Purification and inhibitory profile of phospholipase A₂ inhibitors from Australian elapid sera

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Although the resistance of snakes to their own venom is well known, until now no investigators have examined the serum of Australian snakes. Here we describe the identification and purification of a range of phospholipase A₂ (PLA₂) inhibitors from the serum of Australian elapids. All PLA₂ inhibitors were composed of two protein chains, an α -chain and a β -chain. The α -chains were approx. 22.5 kDa in size and variably glycosylated, whereas the β -chains were approx. 19.8 kDa in size and not glycosylated. Identification of isoforms of the two subunit chains was significant because three of the six sera examined were from single snake specimens. In addition, the glycosylation patterns of the α -chains were thoroughly investigated in these unpooled sera. The functional and structural properties of the purified

inhibitors were studied. Uniquely, a snake PLA₂ inhibitor was found to inhibit human type II PLA₂ enzyme, which has implications for the treatment of the many diseases in which PLA₂ enzymes have been implicated. Further, we demonstrate that the inhibitor forms a non-covalent association with a purified PLA₂ enzyme. Finally, the purified PLA₂ inhibitor was shown to protect *in vivo* against the lethal affects of a homologous PLA₂ enzyme, suggesting a role for PLA₂ inhibitors in the treatment of snake bite victims.

Key words: glycosylation, non-covalent association, protection *in vivo*, snake venom.

INTRODUCTION

Inhibitors of phospholipase A₂ (PLA₂) enzymes have been identified in fungi [1], bacteria [2,3] and plants [4–10] and in human [11,12], bovine [13,14] and pig serum [15]. Additionally, heparin and heparan sulphate [16,17], lipocortin [18–20], lung surfactant protein A [21] and, uniquely, peptides of PLA₂ enzymes themselves [22] have been shown to inhibit PLA₂ enzymic activity.

So far, nine PLA₂ inhibitors (PLIs) have been isolated or identified in the serum of six snakes (not including the present study). These PLIs have been purified from *Naja naja kaouthia* (Thailand cobra [23,24]), *Laticauda semifasciata* (Sea Krait [25]), *Crotalus durissus terrificus* (South American rattlesnake [26–28]), *Trimeresurus flavoviridis* (Habu [29–32]), *Agkistrodon blomhoffii siniticus* (Chinese mamushi [33,34]) and *Bothrops asper* (fer-de-lance [35]). Additionally, an inhibitory serum factor from *Vipera palaestinae* (Palaestian viper) that shares many of the characteristics of PLIs has been identified [36]; however, it has not been clearly shown that this protein inhibits PLA₂ enzymes.

Snake PLIs have many characteristics in common: they are typically large non-covalent, multisubunit complexes with at least one subunit glycosylated. They generally possess an isoelectric point of less than 7 and express various levels of cross-reactivity towards PLA₂ enzymes. Most of these PLIs have been fully sequenced and from the sequence data it is clear that there are three classes of PLI. The first two have similarity with urokinase plasminogen activator receptor, Ly-6 and CD59 (Class A PLIs), and with the carbohydrate-recognition domain of C-

type lectins (Class B PLIs). An additional class of PLI (Class C PLIs), with leucine-rich repeats, is currently defined by the sequence of the PLI from *A. blomhoffii siniticus* [33,37].

Here we report the purification and characterization of six PLIs from a range of Australian elapid sera. Three of the PLIs were purified from the sera of individual snake specimens, enabling a complete characterization of isoforms present in individual sera. All PLIs had similar protein compositions; however, glycosylation patterns were variable. The inhibitory profiles of two PLIs were examined and found to inhibit all PLA₂ enzymes tested, including snake-venom PLA₂, human and pig pancreatic PLA₂ enzymes and bee-venom PLA₂. The ability to protect *in vivo* against a homologous PLA₂ was tested. Finally, the intact PLI complex was found to form a non-covalent complex composed of two non-identical chains; it also formed a non-covalent interaction with a purified PLA₂.

EXPERIMENTAL

Materials

Notechis ater serventyi (Chappell Island tiger snake), *Oxyuranus scutellatus* (coastal taipan) and *Pseudonaja textilis* (common brown snake) sera were obtained from a single specimen of each species. Pooled sera from *N. ater* (Tasmanian tiger snake), *N. scutellatus* (common tiger snake) and *O. microlepidotus* (inland taipan) were obtained from captive specimens at Venom Supplies (Tanunda, South Australia) or World Tiger Snake Farm (Tasmania).

Abbreviations used: ES-MS, electrospray MS; NAI, *Notechis ater* inhibitor; NAsI, *N. ater serventyi* inhibitor; NSI, *N. scutellatus* inhibitor; OA, osteoarthritis; OMI, *Oxyuranus microlepidotus* inhibitor; OSI, *O. scutellatus* inhibitor; PLA₂, phospholipase A₂; PLI, PLA₂ inhibitor; 10pPC, 1-hexadecanoyl-2-(1-prenedecanoyl)-sn-glycero-3-phosphocholine; 10pPG, 1-hexadecanoyl-2-(1-prenedecanoyl)-sn-glycero-3-phosphoglycerol; PTI, *Pseudonaja textilis* inhibitor; RA, rheumatoid arthritis; RP-HPLC, reverse-phase HPLC; SPP, semi-purified preparation; TD, toxic dose; TFA, trifluoroacetic acid.

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Purification

PLIs were purified from whole serum with a DEAE-Sephacel (Pharmacia) column (1.5 cm $\times$ 15 cm). Approx. 10 mg of whole serum was loaded on the column, which had been equilibrated in 0.01 M ammonium acetate, pH 7.0, at a flow rate of 0.5 ml/min. Proteins were eluted with a step gradient of increasing concentrations of 0.1, 0.25, 0.5 and 1.0 M ammonium acetate, pH 7.0, at a flow rate of 0.5 ml/min. The eluate was monitored at 280 nm (UA-6 UV/Vis detector; ISCO) and fractions were collected (Retriever II; ISCO). PLIs were eluted in the 0.5 M peak, which will be referred to as the semi-purified preparation (SPP) and were 90–95% pure.

The PLIs are composed of two chains, an α -chain and a β -chain. The α - and β -chains were purified on a Pharmacia SMART system fitted with a column (2.1 mm $\times$ 100 mm) containing Pharmacia Sephasil C₈ (5 μ m). The column was equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA) in water (buffer A), at a flow rate of 100 μ l/min, proteins were eluted with a gradient of 0.08% (v/v) TFA/80% (v/v) acetonitrile in water (buffer B). The percentage (v/v) of buffer B was increased as follows: 0–2 min, 0%; 2–10 min, 0–55%; 10–50 min, 55–70%; 50–60 min, 70–100%. Software-controlled peak fractionation was used to collect peaks with the integrated fraction collector.

Isoforms were separated with a Pharmacia SMART system fitted with a Pharmacia μ RPC C₂/C₁₈ (2.1 mm $\times$ 100 mm) column. The column was equilibrated in 0.1% (v/v) TFA in water (buffer A), at a flow rate of 50 μ l/min. Purified α - or β -chain was loaded and a gradient of 0.08% (v/v) TFA/80% (v/v) acetonitrile in water (buffer B) was developed as follows: 0–7 min, 0%; 7–20 min, 0–40%; 20–60 min, 40–47%; 60–70 min, 47–100% buffer B. Software-controlled peak fractionation was used to collect peaks with the integrated fraction collector.

Characterization of PLIs

Glycosylation

To investigate the glycosylation of the PLIs, the SPPs were freeze-dried, resuspended in 100 μ l of 20 mM sodium phosphate buffer (pH 7.2)/50 mM EDTA/0.1% SDS and digested with 0.8 unit N-glycosidase F (Boehringer-Mannheim) for 18 h at 37 °C. The α - and β -chains were then separated as outlined above; if isoforms were present, they were also isolated as outlined above.

Proteins were analysed with a Micromass Platform II electrospray single-quadrupole mass spectrometer, which was coupled to a Waters Alliance 2690 HPLC system and controlled by an IBM-compatible computer. The sample was introduced to the mass spectrometer at a flow rate of 10 μ l/min in acetonitrile/water (1:1, v/v). When required, the sample was also diluted in this solvent containing 0.2% (v/v) formic acid to aid ionization of the protein. The MS was calibrated with trypsinogen. The acquisition parameters were as follows: ionization mode ES+, capillary 3.50 kV, HV lens 0.50 kV, skimmer offset 5 V, source temperature 70 °C, ion energy 0.7 V, LM resolution 15.0, HM resolution 15.0, multiplier 650 V. The mass range acquired and the cone voltage were varied to optimize the sensitivity and resolution of the sample. Generally, for the α - and β -chains, the mass range acquired was 1200 to 2600 m/z with a cone voltage ramp of 38 to 60 V over this range.

Complex formation with notexin and molecular mass of native complex

A Superose 12 HR 10/30 (Pharmacia) column was equilibrated with 0.1 M ammonium acetate, pH 7.0, at a flow rate of 0.5 ml/min. The sample was loaded and eluted with a 0.1 M

ammonium acetate (pH 7.0) gradient over 60 min. The eluate was monitored at 214 and 280 nm. Fractions were collected and analysed by SDS/PAGE by the method of Laemmli [38] and stained with silver [39]. 2-Mercaptoethanol was omitted for the non-reducing gel. The following samples were loaded on the column: 100 μ g of notexin (the main toxic PLA₂ enzyme present in the venom of *N. scutatus*), 150 μ g of SPP and 100 μ g of notexin plus 150 μ g of SPP that had been incubated together for 1 h at 20 °C and treated as detailed above. The column was calibrated with standard proteins.

Cross neutralization studies: *N. scutatus* inhibitor (NSI)

The assay used was adapted from the method of Radvanyi et al. [40]. In brief, to 1 ml of assay buffer [50 mM Tris/HCl (pH 7.5)/100 mM NaCl/1 mM EDTA] the following were added sequentially: 16 μ l of a 1:0.6 (v/v) mixture of 10% (w/v) BSA and 1 M CaCl₂, 10 μ l of 0.2 M 1-hexadecanoyl-2-(1-prenedecanoyl)-sn-glycero-3-phosphocholine (10pPC). To this, 35 μ l of a 2.5:1 (v/v) mixture of SPP and venom respectively was added. The SPP (containing NSI) was tested against a range of venoms. The venoms tested were *N. scutatus* (homologous venom), *P. textilis* (common brown snake), *Naja melanoleuca* (forest cobra), *V. russelli* (Russell's viper), *A. bilineatus* (Central American moccasin), *B. alternatus* (víbora de la cruz) and *C. atrox* (western diamondback rattlesnake). Venoms were diluted to yield similar enzymic activities in the assay. The amount of venom (dissolved in saline) added per assay (in a 10 μ l volume) was as follows: *N. scutatus*, 50 ng; *P. textilis*, 500 ng; *N. melanoleuca*, 66.7 ng; *V. russelli*, 666.7 ng; *A. bilineatus*, 500 ng; *B. alternatus*, 1000 ng; *C. atrox*, 1000 ng. Various concentrations of the SPP were tested against each venom. The amounts added to each assay were as follows: 13.9, 3.5, 2.3, 1.1, 0.56, 0.28 and 0.14 μ g. All samples were performed in triplicate and compared with appropriate controls, then plotted as the percentage inhibition relative to negative controls.

Cross neutralization studies: *N. ater* inhibitor (NAI)

Owing to serum supply problems, *N. ater* sera was used for the remaining experiments. The inhibitory activity of NAI was initially tested against notexin (positive control), bee-venom PLA₂ (*Apis mellifera*), pig pancreatic PLA₂ (*Sus scrofa*) and aspirates of osteoarthritis (OA) (three samples) and rheumatoid arthritis (RA) (one sample) synovial fluid. The assay was performed with 1-hexadecanoyl-2-(1-prenedecanoyl)-sn-glycero-3-phosphoglycerol (10pPG) as substrate. Enzymes were diluted to achieve similar levels of activity in the assay. The amount of PLA₂ enzyme (dissolved in saline) added per assay (in a 10 μ l volume) were *N. scutatus* venom, 333 ng; bee-venom PLA₂, 25 ng; pig pancreatic PLA₂, 3330 ng. The amount of PLA₂ enzyme present in the synovial fluid aspirates was not determined. The following dilutions were used in the assay: OA aspirates, undiluted to 1:10 (protein concentration 26.7–34.5 mg/ml); RA, 1:30 (protein concentration 32.3 mg/ml). Dilutions of the SPP varied depending on the PLA₂ tested. Two dilution groups were used for a 7.13 mg/ml solution of the SPP. Group 1 consisted of 12.7, 3.6, 0.5 and 0.3 μ g of SPP per assay; PLA₂ sources challenged with this group were *N. scutatus* venom, pig pancreatic PLA₂ and bee-venom PLA₂. Group 2 consisted of 89.1, 25.5, 12.7 and 3.6 μ g of SPP per assay; PLA₂ sources challenged with this group were all OA and RA samples. Inhibitor dilutions were preincubated with the PLA₂ enzyme in a volume ratio of 2.5:1. All samples were performed in triplicate. Results are expressed as percentages inhibition relative to control values. Protein concen-

trations were determined with the Bradford assay [41], as modified by Bio-Rad.

Protection studies *in vivo*

Neonatal QS mice (3–5 g) were used as the assay system because they are more sensitive than adult mice and to conserve the limited amount of inhibitor available. The toxic dose (TD; essentially LD₁₀₀) for notexin was established. TD was defined as the minimum lethal dose and was found to be 0.75 µg per mouse, administered subcutaneously in saline. The protection studies were performed in triplicate with the following samples; 1 × TD, 2 × TD, 3 × TD and 4 × TD plus SPP (50 µg), notexin control (TD plus saline), SPP control (SPP plus saline) and two negative controls (human serum and BSA, each at 50 µg, plus TD). The samples were mixed with 50 µg of SPP, BSA or human serum as appropriate and incubated for 1 h at 37 °C. A total volume of 100 µl was then injected subcutaneously into each mouse. The mice were incubated on a heating pad maintained at 30 °C for 17 h.

RESULTS

Purification

The DEAE-Sephacel purification profiles for all PLIs from whole sera were similar (results not shown): the PLI eluted in the 0.5 M peak for all serum samples. The α- and β-chains were separated by reverse-phase HPLC (RP-HPLC) (Figure 1), with the α-chains exhibiting a shorter retention time than the β-chains. The separation profile for each PLI seemed similar, except for *P. textilis* inhibitor (PTI) (Figure 1A) in which there was a significant peak that was eluted close to PTIβ. All peaks were collected and analysed by SDS/PAGE (results not shown); only peaks containing proteins with molecular masses consistent with the α- and β-chains of elapid PLIs were examined further. Partial separation of α-chain isoforms was evident for both *O. scutellatus* inhibitor (OSI) and *N. ater serventyi* inhibitor (NASI) (Figures 1B and 1D respectively). From the areas under the

Figure 1 RP-HPLC purification of α- and β-chains

DEAE-Sephacel-purified PLIs were further purified with a Pharmacia Sephasil C₈ (2.1 mm × 100 mm) column at a flow rate of 100 µl/min. The column was equilibrated in 0.1% (v/v) TFA in water and eluted with an increasing concentration of buffer B [0.08% (v/v) TFA/80% (v/v) acetonitrile in water] as indicated by the straight-line graph (right ordinate). The elution positions of the α- and β-chains are indicated. (A) Purification of PTIα and PTIβ. (B) Purification of OSIα and OSIβ. (C) Purification of OMIα and OMIβ. (D) Purification of NASIα and NASIβ. The elution profiles for NAI and NSI were virtually identical to that for NASI and are therefore omitted.

Figure 2 RP-HPLC purification of α- and β-chain isoforms

RP-HPLC-purified α- and β-chains were further purified with a Pharmacia µRPC C₂/C₁₈ (2.1 mm × 100 mm) column at a flow rate of 50 µl/min. The column was equilibrated in 0.1% (v/v) TFA in water and eluted with an increasing concentration of buffer B [0.08% (v/v) TFA/80% (v/v) acetonitrile in water]. (A) Purification of OMIβ isoforms. The peak shoulder is indicated with an arrow. (B) Purification of OMIα isoforms. (C) Purification of NASIα isoforms.

curves at 280 nm the ratio of α-chain to β-chain was estimated as 2:1 for all PLIs. The elution profiles for NAI and NSI were virtually identical with that for NASI and are therefore omitted.

All α- and β-chains (excluding NSI) were analysed by MS; those peaks found to contain a number of proteins were subjected to further purification by RP-HPLC. Purification of the NASI α-chain isoforms and the *O. microlepidotus* inhibitor (OMI) α- and β-chain isoforms is shown in Figure 2. Two β-chain isoforms were detected in the sera of *O. microlepidotus* (Table 1) that were only partly separated by RP-HPLC (Figure 2A). Complete separation was effected for the OMIα isoforms (Figure 2B) as it apparently was also for the NASIα isoforms (Figure 2C). Electrospray-MS (ES-MS) analysis revealed that the peak labelled NASIα_i contained two major proteins, one of 20428 Da and the other of 20578 Da (Table 1); the α-chain 150 Da greater in mass is referred to as NASIα_{iii}. Thus three major α-chain isoforms and one β-chain were detected in the sera collected from a single specimen of *N. ater serventyi*. Two α-chain and two β-chain isoforms were identified in pooled sera from *O. microlepidotus*.

As with NASI, OSI contained three α-chain isoforms and one β-chain (Table 1). Only two α-chain isoforms could be separated by RP-HPLC (results not shown); the third isoform was eluted in the same peak as OSIα_{ii} and identified by ES-MS analysis. PTI, also purified from the serum of a single specimen, contained two α-chain and two β-chain isoforms. Neither the α-chain nor the β-chain isoforms could be adequately separated under the conditions used (results not shown), suggesting a high degree of sequence similarity.

For NAI, two separate batches of pooled sera were analysed to determine variability between batches. It was found that the isoform configuration of the two batches of pooled sera varied (Figure 3). A total of four isoforms were isolated by RP-HPLC, with two isoforms predominating (NAIα_{ii} and NAIα_{iii}) in both batches. It must be emphasized that NAI was purified from a large number of pooled sera and therefore the increased number of isoforms might merely represent individual variation, as has been reported for venom components [42,43]. ES-MS analysis of the purified proteins indicated that five α-chains were in fact present; the peak labelled NAIα_{ii} (Figure 3) contained two proteins that differed in mass by 147 Da (results not shown).

Table 1 ES-MS analysis of RP-HPLC-purified α - and β -chains

All proteins were purified by RP-HPLC and analysed by ES-MS, prior to and following deglycosylation with N-glycosidase. Note that, for clarity, the results with NAI and the five α -chain isoforms have been omitted. However, the molecular masses of the α -chains of NAsI were very similar to those measured for the α -chains of NAI. The β -chain had an identical molecular mass in the venom from both snakes.

PLI chain	Molecular mass (Da)		
	Native	Deglycosylated	Carbohydrate moiety
NAsI α_i	22 428*	20 224	2204*
NAsI α_{ii}	22 596*	20 392	2204*
NAsI α_{iii}	22 578*	20 374	2204*
NAsI β	19 819	—	—
OMI α_i	22 817†	20 411	2406†
OMI α_{ii}	22 881†	20 473	2408†
OMI β_i	19 757	19 765	—
OMI β_{ii}	19 912	19 917	—
OSI α_i	22 585*	20 381	2204*
OSI α_{ii}	22 698*	20 492	2206*
OSI α_{iii}	22 858*	20 652	2206*
OSI β	19 858	—	—
PTI α_i	22 569*	20 373	2196*
PTI α_{ii}	22 502*	20 304	2198*
PTI β_i	19 779	19 782	—
PTI β_{ii}	19 837	19 837	—

* An isoform of molecular mass 657 Da more than that shown also exists.

† Isoforms of molecular masses 205 Da less, 205 Da greater, 450 Da greater and 657 Da greater than that shown also exist.

Figure 3 Purification of NAI α isoforms

NAI α isoforms were purified with a Pharmacia μ RPC C₂/C₁₈ (2.1 mm $\times$ 100 mm) column at a flow rate of 50 μ l/min. The column was equilibrated in 0.1% (v/v) TFA in water and the proteins were eluted with an increasing concentration of buffer B [0.08% (v/v) TFA/80% (v/v) acetonitrile in water] as indicated by the dotted line (right ordinate). The profiles for two serum batches are shown; the thinner line has been shifted upwards by 1 absorbance unit for clarity.

Characterization of PLIs

All α - and β -chains of the PLIs were similar in molecular mass (Table 1). ES-MS analysis indicated the presence of carbohydrate moieties on the α -chain of each PLI. This was investigated by deglycosylation with N-glycosidase and determining the mass of the deglycosylated protein. Two carbohydrate molecular masses of 2204 and 2861 Da were detected on the α -chain of NAI, NAsI, OSI and PTI (Table 1). These masses are characteristic of a biantennary carbohydrate moiety (2204 Da) with the addition of

Table 2 Summary of Australian snake PLI structural data

All sera, except those for *N. ater* and *O. microlepidotus*, were from a single snake.

PLI	No. of α -chains	No. of β -chains	No. of glycoforms	Probable carbohydrate
NAI	5	—	2	Biantennary
NAsI	3	1	2	Biantennary
OMI	2	1	5	Bisecting Biantennary
OSI	3	2	2	Biantennary
PTI	2	1	2	Biantennary
		2	—	

a NeuAc-Hex-HexNAc moiety (mass 657 Da; Hex represents a hexose) to give a mass of 2861 Da [44]. Each protein isoform exists in two glycoforms to increase the number of α -chain masses detected (Table 2). The glycosylation pattern on OMI α was significantly different: a total of five glycoforms were detected (Table 1). The main carbohydrate mass of 2406 Da is characteristic of a bisecting biantennary carbohydrate complex. The carbohydrate mass differences of ± 205 and 657 Da are characteristic of HexNAc and NeuAc-Hex-HexNAc respectively [44], whereas the mass 450 Da is characteristic of NeuAc-Hex. Therefore each protein isoform of OMI α exists in five glycoforms, giving a total of ten different forms of OMI α . No β -chain glycoforms were detected in any PLI; protein isoforms were found only for OMI β and PTI β , of which only PTI was purified from a single snake.

NSI was found to form a non-covalent complex (Figures 4 and 5) that was eluted with a retention time consistent with a native molecular mass of 110 kDa. The molar ratio of α -chain to β -chain is estimated at 2:1, suggesting that the native complex is made up of two α chains and one β chain. The effect of carbohydrate moieties in increasing apparent molecular mass as determined by size-exclusion chromatography is well known [45]. Additionally, NSI binds non-covalently to notexin (13.5 kDa) because this PLA₂ was eluted with the complex at approx. 110 kDa (Figures 4 and 5).

Furthermore, NSI was found to inhibit the enzymic activity of all venom PLA₂ enzymes that it was tested against (Figure 6). Venoms tested were from Australian and Asian elapid, viperid and crotalid snake species. This inhibition was found to be highly efficient (at least 80% in all cases) and concentration-dependent. Generally, the Australian elapid (*N. scutatus* and *P. textilis*) venoms were inhibited strongly by NSI, with a high level of inhibition recorded at the lowest amount of NSI added. In contrast, inhibition of the venoms from the Asian elapid (*N. melanoleuca*), viperid (*V. russelli*) and crotalid (*A. bilineatus*, *B. alternatus* and *C. atrox*) snakes tended to be less efficient and decreased quickly (Figure 6). Whole venoms were tested and as such NSI was capable of inhibiting all of the PLA₂ enzymes that exhibited enzymic activity under the assay conditions used. These venoms contained acidic, basic and neutral PLA₂ enzymes and covered monomeric, dimeric and pentameric structures. Thus NSI inhibits a wide range of PLA₂ enzymes that exhibit both pH and structural variability.

Owing to supply problems with *N. scutatus* sera, the remaining set of experiments were performed with *N. ater* sera (a closely related species). All experiments performed subsequently have

Figure 4 Superose 12 elution profile of NSI incubated with notexin

A solution containing notexin (100 μ g) and NSI (150 μ g, in the form of the SPP) was loaded on a Superose 12 column that had been equilibrated with 0.1 M ammonium acetate, pH 7. See Figure 5 for an SDS/PAGE analysis of the fractions.

Figure 5 SDS/PAGE analysis of fractions collected from the Superose 12 column

The peaks from Figure 4 were collected, analysed by SDS/PAGE and stained with silver. Lane 1, NSI; lane 2, notexin; lane 3, NP2; lane 4 NP3; lane 5, NP5; lane 6, molecular mass markers (sizes are indicated at the right). The locations of the α - and β -chains are indicated. (A) Reduced; (B) non-reduced.

suggested that NSI and NAI are highly similar proteins. NAI was tested against *N. scutatus* venom, pig pancreatic PLA₂ and bee-venom PLA₂. NAI was also tested against human type II PLA₂ in the form of OA or RA synovial fluid aspirates. As before, the tests were designed to highlight inhibition rather than to quantify the affinity of the interaction. NAI strongly inhibited both whole *N. scutatus* venom and bee-venom PLA₂ (Figure 7A). Furthermore, NAI maximally inhibited pig pancreatic PLA₂ at 54.8%. From Figure 7(B) it is apparent that NAI is capable of inhibiting human type II PLA₂ in all of the samples tested. The inhibition was maximal at 41–54% for the OA samples and 29% for the RA sample. As seen above for NSI with the non-Australian snake venoms, the level of inhibition decreased markedly once the concentration of NAI had been lowered.

Significantly, NSI was able to protect *in vivo* against the toxic actions of notexin (Table 3). NSI protected all mice against up to 4 $\times$ TD for notexin and was non-lethal in isolation. Human serum or BSA at the same protein dosage (50 μ g) was not protective.

DISCUSSION

The sera of several snakes have been shown to possess PLIs, most probably as a host defence mechanism against the toxic PLA₂ enzymes produced in the snakes' venom glands. In the present study a total of six PLIs have been purified from the sera of Australian elapids. Significantly, sera from three of the six snakes investigated were from single specimens. This is the first time that PLIs have been reported to be purified from the serum of a single snake. This was pursued so that the presence of isoforms could be satisfactorily demonstrated at the protein level. Three α -chain isoforms were identified in NAsI and OSI, whereas two α -chain isoforms were identified in PTI. Two α - and β -chain isoforms were also identified in OMI; however, this was purified from pooled sera from a few (two to four) captive snakes. From the use of two batches of *N. ater* sera pooled from a large number of snakes, it is evident that batch variation can be seen: up to five α -chain isoforms exist at the protein level (Figure 3). It is therefore likely that different specimens of the same species of snake possess slightly modified α -chains (the β -chain was consistent in all cases). This is not unusual; individual variation in venom components has been well documented [42,43,46].

PTI is the only elapid snake in which multiple forms of the β -chain have been conclusively identified by using sera collected from a single specimen. This result suggests that the β -chain is under less evolutionary pressure than the α -chain. From a structural and functional perspective it is tempting to speculate that the role of the β -chain is structural rather than functional. A model can be envisaged in which the role of the α -chains is to interact with the PLA₂ enzyme, whereas the function of the β -chain is to hold the two α -chains in the correct three-dimensional orientation. This hypothesis will need further examination before a clear conclusion can be drawn.

The suggestion that each PLI purified here was composed of two α -chains and one β -chain in the native complex raises the question as to the subunit composition of the intact PLI. In other words, does the native complex contain multiple copies of the same α -chain or is the intact complex composed of a random mixture of α -chains? The same question can be posed for PTI, in which two β -chain isoforms were identified. This then raises the question of whether or not functional differences exist between the isoforms and hence the intact PLI complex. As yet, it has proved impossible to separate the α - and β -chains without irreversible loss of activity; the same is true for the separation of

Figure 6 Inhibition of various snake-venom PLA₂ activities by NSI

NSI (in the form of the SPP) was tested against a range of whole snake venoms: ■, *N. scutatus*; ▲, *P. textilis*; ▼, *V. russelli*; ◆, *A. bilineatus*; ●, *N. melanoleuca*; □, *C. atrox*; △, *B. alternatus*. Averaged results are represented; error bars are omitted for clarity. See the Experimental section for further details.

Figure 7 Inhibition of PLA₂ activity by NAI

(A) NAI (in the form of the SPP) was tested against *N. scutatus* venom (333 ng per assay; ■), pig pancreatic PLA₂ (3330 ng per assay; ◆) and bee-venom PLA₂ (25 ng per assay; ▲). Results are shown as means ± S.E.M. (B) NAI (in the form of the SPP) was tested against OA and RA synovial fluid aspirates: ■, sample 1 OA; ●, sample 2 OA; ▲, sample 3 OA; ▼, RA. Results are shown as means ± S.E.M. See the Experimental section for further details.

the isoforms. Therefore it is not yet possible to determine the structural or functional role of the α - or β -chain isoforms.

All α -chains were found to be variably glycosylated with N-linked sugars (Table 2), whereas no O-glycosylation was detected. The α -chains of NAI, NAsI, OSI and PTI possess virtually identical glycosylation patterns (Table 1); the mass of the major carbohydrate constituent is characteristic of a biantennary complex. OMIx, in contrast, has a relatively complex glycosylation pattern with a total of five glycoforms (Tables 1 and 2); the mass of the major carbohydrate constituent is characteristic of a bisecting biantennary complex. All other snake PLIs also have at least one chain glycosylated, suggesting the carbohydrate might have a functional role in the inhibitory activity of the PLIs. However, work performed in our laboratory indicates that the carbohydrate moiety has little or no role in the inhibition of enzymic activity [47]. This finding has recently been confirmed for another elapid PLI [25]. For the PLIs that have been sequenced, consensus N-linked glycosylation sites have been identified, or glucosamine has been detected after amino acid analysis of the purified inhibitor [24,27,30,32–34,37]. Furthermore, the masses of the carbohydrates identified, although not examined as thoroughly as here, indicate that the carbohydrate moieties vary from 3 to 5 kDa in molecular mass [23,30], suggestive of a similar glycosylation pattern for Class A and B PLIs. The sole member of Class C PLIs has a total of four consensus N-glycosylation sites and a carbohydrate molecular mass of approx. 9 kDa [37]. The lack of functional activity of these carbohydrate moieties raises the question of why the pattern varies between PLIs. This suggests that the role of the carbohydrate might be related to physiological functions *in vivo*.

From the inhibition studies it is clear that both NSI and NAI are capable of inhibiting all the PLA₂ enzymes that they were tested against; these are the first PLIs to demonstrate such a broad inhibitory profile. Results show that NSI is capable of inhibiting all the snake-venom PLA₂ enzymes tested with a high level of inhibition, at least 80% in all cases. It is perhaps not surprising that NSI inhibits the PLA₂ enzymes of closely related snakes at lower concentrations in comparison with the non-Australian snakes (Figure 6). It is difficult to compare these results directly because the venom concentrations were chosen to display a similar enzymic activity in the assay, so the quantity of venom, and thus that of PLA₂, added varies considerably. Nevertheless, it is apparent NSI inhibits the enzymic activity in a concentration-dependent manner. It is also important to note that the PLA₂ enzymes inhibited encompassed acidic, basic and neutral enzymes and had monomeric, dimeric and pentameric three-dimensional structures. Thus the mode of interaction between the PLI and the PLA₂ either is unique for each category of PLA₂ or, more probably, occurs via a common structural (or sequence) motif.

The results for NAI reveal similar findings. It should be noted that a different substrate (10pPG) was used for this assay because the human type II PLA₂ does not cleave 10pPC in this assay system. Clearly NAI is as effective at inhibiting whole *N. scutatus* venom as NSI (Figures 6 and 7). This indicates that the two proteins share similar properties and that the inhibition is not dependent on the phospholipid substrate used in the assay. NAI also inhibits bee-venom PLA₂ strongly even at the lowest concentration used. This is significant because the three-dimensional structure of bee-venom PLA₂ [48] is quite different from that of notexin [49]. NAI also inhibits pig pancreatic PLA₂, although the level of inhibition observed is not as high as for either bee-venom PLA₂ or *N. scutatus* venom; this is most probably a result of the large amount of pig PLA₂ added to the assay (3.3 µg per assay).

Table 3 Protection studies *in vivo*

Neonatal mice were injected subcutaneously with 100 μ l of each sample and incubated for 17 h at 30 °C, after which survival was recorded. Samples were as follows; 1 $\times$ TD, 2 $\times$ TD, 3 $\times$ TD and 4 $\times$ TD plus 50 μ g of NSI (in the form of the SPP); BSA, BSA (50 μ g) plus TD; human serum, human serum (50 μ g) plus TD; notexin control, TD plus saline; SPP control, SPP plus saline. Preparations were incubated at 37 °C for 1 h before injection. TD for notexin was 0.75 μ g per mouse subcutaneously.

Sample tested	Protection (alive/total)
1 $\times$ TD + SPP	3/3
2 $\times$ TD + SPP	3/3
3 $\times$ TD + SPP	3/3
4 $\times$ TD + SPP	3/3
BSA + TD	0/3
Human serum + TD	0/3
Notexin control (TD)	0/3
SPP control	3/3

For the first time a PLI has been demonstrated to inhibit the enzymic activity of human type II PLA₂, which is found in OA and RA patients [50–52]. All samples of OA synovial fluids and the single RA sample were inhibited by the actions of NAI. This finding suggests that a natural inhibitor such as NAI, or a modified derivative thereof, might prove useful in the treatment of arthritis patients. This also suggests that such natural inhibitors can be used in the wide-ranging diseases in which PLA₂ has been implicated as having a role [53,54].

Protection studies *in vivo* showed that NSI, when preincubated with notexin, was able to protect against the lethal effects of the potent neurotoxin (Table 3). NSI protected against up to four times the TD of notexin. These results suggest a role for this PLI in the treatment of snake bite victims. It is envisaged that the PLI will be used in conjunction with the currently available anti-venenes in an effort to decrease the amount of anti-venene administered and hence to decrease the chance of serious reactions such as anaphylactic shock and serum sickness.

The size of the native complex and the type of interaction with notexin were investigated by size-exclusion chromatography. The size of NSI was estimated as 110 kDa in its native state. Absorbance data from RP-HPLC separation of the subunits suggest that the molar ratio of α -chain to β -chain is 2:1, so a single intact complex of NSI is most probably composed of two α chains and one β chain. The difference between the measured mass of the complex (110 kDa) and the calculated mass (approx. 65 kDa) is believed to be a result of α -chain glycosylation. Glycoproteins are well known for their increase in apparent molecular mass when measured by size-exclusion chromatography, although the possibility cannot be excluded that the complex might be composed of four α chains and two β chains.

The association is non-covalent because the α - and β -chains separate when run on SDS/PAGE under non-reducing conditions (Figures 5A and 5B). Furthermore, NSI formed a non-covalent association with notexin (Figures 4 and 5). It is apparent that any peak containing NSI also contained notexin, highlighting this association. Because the native molecular mass of notexin is 13.5 kDa it must be binding to NSI for it to be eluted with a shortened retention time. Because there was a molar excess of notexin over NSI, the excess notexin was also eluted at its normal elution volume. Clearly the mode of inhibition exhibited by NSI, and presumably the other PLIs characterized here, is therefore to bind directly to the PLA₂ enzyme and

prevent it from hydrolysing the substrate. Whether the NSI/PLA₂ complex is able to bind either monomeric or micellar phospholipid substrate is yet to be determined.

From this study, it is envisaged that snake serum PLIs might prove to be useful as an adjunct to anti-venene treatment or, more importantly, might prove useful in the treatment of the various diseases in which PLA₂ enzymes have been implicated.

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