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Functional site of endogenous phospholipase A_2 inhibitor from python serum

Phospholipase A_2 binding and anti-inflammatory activity

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The functional site of 'phospholipase A_2 inhibitor from python' (PIP) was predicted based on the hypothesis of proline brackets. Using different sources of secretory phospholipase A_2 ($sPLA_2$s) as enzyme, and [^{3}H]arachidonate-labelled *Escherichia coli* as substrate, short synthetic peptides representing the proposed site were examined for their secretory phospholipase A_2 ($sPLA_2$) inhibitory activity. A decapeptide P-PB.III proved to be the most potent of the tested peptides in inhibiting $sPLA_2$ enzymatic activity *in vitro*, and exhibited striking anti-inflammatory effects *in vivo* in a mouse paw oedema model. P-PB.III inhibited the enzymatic activity of class I, II and III PLA_2s, including that of human synovial fluid from arthritis patients. When tested by ELISA, biotinylated P-PB.III interacted positively with various PLA_2s, suggesting that the specific region of PIP corresponding to P-PB.III, is likely to be involved in the PLA_2–PLI interaction. The effect of P-PB.III on the peritoneal inflammatory response after surgical trauma in rats was also examined. P-PB.III effectively reduced the extent of postsurgical peritoneal adhesions as compared to controls. $sPLA_2$ levels at seventh postoperative day in the peritoneal tissue of P-PB.III-treated rats were also significantly reduced ($P < 0.05$) in comparison to those of the untreated controls. The present results shed additional insight on the essential structural elements for PLA_2 binding, and may be useful as a basis for the design of novel therapeutic agents.

Keywords: anti-inflammatory peptide; phospholipase inhibitor from python PIP; protein–protein interaction; phospholipase A_2 inhibitors; postsurgical adhesions.

Secretory phospholipases A_2 ($sPLA_2$s) are enzymes (EC.3.1.1.4) that catalyse the hydrolysis of the *sn*-2 acyl bond of glycerophospholipids to produce free fatty acids and lysophospholipids [1], and are implicated in a range of diseases associated with inflammatory conditions such as arthritis, peritonitis, etc. [2–5]. Furthermore, PLA_2 inhibitors (PLIs) have recently become the subject of much interest due to the potential benefits they could offer in the treatment of inflammation and cell injury.

A number of PLIs have been purified and characterized from a variety of sources, including plant, fungi, and bacteria [6–8]. PLIs that interact with PLA_2s and inhibit their enzymatic activity have been identified in the sera of venomous snakes belonging to Elapidae and Crotalidae families [9–20]. The discovery of specific $sPLA_2$ inhibitors has also been reported in the blood serum of nonvenomous snakes [21,22]. These studies have demonstrated the presence of three different types of PLIs (α, β and γ) in the sera of snakes, which are believed to have a natural defensive role against endogenous snake venom $sPLA_2$s.

Our recent cloning and expression study has revealed that the PLI termed 'phospholipase inhibitor from python (PIP)' possesses potent nonspecies specific antitoxic and anti-inflammatory activities, which have been linked to its ability to inhibit $sPLA_2$ [22]. This inhibitor signifies structural homology with other γ-type snake PLIs [12,14,18] and various mammalian proteins belonging to the 'three fingers' neurotoxin superfamily, including the urokinase-type plasminogen-activator receptor, membrane proteins of the Ly-6 family, and a bone-specific protein RoBo-1 [12,23].

On the basis of sequence homology study, some groups have been able to identify short peptides that act as a surrogate for the larger molecule [24], and their usefulness as potential anti-inflammatory agents have been reported [25]. Short peptides called antiflammins that are synthesized based on the region of highest homology between uteroglobin and lipocortin I, have previously been shown to inhibit PLA_2 [24,25], although there are some reports suggesting that these antiflammins are devoid of PLA_2 inhibitory activity [26,27]. Recently, the importance of proline brackets flanking protein–protein interaction sites has been emphasized in identifying potential functional sites in proteins [28]. Following this hypothesis, we were able to identify the active site on PIP that binds to $sPLA_2$s potently in a nonspecies-specific manner. In the present study, a short oligopeptide, corresponding to the segment of the hypothetical interaction site has been synthesized and examined for its anti-inflammatory activity and PLA_2 binding, with a

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Abbreviations: PLA_2, phospholipase A_2; AIP, anti-inflammatory peptide; IC_{50}, concentration of the inhibitor that inhibits PLA_2 activity by 50%; PIP, phospholipase inhibitor from python; PLI, phospholipase A_2 inhibitor; $sPLA_2$, secretory phospholipase A_2.

Note: a web site is available at http://www.med.nus.edu.sg/ant/anatomy.htm

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view to locate the particular region on PLIs that is responsible for binding to PLA_2.

EXPERIMENTAL PROCEDURES

Materials

All the venoms and PLA_2 toxins used in the experiments were available from the VTRP (Venom and Toxin Research Programme) collections, except for the bee (*Apis mellifera*) venom PLA_2, which was purchased from Sigma Chemical Co. (St Louis, MO, USA). Anti-inflammatory peptide 2 (antiflamin 2), *o*-phenylenediamine dihydrochloride ([2-(biotinamido)ethylamido]-3,3′-dithiodipropionic acid *N*-hydroxysuccinimide ester, and avidin-peroxidase conjugate were purchased from Sigma. UniverSol ES liquid scintillation cocktail was from ICN Biomedicals, Inc., USA; Hylan GF 20 (Synvisc) gel was purchased from Bayer Pte. Ltd (Singapore). All other reagents were of analytical grade or better.

Animals

Swiss albino mice (20–25 g) used for paw oedema assay and the Sprague–Dawley rats (250–320 g) used in the incisional hernia model were purchased from the Laboratory Animals Centre, Sembawang, Singapore, and housed in the Animal Holding Unit of the Department of Anatomy, National University of Singapore for 2 weeks to acclimatize the animals prior to use. Water and food (Glen Forrest Stockfeeders, WA, Australia) were provided *ad libitum* and a 12-h light/12-h dark cycle was maintained. The animals were handled according to the Guidelines of the National Medical Ethics Committee (Singapore), which conform to the World Health Organization's International Guiding Principles for Animal Research [29].

Peptide synthesis

The peptides with the sequences LSLQNGLY and PGLPLSLQNG, designated P-PB.II and PB.III, respectively, were custom-synthesized at the Biotechnology Processing Centre, National University of Singapore, by conventional solid phase techniques using automated ABI 4338 Peptide Synthesizer. The test peptide, designated P-PB.I with the sequence LPGLPLSLQNGLY, and the control peptide designated SP-PB.III, containing the same amino-acid composition as that of P-PB.III, but with the scrambled sequence, QLNPLPGLGS, were synthesized at the Fukuoka Women's University, Japan. All the synthetic peptides were purified by RP-HPLC to more than 95% purity, with yields between 85 and 90%. The sequences were validated by MALDI-MS (Voyager-DESTR BioSpectrometry Workstation).

$SPLA_2$ assay with [^{3}H]arachidonate-labelled *E. coli*

Enzyme activity was assayed according to the described method [30] with minor modifications. Briefly, the reaction mixture contained 200 μL of assay buffer (100 mM Tris/HCl pH 7.5, 25 mM $CaCl_2$), 20 μL of [^{3}H]arachidonate-labelled *E. coli* suspension (0.005 mCi·mL^{-1}; 5.8 μCi·μmol^{-1}, NEN) and 30 μL (10 ng) of daboiatoxin, crotoxin subunit B, β-bungarotoxin, bee venom PLA_2 (Sigma, 1360 U·mg^{-1}), or human synovial fluid, in a total volume of 250 μL. After incubation of the mixture (37 °C, 1 h) and termination of the reaction with 750 μL of chilled NaCl/P_i containing 1% BSA, the microfuge tubes containing the samples were centrifuged (10 000 ***g***, 4 °C) for 15 min, and 500-μL aliquots of the supernatant taken to measure the amount of ^{3}H-labelled arachidonate released from the *E. coli* membrane using liquid scintillation counting (Multipurpose Scintillation Counter LS 6500; Beckman). Appropriate controls without PLA_2 were also included in the assays. To determine the inhibitory activity, daboiatoxin or different source of PLA_2s was preincubated for 1 h at 37 °C with each peptide at varying concentrations (1–250 μM), before addition of the *E. coli* substrate suspension. As controls for the inhibition assays, PLA_2 was preincubated with the assay buffer. All samples, including the controls, were performed in triplicate and plotted as the percentage inhibition relative to negative controls.

IC_{50} determination

IC_{50} was determined by preincubating varying concentrations (1–250 μM) of peptides in a constant volume, against a constant amount of enzyme as described earlier. A sigmoid dose–response curve was generated to allow calculation of the IC_{50} values. All samples were performed in triplicate. Results were analyzed by nonlinear regression with GraphPad PRISM (version 2.01) and expressed as the percentage of inhibition relative to control values.

Biotinylation of peptide

Five-hundred micrograms of peptide P-PB.III (0.36 μmol) was dissolved in 1 mL of 0.1 M $NaHCO_3$ pH 7.5, and the biotinylation reaction was initiated by addition of 60 μL (1.08 μmol) of the biotin disulfide *N*-hydroxysuccinimide ester solution to the peptide solution. The molar ratio of the peptide to biotin used in the reaction was 1 : 3. After incubation of the reaction mixture at 25 °C for 1 h, the reaction was stopped, and unreacted biotinylating agent was removed by dialyzing against 2-L volumes of NaCl/P_i (three changes) at 4 °C using Spectra/Por6 membrane (molecular mass cutoff 1000; SPECTRUM Medical Industries, Inc.). To check the purity, the biotinylated P-PB.III was injected onto a Vydac C_{18} RP-HPLC column and eluted with a linear gradient of solvent A (0.1% trifluoroacetic acid) and solvent B (100% acetonitrile/0.1% trifluoroacetic acid) at a flow rate of 1 mL·min^{-1}. The column eluate was monitored at 215 nm and 1-min fractions were collected. In addition, HPLC-purified biotinylated P-PB.III was subjected to MS analysis.

ELISA

Wells of microtitre plates (Dynex Technologies, Inc., USA) were coated overnight at 4 °C with 100 μL of different sources of either the venom (5 μg·mL^{-1}) or PLA_2 (1 μg·mL^{-1}) in 100 mM carbonate/bicarbonate buffer, pH 9.6. The controls wells were coated with buffer only. All washing steps were carried out at least three times with NaCl/P_i/Tween throughout. The coated plates were washed, and unbound sites were saturated by incubating

for 1 h at 37 °C with 150 μL of 3% fat-free milk powder (Bio-Rad) in $NaCl/P_i$/Tween. After washing, the wells were incubated with 100 μL of biotinylated P-PB.III (1 $\mu g \cdot mL^{-1}$) in $NaCl/P_i$/Tween for 1 h at 37 °C, washed again and incubated further for 1 h at 37 °C with 100 μL of Avidin-peroxidase conjugate (Affinity purified, Sigma) at a dilution of 1 : 2000 in $NaCl/P_i$/Tween. After washing, 100 μL of substrate solution (0.5 $g \cdot L^{-1}$ *o*-phenylenediamine di-HCl/ 0.02% H_2O_2; Sigma) was added to each well and the enzymatic reaction stopped by adding 50 μL of 2 M H_2SO_4 prior to reading the absorbance at 490 nm (Emax Precision Microplate Reader, Molecular Devices).

Effect of active peptide on PLA_2-induced mouse paw oedema

The oedema produced by the crude venom or purified PLA_2s from *Daboia russelli siamensis* venom or bee venom, was assayed according to the method described [31]. Male Swiss albino mice (20–25 g) in groups of four were injected subcutaneously into the footpad of the left hind paw with the indicated amounts of venom or PLA_2s (5 μg venom; 1 μg daboiatoxin or bee venom PLA_2) in a total volume of 25 μL of sterile $NaCl/P_i$. At 45 min thereafter, the mice were euthanized using CO_2 insuffulation, and both hind limbs disarticulated at the ankle joint were individually weighed. The increase in weight due to oedema was calculated by subtracting the weight of each nontreated right hind limb. To study the effect on PLA_2-induced paw oedema, venom (5 μg) or PLA_2s (1 μg) were preincubated with varying concentrations of the inhibitors (PIP, 0.5, 1 nmol; P-PB.III, 50, 100 nmol; AIP-2, 92 nmol), in a total volume of 25 μL prior to injection. Inhibitory effects were assessed by comparing the paw oedema of inhibitor-treated groups to that of nontreated groups. Inhibitors alone or $NaCl/P_i$ alone were injected as controls.

Effect of active peptide on postsurgical peritoneal adhesions

An *in vivo* incisional hernia model [32] was used to assess the potential therapeutic application of the active peptide P-PB.III in reducing the formation of postsurgical peritoneal adhesions in male Sprague–Dawley rats (250–320 g). Under light ether anesthesia and by means of a midline laparotomy incision, a ventral abdominal defect (15 × 25 mm) was created in each of the 30 rats, which were divided into four groups. The caecum was located, externalized and the serosal surface abraded, using dry gauze until subserosal punctate hemorrhage was seen. A polypropylene mesh (Surgipromesh, Autosuture Co.) was then sutured to the abdominal defect. Prior to closure of the abdominal skin, a hyaluronate-based gel (Hylan GF 20), either alone or with an anti-inflammatory peptide, was administered intraperitoneally over the abraded caecum. Group I ($n = 12$) contained only the mesh to serve as a control; group II ($n = 6$) contained exclusively the gel, while groups III ($n = 6$) and IV ($n = 6$) contained the gel spiked with 0.16 μmol each of the anti-inflammatory peptides, P-PB.III and AIP-2, respectively. On postoperative day 7, a re-laparotomy was performed and peritoneal adhesions were graded using a method previously described [33].

Peritoneal tissue $sPLA_2$ activity

The peritoneal tissue specimens collected from each rat at day 0 and on postoperative day 7 were stored immediately at −80 °C until the time of analysis. Approximately 150–250 mg (wet weight) of the peritoneal tissues were weighed and homogenized in 2 mL of $NaCl/P_i$ using Heidolph DIAX900 homogeniser (Germany). Supernatant collected after centrifugation (20 000 ***g***) at 4 °C for 20 min was used for measurement of total protein [34] and PLA_2 activity [30]. For each sample, the mean and standard deviations were obtained for replicates ($n = 3$).

Statistical analysis

The results from the paw oedema experiment in mice were analyzed by a one-tailed Student's *t*-test for groups of unpaired observations. Significance was taken at $P < 0.05$. The statistical significance of the effects of the peptides was also confirmed by one-way ANOVA.

Wilcoxon rank sum test was used for analyzing differences in peritoneal tissue PLA_2 activity at two different time points, day 0 (at the time of surgery) and day 7 (after surgical trauma). The significance of the difference in the postoperative peritoneal tissue PLA_2 activity at day 7 between the P-PB.III-treated and untreated groups were analyzed by nonparametric Mann–Whitney *U*-test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

PIP has significant amino-acid sequence homology with other snake PLIs

The nonredundant BLASTP alignment of the amino-acid sequence of a mature PIP monomer with the database sequences whose match satisfies the preset *E* value of 0.001 is shown in Fig. 1. The mature PIP protein contains 16 cysteine residues all of which align perfectly in the database matched sequences. It has the highest sequence identity (57–61%) to the mature PLIs from the sera of Crotalidae snakes, *Agkistrodon blomhoffii siniticus* [14], *Crotalus durissus terrificus* [11,13], and *Trimeresurus flavoviridis* (*Protobothrops flavoviridis*) [9,15], with sequence identities of 61, 60 and 57%, respectively. PIP also has a significant (57%) homology to the sequences of mature PLIs of a nonvenomous snake *Elaphe quadrivirgata* [21], and also to those of the PLIs from the sera of Australian Elapidaes, *Notechis ater*, *Notechis scutatus*, and *Oxyuranus scutellatus* [19], with sequence identities in the vicinity of 56%.

The potential interaction site on PIP is predicted by searching for proline residues that mark the flanks of protein–protein interaction sites

The amino-acid sequence of PIP (Fig. 1) shows four proline residues at positions 85, 87, 90 and 100. As the residues at position 85 and 100, and 90 and 100, respectively, served as the flanking prolines enclosing a small segment of the PIP in each case, we predicted that the segments, LPGLPLSLQN GLY (P-PB.I) and/or LSLQNGLY (P-PB.II), might indicate possible interaction site for PIP with $sPLA_2$. Both these peptides displayed *in vitro* PLA_2 inhibitory activity, but

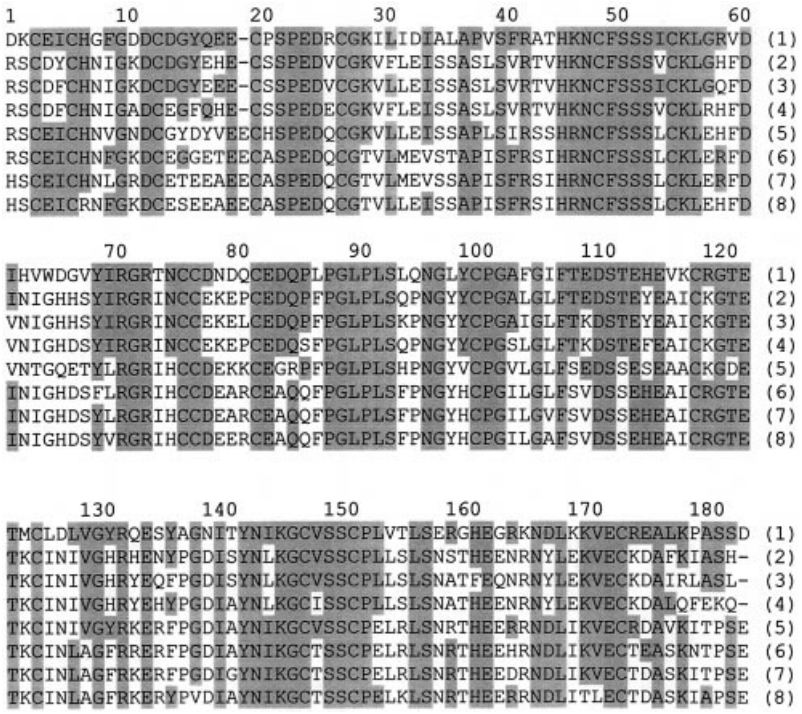

Fig. 1. Alignment of the mature PIP monomer with the database sequences. The *E* value was preset at 0.001 for matching the amino-acid sequences. The shaded boxes indicate residues identical to those of PIP. (1) *Python reticulatus* PIP; (2) *Agkistrodon blomhoffii siniticus* PLIγ; (3) *Crotalus d. terrificus* CNF; (4) *Protobothrops flavoviridis* PLIγ; (5) *Elaphe quadrivirgata* PLIγ; (6) *Notechis ater* α subunit isoform NAI-3 A; (7) *Notechis scutatus* α chain iii; (8) *Oxyuranus scutellatus* α subunit isoform OSI-1 A.

P-PB.I was the only peptide that was found to possess remarkable *in vivo* anti-inflammatory activity, while P-PB.II was less active. With P-PB.I being more active than P-PB.II, it was assumed that the bioactivity might be related mainly to that particular segment of PIP. The third peptide P-PB.III with the sequence PGLPLSLQNG, which represents the shorter segment of the proposed site, exhibited the strongest anti-PLA_2 and anti-inflammatory activities, while the scrambled peptide SP-PB.III was found to be totally devoid of PLA_2-inhibitory activity (Table 1).

Synthetic peptides derived from the hypothetical interaction site inhibit PLA_2 enzyme activity and bind to $sPLA_2$s

The dose–response relationships for the synthetic peptides and the full-length recombinant PIP were determined and are shown in Fig. 2. The 13-residue peptide P-PB.I, which corresponds to PIP residues 86–98, is a strong inhibitor against the PLA_2 activity of daboiatoxin (IC_{50} 37.82 ± 2.40 μM), while the octapeptide P-PB.II, is less potent (IC_{50} 45.09 ± 1.14 μM). Among the three synthetic peptides examined for PLA_2 inhibitory activity, the decapeptide P-PB.III, corresponding to PIP residues 87–96, is the strongest inhibitor that possesses PLA_2 inhibitory potency (IC_{50} 22.65 ± 2.91 μM) equivalent to that of the recombinant inhibitor PIP (IC_{50} 19.51 ± 2.06 μM). P-PB.III dose-dependently inhibits the enzyme activity of a variety of $sPLA_2$ sources from snakes, bee and human, over a wide concentration range (1–250 μM), while the scrambled peptide SP-PB.III, fails to inhibit $sPLA_2$ at any concentration tested. Biotinylation of the active peptide, P-PB.III does not seem to result in considerable loss of inhibitory potency as judged by similar IC_{50} values obtained for the native P-PB.III (IC_{50} = 22.6 ± 2.9 μM) and its biotinylated product (IC_{50} = 25.8 ± 3.1 μM) in the binding assays.

The experimental evidence of the fact that P-PB.III interacts with $sPLA_2$ is demonstrated by ELISA and shown in Fig. 3. The purity of the biotinylated P-PB.III as evaluated by RP-HPLC was 95%, and the determined

Table 1. Amino-acid sequences and properties of peptides derived from the predicted site. Test peptides P-PB-I, II, III and the control scrambled peptide S-PB.III were synthesized by solid phase techniques. Experimental details are described in the Experimental procedures. PLA_2 inhibition indicates maximal enzyme inhibition towards daboiatoxin seen at a fixed peptide concentration (100 μM). IC_{50} values were calculated from the corresponding dose–response curves shown in Fig. 2, by nonlinear regression analysis with GraphPad PRISM (version 2.01). Anti-inflammatory activity was assessed by daboiatoxin-induced mouse paw oedema experiments. Values reported are the mean of triplicate experiments.

Code no.	Sequence	Length	M_r	PLA_2 inhibition (%)	IC_{50} (μM)	Anti-inflammatory activity
P-PB.I	LPGLPLSLQNGLY	13	1385	70.71	37.82	(+) moderate
P-PB.II	LSLQNGLY	8	1018	51.69	45.09	(–) negative
P-PB.III	PGLPLSLQNG	10	995	91.60	22.60	(+ +) strong
SP-PB.III	GLNPLPGLQS	10	995	0	–	Not tested

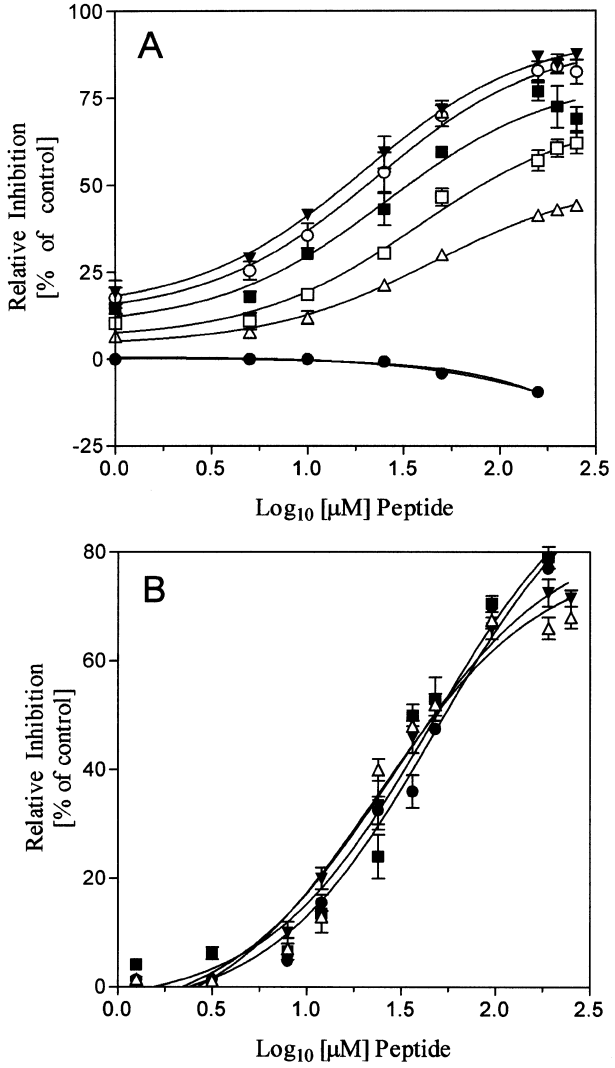

Fig. 2. Phospholipase A$_2$ inhibition curves for PIP and various synthetic peptides. (A) Inhibition profiles against daboiatoxin PLA$_2$ activity: PIP (▼); PIP-derived test peptides, P-PB.III (○), P-PB.I (□), P-PB.II (△); control scrambled peptide, SP-PB.III (●); Biotinylated P-PB.III (■). (B) Inhibition profiles of the active peptide P-PB.III against enzymatic activity of various sources of sPLA$_2$s – β-bungarotoxin (△), crotoxin B (▼), bee venom PLA$_2$ (■), human synovial fluid (●). Results are the mean ± SD. IC$_{50}$ values were graphically determined from the inhibition curves, constructed on the basis of the *in vitro* results of ^{3}H-labelled *E. coli* membrane assays.

mass was 1118 Da. Based on the mass (998 Da) of the intact peptide and that of the biotinylated P-PB.III, 0.12 mol biotin was apparently bound to 1 mol of P-PB.III.

Most venoms and PLA$_2$s examined, reacted positively with the biotinylated P-PB.III, although the results vary depending upon the type of PLA$_2$ used. P-PB.III interacts very strongly with group I PLA$_2$ toxin, β-bungarotoxin, but binds moderately to group II PLA$_2$ toxins like daboiatoxin, mojave toxin subunit B, ammodytoxin A and crotoxin. It gives strong positive ELISA reaction with the enzymatically active basic subunit of crotoxin while its binding to the non-PLA$_2$ acidic subunit of crotoxin is negligible. Interestingly, the biotinylated peptide P-PB.III

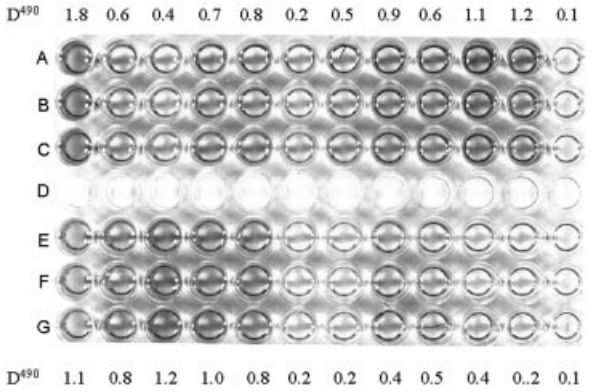

Fig. 3. Binding of P-PB.III to various sources of sPLA$_2$ in ELISA. Biotinylated P-PB.III was directed against different sources of sPLA$_2$ or crude venom coated on microtitre plate wells. Bound biotinylated peptide in each well was detected with avidin–peroxidase conjugate and color development with a substrate solution as described in the Experimental procedures. All samples were measured in triplicates and the mean signals (A_{490}) are shown over each well area. Rows (A–C), from left to right: β-bungarotoxin, mulgatoxin, taipoxin, crotoxin, crotoxin B, crotoxin A, ammodytoxin A, daboiatoxin, mojave toxin B, bovine pancreatic PLA$_2$, human synovial fluid, blank. Rows (E–G), from left to right: venoms of *N. siamensis*, *P. australis*, *O. hannah*, *N. kaouthia*, *B. multicinctus*, *E. carinatus*, *C. rhodostoma*, *D. siamensis* (Myanmar), *D. russelli* (India), *D. pulchella* (Sri Lanka), *D. siamensis* (Thailand), blank.

also reacted strongly with the human synovial fluid collected from arthritic patients.

The synthetic peptide corresponding to the active site has marked anti-inflammatory activity

The anti-inflammatory effects of P-PB.III, in comparison to those of the full-length recombinant PIP and the anti-inflammatory peptide (antiflammin 2) is reported in Table 2. Co-injection of P-PB.III, either with the venom, toxic PLA$_2$ (daboiatoxin), or the bee venom PLA$_2$ into the mouse footpad significantly ($P < 0.01$) inhibits the formation of inflammatory oedema over two different dose ranges (50, 100 nmol), with a higher suppression of the inflammatory response seen at a higher dose. In contrast, AIP-2, is less potent then P-PB.III. Comparison of the dose–responses of the recombinant PIP (0.5, 1 nmol) and P-PB.III (50, 100 nmol) by one-way ANOVA shows that there is no significant difference ($P < 0.05$) between the two forms of inhibitor, thus providing evidence that the peptide P-PB.III retains much of the anti-inflammatory property of the intact parent PIP molecule. Although P-PB.III (100 μg) is as potent as PIP (100 μg) on basis of mass, it is much less potent (≈ 100 fold) on a molar basis.

Intraperitoneal administration of P-PB.III reduces peritoneal tissue PLA$_2$ activity and modulates peritoneal inflammatory response after surgical trauma

With the aim of investigating the potential therapeutic application of P-PB.III, the effect of the peptide in reducing peritoneal inflammatory response was studied in an *in vivo*

Table 2. Anti-inflammatory effect of inhibitors on PLA_2-induced mouse paw oedema. Experimental details are described in the Experimental procedures. Inhibitory effects were expressed as percentage inhibition of paw oedema, and were assessed by comparing the paw oedema (increase of wt. in mg) of mice receiving (PLA_2 + inhibitor) to those receiving PLA_2 alone. The results (mean ± SD; $n = 4$) were analyzed by a one-tailed Student's *t*-test for groups of unpaired observations (significance taken at minimum of $P < 0.05$). PIP, phospholipase inhibitor from python; P-PB.III, active peptide; AIP-2, anti-inflammatory peptide-2 from Sigma.

Treatment	nmol (μg)	Oedema (mg)	% Inhibition
D.r. siamensis venom	– (5)	117 ± 20	–
+ PIP	1 (100)	29 ± 1 ($P < 0.01$)	74.7 ± 0.8
+ P-PB.III	100 (100)	33 ± 6 ($P < 0.01$)	71.1 ± 5.5
+ AIP-2	92 (100)	76 ± 11 ($P < 0.05$)	35.0 ± 9.7
Daboiatoxin PLA_2	(1)	166 ± 9	–
+ PIP	0.5 (50)	18 ± 5 ($P < 0.01$)	89.2 ± 3.0
+ PIP	1.0 (100)	13 ± 3 ($P < 0.01$)	92.2 ± 1.8
+ P-PB.III	50 (50)	63 ± 7 ($P < 0.01$)	62.1 ± 4.0
+ P-PB.III	100 (100)	35 ± 6 ($P < 0.01$)	79.0 ± 3.6
+ AIP-2	92 (100)	108 ± 11 ($P < 0.01$)	35.3 ± 6.5
Bee venom PLA_2	(1)	89 ± 6	–
+ PIP	0.5 (50)	52 ± 3 ($P < 0.01$)	39.9 ± 3.8
+ PIP	1.0 (100)	19 ± 2 ($P < 0.01$)	78.1 ± 2.1
+ P-PB.III	50 (50)	42 ± 4 ($P < 0.01$)	51.7 ± 4.2
+ P-PB.III	100 (100)	31 ± 4 ($P < 0.01$)	63.8 ± 4.8
+ AIP-2	92 (100)	60 ± 7 ($P < 0.01$)	33.6 ± 3.9
PIP alone	1.0 (100)	9 ± 8	–
P-PB.III alone	100 (100)	13 ± 2	–
AIP-2 alone	92 (100)	9 ± 4	–

Table 3. Effect of anti-inflammatory peptides on peritoneal inflammatory response in individual rats after surgical trauma. Experimental details are described in the Experimental procedures. Values reported are the means ± SD, where $n = 6$–12 rats. One-tailed Student's *t*-test for groups of unpaired observations was done with significance tested at $P < 0.05$: a vs. b, not significant ($P > 0.05$); a vs. c, significant ($P < 0.05$); a vs. d, not significant ($P > 0.05$). The effects of P-PB.III and AIP-2 were confirmed by one-way ANOVA.

		Adhesion score	
Group no.	Rat no.	Grade	Mean ± SD
I (control)	255–266	4	4.0 ± 0 [a] ($n = 12$)
II (with gel only)	267	4	3.16 ± 2.82 [b] ($n = 6$)
	268	4	
	271	4	
	270	3	
	269	2	
	272	2	
III (with gel + P-PB.III)	275	1	2.00 ± 0.82 [c] ($n = 6$)
	276	1	
	278	2	
	273	2	
	274	3	
	277	3	
IV (with gel + AIP-2)	279	4	3.30 ± 1.07 [d] ($n = 6$)
	284	4	
	281	4	
	280	3	
	283	3	
	282	2	

incisional hernia model in rats. Most animals in the control group (group I) developed dense adhesions, while in the experimental groups (groups II–IV), relatively fewer postsurgical peritoneal adhesions were seen. Intraperitoneal administration of P-PB.III along with the gel to the site of peritoneal injury significantly reduced the peritoneal inflammatory response with fewer postsurgical adhesions ($P < 0.05$), whereas either the gel alone or the gel with AIP-2, was found to be relatively less potent in reducing the postsurgical peritoneal adhesions. Table 3 depicts adhesion grades in individual rats as analyzed by an independent observer who was blinded about the treatment and nontreatment groups.

At day 7 following surgical trauma, the PLA_2 activity of the peritoneal tissue extracts of control rats markedly increased ($P = 0.028$) over the basal levels found at day 0 (Fig. 4A). In contrast, no significant difference in the peritoneal PLA_2 activity ($P > 0.05$) was found between those two levels (day 0 vs. day 7) in the P-PB.III-treated rats (Fig. 4B). Moreover, when the peritoneal tissue PLA_2 levels of P-PB.III-treated (Fig. 4B) and untreated (Fig. 4A) rats at day 7 following surgical trauma were compared, there was a highly significant difference ($P = 0.025$) observed between the controls and the inhibitor-treated animals (Fig. 4A vs. 4B). These results suggest that the active peptide P-PB.III can afford an effective *in vivo* inhibition of total catalytic PLA_2 activity which is apparently increased as a result of trauma after surgery.

DISCUSSION

Identification of a protein–protein interaction site is an important step that has significant potential to clarify structure–function relationships of protein and drug designs. Through a survey of a database of protein–protein

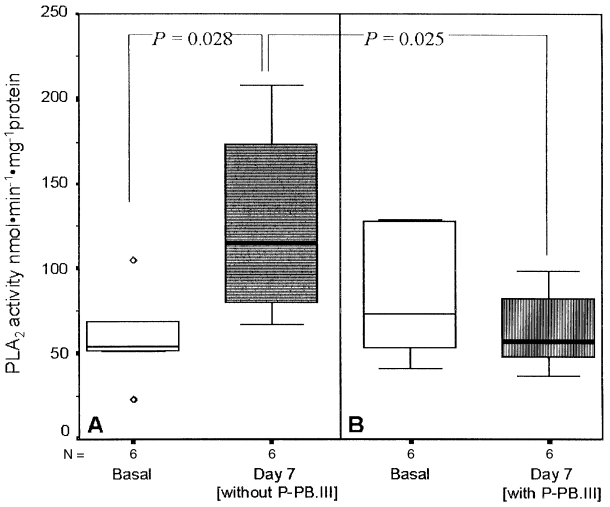

Fig. 4. Box plots of the PLA_2 activity in extracts of rat peritoneal tissue obtained at the time of surgery (basal) and at day 7 following surgical trauma. (A) Control group ($N = 6$) without P-PB.III treatment; (B) Experimental group ($N = 6$) with P-PB.III treatment. Data are presented as median (solid line across the box), 25th and 75th percentiles. Significant differences are indicated by the P values. Outliers are represented by circles; open and hatched boxes represent basal and day 7 samples, respectively.

interaction sites, a unique prediction method to identify those sites has previously been proposed, based on the observation that proline is the most common residue found in the flanking segments of interaction sites [28]. In the present study, we have recognized a proline-rich cluster corresponding to residues 85–100 of PIP and other database sequences in the alignment. Because this proline-rich segment is highly conserved amongst members of the snake serum PLI family, it is a distinguishing feature, and is therefore believed to contribute to the biological activity specifically associated with the snake PLI family.

Hence, using the proline bracket method for predicting interaction sites, we have been able to identify the functional site of PIP belonging to the three fingers neurotoxin superfamily. The present findings provide evidence that the mode of interaction between the PLI and the PLA_2 occurs via a common sequence motif represented by the peptide P-PB.III. This decapeptide displays a diverse inhibitory profile against the enzymatic activity of all types of PLA_2s examined, including that of human secretory PLA_2 present in the synovial fluid of subjects suffering from arthritis. Using a monoclonal antibody specific against human synovial $sPLA_2$ (Calbiochem, USA), we found that $sPLA_2$ activity detected in the synovial fluid was inhibited (data not shown), thus confirming that the enzyme contained in the synovial fluid was in fact, a human group II $sPLA_2$. To ensure that the inhibition displayed by the active peptide against $sPLA_2$s was specific and not artefactual, a dose–response experiment was performed with the peptide P-PB.III, as well as with the control peptide S-P-PB.III, that had scrambled sequence. The peptide P-PB.III inhibited most types of $sPLA_2$s examined, including human synovial $sPLA_2$, while the scrambled peptide was noninhibitory, confirming that the inhibition was not nonspecific. The active peptide also binds to different sources of PLA_2s tested in ELISA. Whatever the species of $sPLA_2$ origin, the wide spectrum of binding to $sPLA_2$s and inhibition of the enzyme activity displayed by the active peptide, coupled with the striking anti-inflammatory effects it possessed, outlines the potential therapeutic usefulness of this inhibitor as an anti-inflammatory agent.

The domain of $sPLA_2$ or PLI involved in inhibitor binding has yet to be fully elucidated although some structural information suggests that the three-finger motifs of PLIs are important for interaction between γ-type inhibitors and PLA_2s [35,36]. The broad spectrum of inhibition seen with the PIP-derived peptide in this study suggests that like PIP and other γ-type inhibitors, it could probably recognize the Ca_2^+-binding loop, which is a common structural element conserved among all groups of secretory PLA_2s, including human synovial $sPLA_2$ [37]. Previous data on epitope mapping and studies with synthetic peptides also suggest that the conserved core region of PLA_2 including most of the Ca_2^+-binding loop may be a potential target for developing selective inhibitors of $sPLA_2$s [38]. Based on ELISA results, it appears that P-PB.III binds directly to $sPLA_2$, perhaps through the residues on the Ca_2^+-binding loop. However, it is highly unlikely that its binding to $sPLA_2$ could involve nonspecific electrostatic interaction, as no charged amino-acid residues, other than the polar and nonpolar residues, are present in the sequence of P-PB.III.

The present results show the oedema-reducing activity of the active peptide, which appears to act via inhibition of PLA_2 activity, and confirms the decapeptide P-PB.III as a potent anti-inflammatory peptide that has potential therapeutic applications, especially for PLA_2-related inflammatory conditions. The *in vivo* postsurgical peritoneal adhesion model in rats indicates that the intraperitoneal administration of the peptide directly to the site of peritoneal injury can reduce the formation of postsurgical adhesions by a mechanism that could involve inhibition of the activation of endogenous $sPLA_2$ [2] and through reduction in the peritoneal inflammatory response that occurs after surgery. These results strongly support that the predicted region indeed plays an important role in the interaction between $sPLA_2$ and the endogenous PLIs of snakes. At present, a crystallographic study is in progress to understand the structural details of PLA_2–PLI interaction.

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