (50 nM and 100 nM) on amidolytic activity of FXa (0.15 μM) at 37°C, pH 7.4. doi:10.1371/journal.pone.0101334.g004

Table 2. Kinetics of inhibition of FXa (0.15 μM) and thrombin (0.03 NIH U/ml) by Nk-PLA$_2$α and Nk-PLA$_2$β at 37°C, pH 7.4, respectively.

Parameters	Factor Xa inhibition			Thrombin inhibition		
	Nk-PLA$_2$α (nM)			Nk-PLA$_2$β (nM)		
	0	50	100	0	50	100
Vmax (μmol pNA min^{-1})	15.05	12.72	2.76	1.68	0.378	0.054
Km (mM)	0.33	0.30	0.17	1.39	0.56	0.24
Kcat (min^{-1})	693	586	127	10	2.2	0.32

The kinetic parameters (*Km*, *Vmax*, and *Kcat*) values were determined from Michaelis-Menten plot as described in the text. The chromogenic substrates (0.2 mM) used for the amidolytic activity assay of thrombin and FXa were T1637 and F3301, respectively. The values are mean of triplicate determinations and SD was found within 10% of the mean value.

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to a significantly (p<0.01) different extent. However, at 1:10 antigen (PLA$_2$): antivenom (protein:protein) ratio the catalytic activity of both the enzymes were inhibited to 62 to 65% of their original activity whereas their anticoagulant activity was not affected (Table 3). At an antigen (PLA$_2$): antivenom ratio of 1:200, the monovalent antivenom also failed to neutralize the thrombin inhibitory activity of these PLA$_2$ enzymes (Table 3). Interestingly, heparin showed dissimilar effect in neutralizing the catalytic property of Nk-PLA$_2$α and Nk-PLA$_2$β isoenzymes; nevertheless, FXa or thrombin inhibitory properties of these PLA$_2$ isoenzymes in presence of heparin remained unaffected (Table 3).

Discussion

The LC-MS/MS analysis of both these PLA$_2$ isoenzymes has suggested their identity with PLA$_2$ enzymes isolated from cobra venom. The molecular mass of Nk-PLA$_2$α (13,463.91 Da) and Nk-PLA$_2$β (13,282.38 Da) is very close to the molecular mass of previously reported PLA$_2$ isoenzymes NK-PLA$_2$-I (13.6 KDa) and Nk-PLA$_2$-A (13,619.36 Da), and Nk-PLA$_2$-II (13,346.19 Da) and Nk-PLA$_2$-B (13,303.05 Da), isolated from venom samples of *N. kaouthia* of different geographical locations [5–7]. Moreover, the active sites of Nk-PLA$_2$α and Nk-PLA$_2$β, like classical PLA$_2$ enzymes from snake venom, also contain histidine which is considered as the most crucial residue for phospholipids hydrolysis [3,5–8,10,18]. These combined data suggest that Nk-PLA$_2$α and Nk-PLA$_2$β are PLA$_2$ isoenzymes purified from *N. kaouthia* venom of eastern India origin.

The PLA$_2$ enzymes on the basis of their potencies to prolong the re-calcification time of PPP have been classified into weak or strong anticoagulant enzymes [2,19]. The anticoagulant strength of Nk-PLA$_2$α or Nk-PLA$_2$β categorized them to strong anticoagulant PLA$_2$ enzymes [2–3,8,10]. The significantly higher anticoagulant activity of Nk-PLA$_2$β than that of Nk-PLA$_2$α suggests that these PLA$_2$ isoenzymes have evolved to exert pharmacological effects by different mechanisms in snakebite victims. Recently Saul et al [20] have also reported the presence of two PLA$_2$ isoforms in venom of *Vipera ammodytes ammodytes*, which differ in sequence by only two amino acid residues (Phe[124] is replaced by Ile[124] and Lys[128] is replaced by Glu[128]). These natural PLA$_2$ isoforms exhibit significant differences in toxicity and anticoagulant potencies [20]. Comparison of primary structures of both the Nk-PLA$_2$s, which is our next goal of study, may shed light on their structural motifs correlated to the differences in their biological activity.

Snake venom PLA$_2$s enzymes most likely prolong the blood coagulation through hydrolysis of pro-coagulant phospholipids of plasma rendering them unavailable for initiating the coagulation process [2–3,8,10]. The role of catalytic activity in anticoagulant
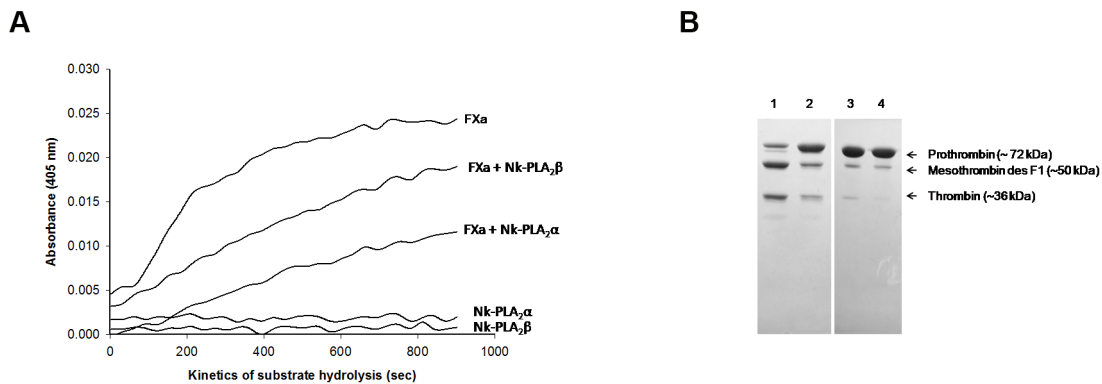

Figure 5. Inhibition of prothrombin activation by FXa pre-treated with NkPLA$_2$ isoenzymes. (a) The FXa (20 nM) was pre-incubated with Nk-PLA$_2$α (1.0 μg), or Nk-PLA$_2$β (1.0 μg), or buffer (control) for 60 min at 37°C, pH 7.4. The prothrombin (1.4 μM) activation by FXa (treated or control) was determined by formation of thrombin by using its chromogenic substrate T1637 (0.2 mM). (b) SDS-PAGE (15%) analysis (reduced conditions) of affect of Nk-PLA$_2$α and Nk-PLA$_2$β on inhibition of prothrombin activation by FXa. Before addition of prothrombin (10.0 μg), FXa (20 nM) was pre-incubated with Nk-PLA$_2$α or Nk-PLA$_2$β for 60 min at 37°C, pH 7.4. Lane 1, prothrombin treated with FXa for 60 min at 37°C (control); lane 2, prothrombin treated with FXa (pre-incubated with 2.0 μg of Nk-PLA$_2$β) for 60 min at 37°C; lanes 3 and 4, prothrombin treated with FXa (pre-incubated with 1 and 2 μg of Nk-PLA$_2$α, respectively) for 60 min at 37°C. The experiment was repeated three times to assure the reproducibility.
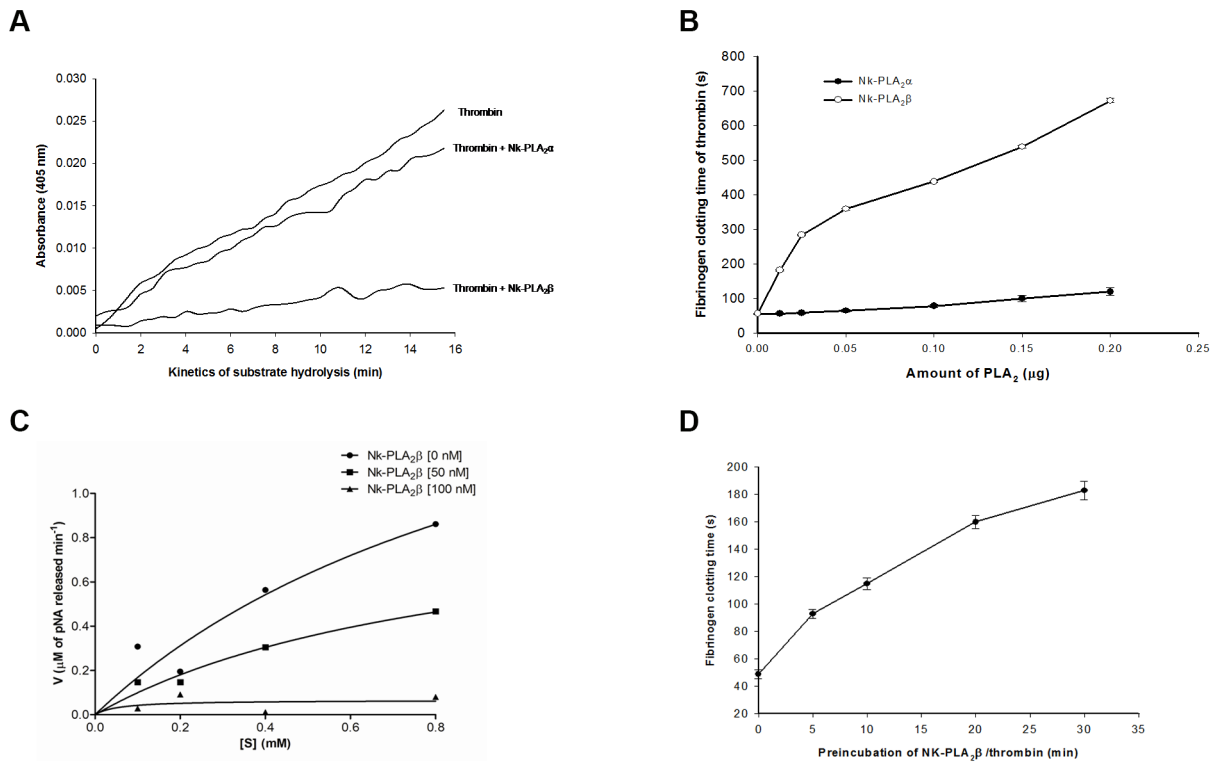doi:10.1371/journal.pone.0101334.g005

Figure 6. Inhibitory effect of Nk-PLA$_2$α and Nk-PLA$_2$β on amidolytic activity and fibrinogen clotting property of thrombin. (a) The thrombin (0.03 NIH U/ml) was pre-incubated with Nk-PLA$_2$α (1.0 μg), or Nk-PLA$_2$β (1.0 μg), or buffer (control) for 30 min at 37°C, pH 7.4 before addition of its chromogenic substrate T1637 (0.2 mM). The values are mean of triplicate determination. **(b)** Inhibition of fibrinogen clotting activity of thrombin through different doses (0–0.2 μg) of Nk-PLA$_2$α or Nk-PLA$_2$β. Before the addition of fibrinogen (final concentration ~7.5 μM in 20 mM K-phosphate buffer, pH 7.4), thrombin (0.03 NIH U/ml) was pre-incubated with different doses of PLA$_2$ (dissolved in 20 mM K-phosphate buffer, pH 7.4) for 30 min at 37°C. Values are mean ± SD of triplicate determination. **(c)** Michaelis-Menten plot to show the Inhibition of amidolytic activity of thrombin (0.03 NIH U/ml) through two different concentrations (50 nM and 100 nM) of Nk-PLA$_2$β. Before the addition of substrate T1637 (0.2 mM), thrombin was pre-incubated with two different doses of the above PLA$_2$ in 20 mM Tris-HCl, pH 7.4 for 30 min at 37°C **(d)** The effect of pre-incubation of Nk-PLA$_2$β/thrombin on inhibition of fibrinogen clotting activity of thrombin. A fixed amount (0.05 μg) of Nk-PLA$_2$β was pre-incubated with thrombin (0.03 NIH U/ml) for 0–30 min at 37°C and then assayed for fibrinogen clotting activity. The values are mean ± SD of triplicate determinations.
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mechanism is evident from the fact that Nk-PLA$_2$β compared to Nk-PLA$_2$α demonstrating superior PC binding as well as PC hydrolysis property also shows higher anticoagulant potency [2–3,8]. Furthermore, inhibition study with heparin and pBPB also supports the role of catalytic activity in anticoagulant property of NK-PLA$_2$ isoenzymes. It has been mentioned that very low but specific plasma phospholipids hydrolysis is the characteristics feature of strongly anticoagulant PLA$_2$s whereas non specific, non-anticoagulant PLA$_2$ enzymes hydrolyze the plasma phospholipids indiscriminately and therefore, they fail to retard the blood coagulation [2–3,10,17]. Furthermore, these results are in accordance with our previous observations showing strong anticoagulant PLA$_2$ enzymes affect blood coagulation by mechanism that is independent of phospholipids hydrolysis [3,8]. Differential inhibition of catalytic activity and anticoagulant property (pharmacological activity) of Nk-PLA$_2$α and Nk-PLA$_2$β by monovalent antivenom, pBPB (histidine modifier, PLA$_2$ inhibitor) and heparin suggests that there are separate or perhaps overlapping sites in these PLA$_2$ molecules that contribute to these activities [2–3,21]. Studies have indicated that heparin acts as a competitive inhibitor for human secretory class II PLA$_2$ and closely related snake venom PLA$_2$ enzymes [22].

The non-enzymatic mechanism of anticoagulant action of snake venom PLA$_2$ has been executed by competing with blood clotting factors such as thrombin, FXa, Va, or prothrombinase complex in the lipid surface [2–3,8,11–12,20]. The present study strongly indicates that Nk-PLA$_2$α or Nk-PLA$_2$β prolongs the blood coagulation by targeting different coagulation factors. The anticoagulant mechanism of Nk-PLA$_2$β is partially dependent on FXa inhibition suggesting FXa is the major pharmacological target for Nk-PLA$_2$α to disrupt the coagulation cascade through a non-catalytic, phospholipids independent mechanism. This significant difference in potency of FXa inhibition by two PLA$_2$ isoenzymes purified from the same snake venom is in accordance with the report of Saul et al. [20]. It has been shown that a point mutation of Lys[128] to Glu[128] perturbs the interaction of PLA$_2$ isoform with FXa results in a 10-fold decrease in the affinity of mutated enzyme for FXa [20]. Therefore, it may be anticipated that small surface residue changes in snake venom isoenzymes can give rise to very significant changes in their biological activity [15,20,23]. The kinetics of inhibition suggests that the Nk-PLA$_2$α binds to the enzyme (FXa)-substrate complex (uncompetitive mode of inhibition) rather than competing with the substrate for binding at the active site of the enzyme. In contrast, strongly anticoagulant PLA$_2$ enzyme CM-IV purified from *N. nigricollis* venom is a non-competitive inhibitor of FXa [24]. Furthermore, inhibition of FXa by Nk-PLA$_2$α is more pronounced compared to CM-IV ($Ki = 250$ nM) [24]. Moreover, the mechanism of FXa
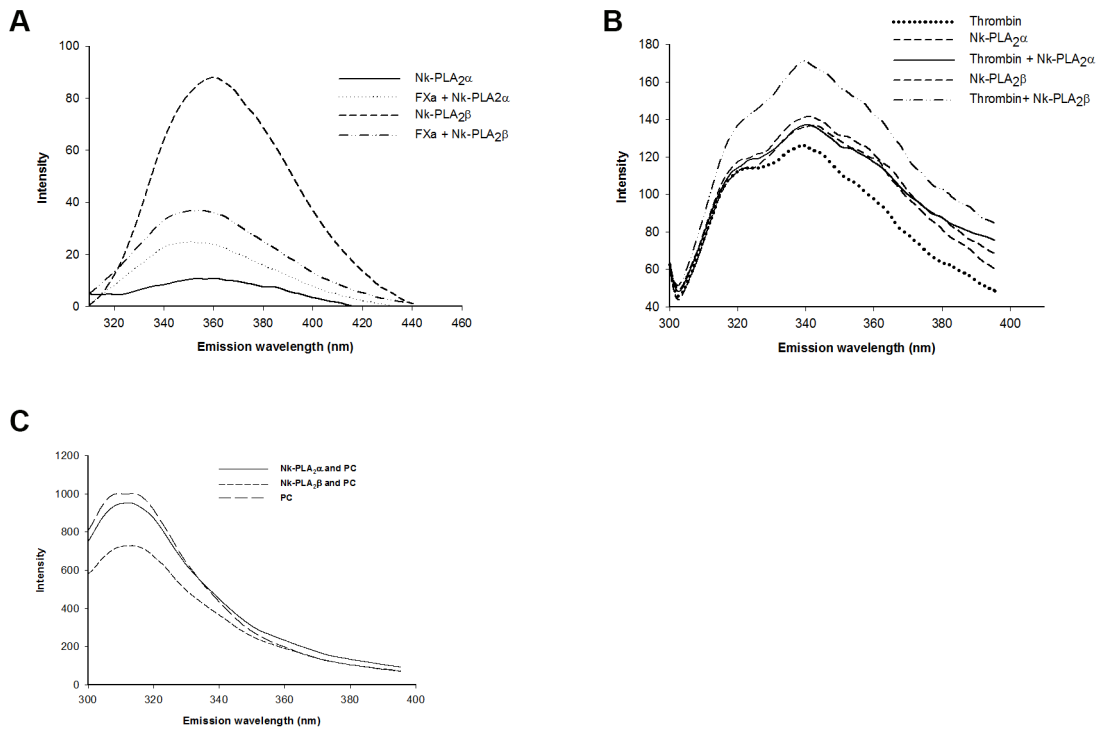
Figure 7. Spectrofluorometric assay of the interaction of Nk-PLA$_2$ isoenzymes with FXa, thrombin and PC. (a) FXa incubated with Nk-PLA$_2\alpha$ or Nk-PLA$_2\beta$ (at 1:10 ratio), (b) interaction of thrombin with Nk-PLA$_2\alpha$ or Nk-PLA$_2\beta$ (at 1:10 ratio), (c) PC with Nk-PLA$_2\alpha$/Nk-PLA$_2\beta$ (at 10:1 ratio) in presence of 0.5 mM EDTA (to prevent the phospholipids hydrolysis). The data shown above represent a typical experiment; however, the experiments were repeated three times to assure the reproducibility.
doi:10.1371/journal.pone.0101334.g007

inhibition by Nk-PLA$_2\alpha$ or Nk-PLA$_2\beta$ and human group II secretory PLA$_2$ (hsPLA$_2$) seems to be different because hsPLA$_2$ does not inhibit the amidolytic activity of FXa [25].

The Nk-PLA$_2\beta$ exerts potent anticoagulant activity via targeting thrombin without the requirement of any cofactor. Till date, only two snake venom PLA$_2$ enzymes, one purified from venom of *N. haje* [11] and the other from the venom of *Daboia russelii russelii* [3], have been demonstrated to prolong PPP clotting via thrombin inhibition. The *Ki* value indicates that Nk-PLA$_2\beta$ is a more potent

inhibitor of thrombin compared to the previously reported thrombin-inhibitor PLA$_2$s isolated from snake venoms [3,11]. Furthermore, Nk-PLA$_2\beta$ inhibited thrombin by uncompetitive mechanism whereas TI-Nh, a PLA$_2$ purified from *N. haje* venom is a mixed-type inhibitor of thrombin [11]. Therefore, the mechanism and strength of thrombin inhibition by PLA$_2$ enzymes purified from venoms of *N. haje* and *N. kaouthia* are different. The spectrofluorometric data also vouch the interaction of Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$ with FXa and thrombin [3,8–9]. However,

Table 3. Effect of pBPB and monovalent antivenom on catalytic, anticoagulant and thrombin inhibitory activity of Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$.

Inhibitor/antivenom	Percent inhibition					
	Catalytic		Anticoagulant		FXa inhibition	Thrombin inhibition
	Nk-PLA$_2\alpha$	Nk-PLA$_2\beta$	Nk-PLA$_2\alpha$	Nk-PLA$_2\beta$	Nk-PLA$_2\alpha$	Nk-PLA$_2\beta$
pBPB (5 mM)	68.7±2.1	81.6±3.2[a]	42±2.2	51.8±3.4[b]	ND	ND
Heparin (1 IU)	23.7±2.1	6.8±1.0[a]	16.1±1.8	0[a]	0	0
Monovalent antivenom						
1:10	65.0±3.3	62.0±4.2	0	0	0	0
1:100	98±1.0	96±2.1	0	0	0	0
1:200	100	100	21.3±1.5	19.8±2.1	18.7±2.0	13.5±1.7[b]

The activity of enzymes in absence of inhibitor/antivenom was considered as 100% activity and the other values were compared with that. The values are mean ± S. D. of triplicate determinations. ND: not determined; pBPB: p-bromophenacyl bromide. Significance of difference with respect to Nk-PLA$_2\alpha$;
[a]p<0.01,
[b]P<0.05.
doi:10.1371/journal.pone.0101334.t003

the significantly different strength of binding of these PLA$_2$ isoenzymes to thrombin or FXa reinforces the fact that these PLA$_2$ isoenzymes have different pharmacological targets in order to exert their anticoagulant action [2]. Moreover, like RVAPLA$_2$ isolated from *D. r. russelii* venom [3], the binding of Nk-PLA$_2\beta$ to thrombin is not a very rapid event. In a sharp contrast, a Kunitz-type protease inhibitor isolated from *D. r. russelii* venom binds to thrombin quickly [26].

The final stage of the blood coagulation cascade is catalyzed by thrombin, a 36 kDa trypsin-like serine protease, which is synthesized in the liver as its precursor prothrombin. Inactive prothrombin is proteolytically cleaved by the prothrombinase complex involving the activated factor X (FXa)/Va to form thrombin which is responsible for physiologic homeostasis and pathologic thrombosis [27]. Thrombin and FXa are, therefore, important pharmaceutical targets for the treatment and prevention of thrombotic disorder, a leading cause of cardiovascular death throughout the world [28–29]. Furthermore, platelet aggregation inhibitor aspirin prevents intravascular clot formation and it has therefore been used as prophylaxis to prevent myocardial infarction and stroke for more than 100 years now. However, several limitations of aspirin have been reported and the best dose of aspirin for the prevention of cardiovascular disease has not been well established [30]. The Nk-PLA$_2\beta$ by virtue of its thrombin inhibitory property partially prevented the thrombin-mediated aggregation of human platelets which may have therapeutic implications for averting unwanted clot formation.

Despite their widespread use, the anticoagulant drugs heparin and warfarin are associated with significant complications [28–29].

This underscores the search for new, safe and potent anticoagulant drugs that do not depend on vitamin K antagonism [29]. Notably, the cardiovascular therapeutic area has been undergoing unprecedented changes with the clinical introduction of new drugs targeting thrombin, factor Xa and inhibition of platelet aggregation. The direct thrombin inhibitors compared to indirect thrombin inhibitor such as heparin may offer a more specific anticoagulant effect [26]. Furthermore, our previous studies had vouched the non-toxic nature of *N. kaouthia* PLA$_2$ isoenzymes [5,7]. Taken together, it may be anticipated that future therapeutic application of Nk-PLA$_2\alpha$ and Nk-PLA$_2\beta$ as combined platelet aggregation inhibitors and anticoagulant agents for the treatment and/or prevention of cardiovascular disorder is highly promising [13,28].

Acknowledgments

Author Contributions

Conceived and designed the experiments: AKM. Performed the experiments: AKM BK RT. Analyzed the data: AKM. Contributed reagents/materials/analysis tools: AKM. Wrote the paper: AKM.

References

1. Mukherjee AK, Maity CR (2002) Biochemical composition, lethality and pathophysiology of venom from two cobras- *Naja naja* and *Naja kaouthia*. Comp Biochem Physiol 131(B): 125–132.
2. Kini RM (2006) Anticoagulant proteins from snake venoms: structure, function and mechanism. Biochem J 397: 377–387.
3. Mukherjee AK (2014) A major phospholipase A$_2$ from *Daboia russelii russelii* venom shows potent anticoagulant action via thrombin inhibition and binding with plasma phospholipids. Biochimie 99, 153–161
4. Pungerčar J, Križaj I (2007) Understanding the molecular mechanism underlying the presynaptic toxicity of secreted phospholipase A$_2$. Toxicon 50: 871–892.
5. Doley R, Mukherjee AK (2003) Purification and characterization of an anticoagulant phospholipase A$_2$ from Indian monocled cobra (*Naja kaouthia*) venom. Toxicon 41: 81–91.
6. Doley R, King GF, Mukherjee AK (2004) Differential hydrolysis of erythrocyte and mitochondrial membrane phospholipids by two phospholipase A$_2$ isoenzymes (NK-PLA$_2$-I and NK-PLA$_2$-II) from Indian monocled cobra *Naja kaouthia* venom. Arch Biochem Biophys 425: 1–13.
7. Mukherjee AK (2007) Correlation between the phospholipids domains of the target cell membrane and the extent of *Naja kauthia* PLA$_2$-induced membrane damage: Evidence of distinct catalytic and cytotoxic sites in PLA$_2$ molecule. Biochim Biophys Acta 1770: 187–195.
8. Saikia D, Thakur R, Mukherjee AK (2011) An acidic phospholipase A$_2$ (RVVA-PLA$_2$-I) purified from *Daboia russelli* venom exerts its anticoagulant activity by enzymatic hydrolysis of plasma phospholipids and by non-enzymatic inhibition of factor Xa in a phospholipids/Ca^{2+} independent manner. Toxicon 57: 841–850.
9. Saikia D, Bordoloi NK, Chattopadhyay P, Chocklingam S, Ghosh SS, et al. (2012) Differential mode of attack on membrane phospholipids by an acidic phospholipase A$_2$ (RVVA-PLA$_2$-I) from *Daboia russelli* venom. Biochim Biophys Acta–Biomembrane 12: 3149–3157.
10. Saikia D, Majumdar S, Mukherjee AK (2013) Mechanism of *in vivo* anticoagulant and haemolytic activity by a neutral phospholipase A$_2$ purified from *Daboia russelii* venom: Correlation with clinical manifestations in Russell's Viper envenomed patients. Toxicon 76: 291–300.
11. Osipov AV, Filkin SY, Makarova YV, Tsetlin VI, Utkin YN (2010) A new type of thrombin inhibitor, non-cytotoxic phospholipase A$_2$, from the *Naja haje* cobra venom. Toxicon 55:186–194.
12. Zingali RB (2007) Interaction of snake-venom proteins with blood coagulation factors: mechanisms of anticoagulant activity. Toxin Rev 26: 25–46.
13. Koh CY, Kini RM (2012) From snake venom toxins to therapeutics-cardiovascular examples. Toxicon 59: 497–506.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193: 265–275.
15. Mukherjee AK, Mackessy SP (2013) Biochemical and pharmacological properties of a new thrombin-like serine protease (Russelobin) from the venom of Russell's Viper (*Daboia russelii russelii*) and assessment of its therapeutic potential. Biochim Biophys Acta 1830(6): 3476–3488.
16. Bjork I, Lindahl U (1982) Mechanism of the anticoagulant action of heparin. Mol Cell Biochem 48: 161–182
17. Condrea E, Yang C-C, Rosenberg P (1981) Lack of correlation between anticoagulant activity and phospholipid hydrolysis by snake venom phospholipases A$_2$. Thromb Haemost 45: 82–85.
18. Diaz-Oreiro C, Gutierrez JM (1997) Chemical modification of histidine and lysine residues of myotoxic phospholipases A$_2$ isolated from *Bothrops asper* and *Bothrops godmani* snake venoms: Effects on enzymatic and pharmacological properties Toxicon 35: 241–252.
19. Verheij HM, Boffa MC, Rothen C, Bryckaert MC, Verger R, et al (1980) Correlation of enzymatic activity and anticoagulant properties of phospholipase A$_2$. Eur J Biochem 112: 25–32.
20. Saul FA, Prijatelj-Znidarsic P, Vulliez-le Normand B, Villette B, Raynal B, et al. (2010) Comparative structural studies of two natural isoforms of ammodytoxin, phospholipases A$_2$ from *Vipera ammodytes ammodytes* which differ in neurotoxicity and anticoagulant activity. J Struct Biol 169: 360–369.
21. Lomonte B, Tarkowski A, Bagge U, Hanson LA (1994) Neutralization of the cytolytic and myotoxic activities of phospholipases A2 from *Bothrops asper* snake venom by glycosaminoglycans of the heparin/heparan sulfate family. Biochem Pharmacol 47:1509–1518.
22. Dua R, Cho W (1994) Inhibition of human secretory class II phospholipase A$_2$ by heparin. Eur J Biochem 221: 481–490.
23. Faure G, Saul F (2011) Structural and functional characterization of anticoagulant, FXa-binding Viperidae snake venom phospholipases A$_2$. Acta Chim Slov 58: 671–677.
24. Stefansson S, Kini RM, Evans HJ (1990) The basic phospholipase A$_2$ from *Naja nigricollis* venom inhibits the prothrombinase complex by a novel nonenzymatic mechanism. Biochemistry 29: 7742–7746.
25. Mounier CM, Hackeng TM, Schaeffer F, Faure G, Bon C, et al. (1998) Inhibition of prothrombinase by human secretory phospholipase A$_2$ involves binding to factor Xa. J Biol Chem 273: 23764–23772.
26. Mukherjee AK, Mackessy SP, Dutta S (2014) Characterization of a Kunitz-type protease inhibitor peptide (Rusvikunin) purified from *Daboia russelii russelii* venom. Int J Biol Macromol 67: 154–162
27. Fenton JW, Ofosu FA, Brezniak DV, Hassouna HI (1993) Understanding thrombin and hemostasis. Hematol Oncol Clin North Am 7: 1107–1119.

28. Hylek EM, Solarz DE (2004) Dual antiplatelet and oral anticoagulant therapy. Increasing use and precautions for a hazardous combination. Cardiovasc Interven 1: 62–64.

29. Jacomella V, Corti N, Husmann M (2013) Novel anticoagulants in the therapy of peripheral arterial and coronary artery disease. Curr Opin Pharmacol 13: 1–7.

30. Pulcinelli FM, Pignatelli P, Celestini A, Riondino S, Gazzaniga PP, et al. (2004) Inhibition of platelet aggregation by aspirin progressively decreases in long-term treated patients. J Am Coll Cardiol 43: 979–984.