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EVOLUTIONARY REDUCTION OF ENZYMATIC ACTIVITIES OF SNAKE VENOM PHOSPHOLIPASES A_2

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The reaction mechanism of the 14 kDa secreted phospholipases A_2 (PLAs) and examples of venom PLAs with diminished catalytic activities are reviewed. Evolutionary strategies to reduce the venom PLA catalytic power and new function gains are discussed. Down-regulations of the enzymatic activities appear to be due to: 1) retention of interfacial binding, but with selective alternation of active site residues in basic PLAs; 2) mutations at both the interfacial binding sites and catalytic sites of strong anticoagulating PLAs which bind to the coagulation factor; and 3) either substitution or truncation of the interface binding sites in acidic subunits of heterodimeric PLA-neurotoxins to generate chaperon like molecules.

Keywords: Phospholipase A2, Snake venom toxin, Interface binding, Catalytic site, Oxyanion hole, Regulation

Introduction

Phospholipases A_2 (PLAs) are major components in snake venoms and usually exist in multiple isoforms or paralogs. These relatively small (14 kDa, EC3.1.1.4) secreted hydrolases are calcium dependent enzymes, which hydrolyze the *sn-2* ester of glycerophospholipid to produce a fatty acid and a lysophospholipid (Six and Dennis, 2000; Gubensek and Kordis, 1997). The venom PLAs have been classified according to minor structural differences as group IA, IB, IIA and IIB. The former two are present in elapid and hydrophiid venoms while the latter two are present in viperid venoms. Up to 300 venom PLA_2 s have been so far sequenced (Kini et al., 1997; Danse et al., 1997). They show >50% sequence identity and have a conserved 3D-protein

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scaffold (Scott, 1997), but their catalytic activities differ greatly and are not necessarily related to the toxicity (Rosenberg, 1997). The venom PLA families induce a wide spectrum of toxic or pharmacological effects, and some possibly play more than one roles. Thus, their structure–function relationships are subtle and intriguing challenges.

The PLAs gain their full activity only when they bind to phospholipid micelles or membranes, an effect known as interfacial activation (Gelb et al., 1995; Qin et al., 2005). X-ray structures are available for more than 20 of the venom PLAs, both in free form and as complex with catalytic site inhibitors (Kini, 2005; White et al., 1990; Ward et al., 1998; Pan et al., 2002; Murakami et al., 2006). NMR and other spectroscopic studies identified conformational changes in the PLAs upon binding to phospholipid micelles or membranes, i.e. the first few residues of the N-terminus are flexible in solution, but upon micelle binding its α-amino group is engaged in H-bonding to the active site (Vandenberg, 1995a,b). Membrane potential may also affect PLA enzymatic activity (Thuren et al., 1987; Ward et al., 1998). It was shown that membrane binding of group IIA PLAs is accompanied with destabilization of the N-terminal α-helices, while the α-helices of the group IB PLAs adopted more fixed conformation upon binding the phospholipid micelles (Qin et al., 2005).

The collar on the surface of the PLA molecule, that surrounds the slot entrance of monomeric substrate (i.e., residues 3, 6, 7, 19, 20, 31, 64–69, 113, 116, 122), appears to function as the interface binding surface (White et al., 1990; Koduri et al., 1997; Scott, 1997; Qin et al., 2005). Results from study by Fourier transform infrared spectroscopy (FT-IR) (Qin et al., 2005) suggested that the enzyme probably forms a water tight seal with the interface, and the phospholipid must travel about 7 Å into the catalytic site slot to reach the catalytic network. The substrate's acyl chains contact hydrophobic amino acids including residues 4, 5, and 9 that line the walls of the slot (White et al., 1990; Gelb et al., 1995; Pan et al., 2002; Qin et al., 2005). The conserved catalytic site residues of PLAs have been probed by mutagenesis and 3D structural studies (Thunnissen et al., 1992; Gelb et al., 1995); an aspartate-histidine-water catalytic triad was identified, that is analogous to the triad found in serine proteases except that the serine is replaced by water molecules hydrogen bonded

to the imidazole ring of His48. It was proposed that water is the attacking nucleophile and the tetrahedral intermediate formed at the substrate's *sn-2* ester in the transition state is stabilized by an "oxyanion hole" near the main chain amide group of Gly30. The active site Ca^{+2} ion is coordinated by Asp49 and backbone carbonyl groups from a Gly-rich binding loop at positions 26–32 of the PLA.

The present review focuses on natural structural alternations of venom PLAs, which lead to toxins with low catalytic activities. Major examples of catalytically inactive variants of PLA during venoms evolution of elapid or viperid species will be discussed in categories.

Materials and Methods

Venoms and Other Materials

Bungarus fasciatus venom (Thailand origin) was purchased from Miami Serpentarium Labs. *Pseudechis australis* and *Pseudechis porphyriacus* were purchased from Australian Reptile Park (Gosford, New South Wales, Australia). Synthetic L-dipalmitoyl glycerophosphocholine was purchased from Avanti Polar Lipids (Alabaster, AL). Other chemicals were from Merck, Aldrich or Sigma.

Purification of Venom PLA

The venom PLAs of *B. fasciatus* were purified using the methods of Liu et al. (1988, 1989, 1990, 1994). After repeated centrifugations at 12,000 *g* for 5 min, aliquots of 100 μl dissolved venom (15 mg) were injected into a gel-filtration column (Superdex75, HR10/30) on a Fast protein liquid chromatography system. The proteins were eluted in 0.1 *M* ammonium acetate (pH 6.4) at room temperature. Pseudexin A and B from *P. porphyriacus*, and PLAs Pa-11 and Pa-13 from *P. australis* were purified according to the published methods (Nishida et al., 1985; Schmidt and Middlebrook, 1989; Tabasaki et al., 1990). The PLAs were further purified by high-performance liquid chromatography (HPLC) on a RP-C18 column (Wang et al., 1999, 2005; Tsai et al., 2001). Fractions

containing PLA activities were separately collected and freeze-dried in a vacuum centrifuge device (Labconco, CA).

Enzymatic Activities and Kinetic Parameters

The concentration of PLA_2 in the stock solution was determined by the absorbance at 280 nm, assuming an extinction coefficient of 1.5 at 1.0 mg/ml of the protein. Hydrolytic activities of PLA_2 towards mixed micelles of L-dipalmitoyl phosphatidhlcholine and deoxycholate or Triton X-100 were assayed at pH 7.4 and 37 °C on a pH-stat apparatus (Radiometer, RTS 822, Denmark). The initial enzymatic rate was corrected for the spontaneous rate. Binding affinity of Ca^{+2} ion to the PLA was also determined by plots of $1/V_{max}$ versus $CaCl_2$ concentration.

Results and Discussion

Evolutionary Mechanisms of PLA Inactivation

Normal reactions of PLAs include three steps. First, the enzyme binds to the interface and conveys a phospholipid molecule to the active site slot; second, the scissile ester bond of monomeric substrate is hydrolyzed by the catalytic triad with transition-state stabilization of the carbonyl oxyanion, and finally, the release of fatty acid and lysophospholipid product (White et al., 1990; Gelb et al., 1995; Pan et al., 2002; Qin et al., 2005). Thus, strategic inactivation of venom PLAs are usually related to any one or combination of the following mutations at: a) the structural motifs that recognize the interface; b) the structural features that control substrate binding; and c) the catalytic residues directly involved in the ester hydrolysis. I will discuss examples of natural PLA inactivation found in literature or recently studied by us in the following section.

A. Reduced Catalysis with Retention of Interfacial Binding

BASIC CATALYTICALLY INACTIVE G6/W6 PLAS OF CROTALID VENOM

Since D48 is normally the major side chain of PLA responsible for binding the active site Ca^{+2} ion, the PLAs with D49 K and Y28 N mutations can not bind Ca^{+2} at the active

site, and thus lose hydrolytic activities (Lomante et al., 2003). The K49-PLA-homologs are the major form of venom PLAs of New World non-rattlers, e.g. *Bothrops* and *Agkistrodon*, and are apparently present in most venom species of Asian pit vipers, especially *Protobothrops* and *Trimeresurus* (Wang et al., 1999, 2005; Tsai et al., 2001). The venom contents are usually high, consistent with their noncatalytic roles. In general, this family of basic PLA homologs (pI > 9) share 75% sequence identity. Furthermore, it was recently found that the K49R substitution occurred in this family of PLAs from *Protobothrops* (Wang et al., 1996; Tsai et al., 2004), and the major venom PLA of Malayan pitviper (*Calloselasma rhodostoma*) is an inactive PLA homolog with W6D49 substitution (Tsai et al., 2000). Besides biomembranes, these versatile toxins are capable of binding to polyanionic heparin or lipopolysaccharides. They also show strong myotoxic or cytotoxic and bactericidal activities, and may induce edema (Liu et al., 1991; Lee et al., 2001) and inflammation (Landucci et al., 2000; Zuliani et al., 2005). It has been shown that necrosis and apoptosis induced by them are associated with increments in cytosolic Ca^{2+} levels following plasma membrane perturbation, and possibly with the involvement of mitochondria (Mora et al., 2006).

In addition to position 49, other alterations at the N-terminal region of the G6W6 basic proteins such as D4L5Q11 (which are normally Q4F5K11 in most crotalid D49-PLAs), probably impede the binding of phospholipid substrates to the hydrophobic wall of the K49-PLA family (Wang et al., 1996, 2005; Lomonte et al., 2003). Thus, the interfacial binding of K49-PLAs probably is not followed by entry of single substrates.

MYOTOXIC SER49 PLA IN VIPERINAE VENOM

Myotoxic PLAs are common components in viperid snake venom. Myotoxicity has been defined as the ability of proteins to induce skeletal muscle necrosis *in vivo* upon intramuscular injection, or *in vitro* upon incubation with differentiated skeletal muscle. Like the K49-PLA of pit viper venoms, G6S49-PLA homologs with impeded catalysis were found in the venom of some true vipers, especially African *Echis* (Polgar et al., 1996) and some European *Vipera* (Krizaj et al., 1991). Because binding of a single phospholipid molecule to the active site requires not only that the enzyme be bound to the interface, but also that the enzyme

contain calcium, as shown by direct binding studies and inferred from X-ray structures, these group II PLAs without D49 may not bind substrate (Yu et al., 1993), and should have no direct catalytic activities.

Pro31-PLAs of elapid venom

Most if not all elapid venom PLAs have a conserved D49 except that an inactive PLA with A49 (as well as N28D30) substitution was found in low content from the pooled venom of *B. fasciatus* (Liu et al., 1992). Instead, weakly catalytic or inactive forms of venom PLAs with a unique Pro31 residue are present in many elapid venom species, while other group I PLAs with higher catalytic activities have either Lys, Arg or Leu at position 31 (Liu and Lo, 1994). These P31-PLAs were found in the venom of Asian golden krait *B. fasciatus* (Liu and Lo, 1994; Liu et al., 1988, 1989, 1990), Australian *Pseudechis* (Nishida et al., 1985; Schmidt and Middlrbrook, 1989; Liu et al., 1990, Takasaki et al., 1990) and certain Hydrophiid species (e.g. *Laticauda* species) (Takasaki et al., 1988). Amino acid sequences of the basic P31-PLAs with low catalytic activities were aligned in Fig. 1. Many of them are abundant venom components, e.g. three P31-PLA isoforms (BfVa, BfVb-2 and BfVI) together comprised 45–55% of *B. fasciatus* venom protein content (Liu et al., 1990), and pseudexin B is about 15% of the protein in *P. porphyriacus* venom (Schmidt and Middlebrook, 1989). Notably, some of the nonenzymatic acidic subunits of highly potent taipoxin and texilotoxin in Australia elapid venoms (Fig. 1), and some inactive K49-PLAs of crotalid venom also contain P31 substitution (Tsai et al., 2001; Wang et al., 2005).

All these basic P31-PLAs have been reported to have no or very low enzyme activities toward various kind of aggregated and monodispersed substrates *in vitro*, but they may be more lethal than other PLA isoforms with higher catalytic activities. As shown in Table 1 the P31-PLAs Bf Va, Bf Vb-2 and Bf VI have much lower catalytic rate than Bf X-1 (containing K31), although all of them hydrolyzed phospholipid (lecithin) at *sn-2* ester position in a Ca^{+2}-dependent reaction (Chang et al., 1983). The functions of the P31-PLAs have been reported to be like cobra cardiotoxins, myotoxins or membrane-acting cytotoxins, which cause membrane structure change or depolarization

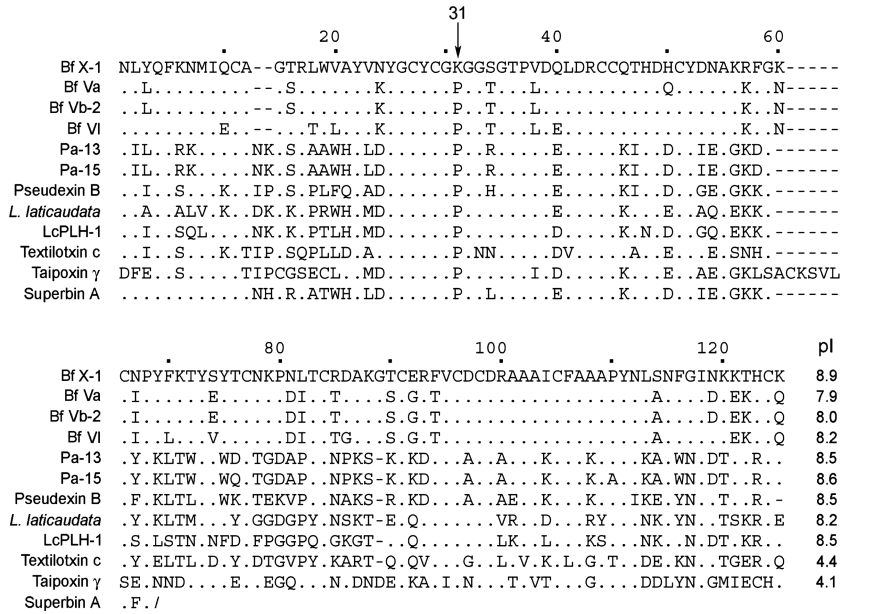

FIGURE 1 Sequence alignment of Elapid venom PLAs with Pro31 substitution. Single-letter codes of amino acids are used, and residues identical to those of a reference PLA, Bf X-1, are denoted with dots; gaps are marked with hyphens. Previous numbering system is adopted (Renetseder et al., 1985). GenBank or Swiss-prot accession numbers and the venom species for the PLAs are: *Bungarus fasciatus*: Bf X-1, P14615; Bf Va, P00628; Bf Vb-2, P00629; Bf VI, P00627; *Pseudechis australis*: Pa13, AAZ22665; Pa15, P20257; *Pseudechis porphyriacus*: Pseudexin B, P20259; *Laticauda laticaudata*: PLA-homolog, BAB72248; *Laticauda colubrine*: LcPLH-I, P10117; *Oxyuranus scutellatus*: Taipoxin γ, P00616; *Pseudonaja textiles*: Textilotoxin-C, AAZ22646; *Austrelaps superbus*: Superbin A, P59067.

(Shiaulin et al., 1975; Xu, 1986; Qi et al., 1989). It seems logical to speculate that the basic membrane toxins may be synergistic with other more active acidic PLAs (e.g., Bf X-1) to cause a severe cytotoxic effect or necrosis. Recently, a Tyr31-PLA without enzyme activity was isolated from *Bungarus caeruleus*, and the PLA existed as homodimer containing two mannose molecules H-binding to both Y31 and D49 (Singha et al., 2005).

Previous studies on pancreatic PLA mutants have revealed that replacements of L31 to other amino acids reduced the enzymatic activities considerably (Kuipen et al., 1990; Yu et al., 2000). Apparently, P31 in the Ca^{+2}-binding loop of PLA could

TABLE 1 Catalytic activities and lethal effects of *B. fasciatus* PLAs. Substrate used were: (a) micelles of 3 mM dPPC plus 3 mM sodium deoxycholate; (b) 3 mM dPPC plus 6 mM triton X-100; (c) micelles of egg lecithin and deoxycholate, with 5 mM $CaCl_2$ and 0.1 M NaCl at 37°C

		Specific activity, mmc/min/g enzyme			
PLA	Residue 31	(a)	(b)	(c)	(i.p.) LD_{50} μg/g mice
Bf Va	Pro	10.2	38	0.4	4.7
Bf Vb-2	Pro	5.9	27	1.6	4.7
Bf VI	Pro	5.8	10	0.3	3.6
Bf X-1	Lys	50	320	42	>50
Bf III	Lys	152	1000	n.d.	>50

affect the correct configuration of an oxyanion-hole involving backbone NH of Gly-30. In other esterases such as lipases, the contribution of oxyanion hole to the transition-state stabilization may be up to 20 kJ/mole, which account to an increase of catalytic rates by 100–1000 fold (Magnusson et al., 2005).

It is expected that the concentration of calcium required for maximal PLA activity will depend on the fraction of enzyme bound to the membrane and the affinity of the enzyme for the phospholipid substrate. In order to find whether the Ca^{+2}-binding affinities or the turn over rate of P31-PLAs is inferior, we carried out kinetic analyses on these P31 PLAs. Our results showed that the P31-PLAs may bind Ca^{+2} strongly, with affinity constants of 13–49 μM (Fig. 2) which are better than many other venom PLAs with higher catalytic power (affinity constants > 100 μM). The P31-PLAs prefer the zwitterionic micelles (Triton X-100) than the anionic micelles (deoxycholate) substrates. The k_{cat} values of Bf VI calculated from double reciprocal plots are about 10-fold lower than that of Bf X-1 (a K31-PLA isoform), while the apparent K_M values of these two PLAs are less affected (Tsai, I. H., et al., 2007). It is very likely that the P31 substitution may disarrange or distort the structure of oxyanion hole essential for transition state stabilization, thus reducing k_{cat} by about 10-folds.

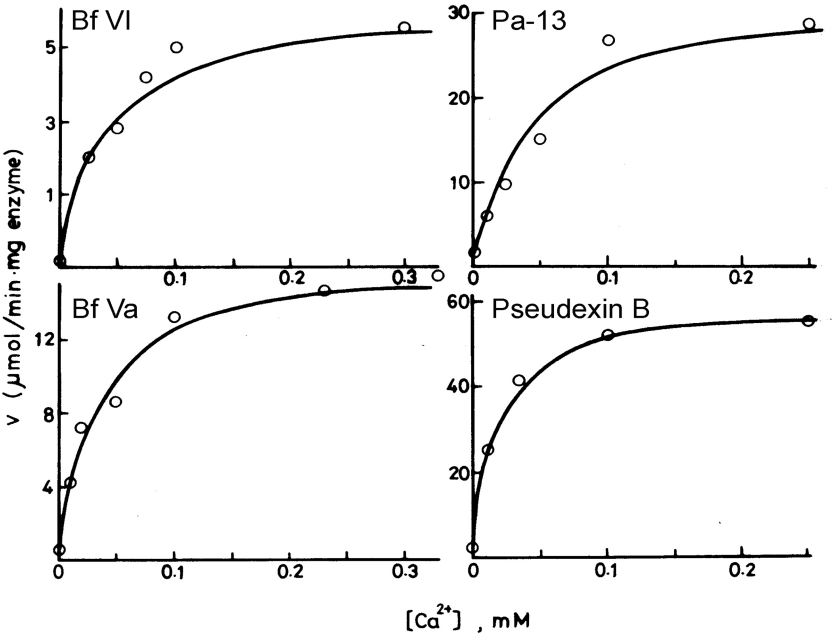

FIGURE 2 Dependence of hydrolysis rates of four venom P31-PLA on Ca^{+2} concentrations. The micellar substrates used was 3 m*M* *L*-dipalmitoyl phosphatidylcholine and 6 m*M* triton X-100, and initial rate of the enzymatic reactions were monitored at pH 7.2 and 37 °C on a pH-stat apparatus in presence of 0.14–0.20 μM of the PLAs.

B. Basic Anticoagulating PLA with Altered Interfacial Binding

Anticoagulating PLAs are present in many genera of venomous snakes; their target mode and mechanism has been reviewed extensively (Kini, 2005). Anticoagulating effects of venom PLAs are often not related to the enzymatic activities (Condrea et al., 1982; Inada et al., 1994; Mounier et al., 2000; Zhong et al., 2002). Substitutions at both interfacial binding and catalysis sites appear to happen in the strong anticoagulating PLA-CMIV of *Naja nigricollis* venom (Kini, 2005). This group of PLA bear sequence similarity to factor V and tissue factor at region 54–71 (Kini, 2005; Jabeen et al., 2005), and may bind coagulation factor FXa in a hydrolysis independent mode and thus inhibit prothrombinase activation.

The residues of the i-face of PLAs are in an ensemble of conformations (Gelb et al., 1995), which apparently are effective

in accommodating diverse binding targets such as coagulation factors. It appears that specific substitutions that may be unique to each PLA2, *i.e.* the N-and C-terminal part of human group IIA PLA, β-wing region and the C-terminal part of ammodytoxin (i.e., residues R72, K74, H76, R77) (Han et al., 1997), and residues E53, W70, T56 and D67 in basic venom PLA of *Agkistrodon halys* Pallas (Zhong et al., 2002), are responsible for their anticoagulating properties. In general, these substitutions altered interfacial binding sites (the i-face) of the PLA and enable it to bind coagulation factors.

Recently, new types of D49 substitutions to neutral amino acids (Asn and Gln) have been found in strong anticoagulating PLAs of many crotalid venom species. For example, R6N49-PLAs were identified in the venom of Asian pit vipers including: *Trimeresurus stejnegeri, Trimeresurus popeorum* (Tsai et al., 2004), *Trimeresurus albolabris* (Rojnuckarin et al., 2006), *Protobothrops mucrosquamatus* (Wei et al., 2006) and possibly some other *Protobothrops* (Tsai et al., 2004). A PLA with special R6Q49 substitution was identified in the venom of *Gloydius blomhoffii ussurensis* (Bao et al., 2005). These PLAs thus cannot bind Ca^{+2} and are inactive enzymes. It is possible that R6 and basic residues at positions 7–15 also contribute to the lower catalytic activity of these anticoagulating PLAs since mutagenesis of E6R in an acidic PLA from *Agkistrodon P. piscivorus* resulted in lower k_{cat} value (Prijatelj et al. 2006).

C. Truncation of Interface Binding Sites of Crotoxin-A Precursor to Generate Chaperon Subunit of Rattlesnake Neurotoxins

For all the neurotoxic PLA enzymes the enzymatic activity has been known to be a necessary condition for the neurotoxicity (Yang, 1997; Kattah et al., 2002; Chen et al., 2004). Recently, it has been shown that the product of the PLA reaction have equivalent effects of snake PLA_2 neurotoxins (Rigoni et al., 2005). However, for different venom neurotoxic PLAs catalytic powers and toxicities are not well correlated. Detailed mechanisms and the roles of ester-hydrolysis remain to be solved for neurotoxins, which result in the blockade of neurotransmitter exocytosis.

Crotalid venom evolved neurotoxic PLAs either in monomeric form (e.g., Agkistrodotoxin from *Gloydius brevicaudus*

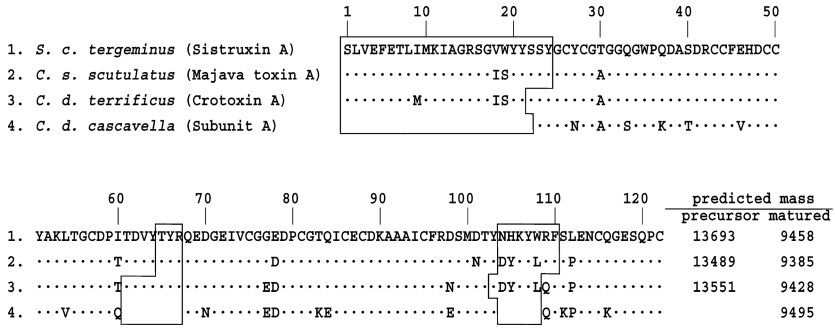

FIGURE 3 Amino acid sequence alignment of crotoxin A-like subunits from *Crotalus* and *Sistrurus* venoms. Single-letter codes of amino acids are used, and residues identical to those in the top line are denoted with dots. Three regions, which are removed by proteolysis during the post-translational processing are blocked.

venom) or in heterodimeric form (e.g., crotoxin). Crotoxin-like toxins are present only in venom of neurotoxic rattlesnakes, which possibly evolved from Asian *Gloydius* (Chen et al., 2004). Expression of a crotoxin A (i.e., crotapotin) gene is a venom marker of the neurotoxic *Crotalus* and *Sistrurus* (Wooldridge et al., 2001). The N-terminal region of PLA has been known to be essential for maintaining the active site H—bond network and its interfacial binding (Han et al., 1997; Qin et al., 2005). Thus, specific deletion of three interfacial peptides at 1–23, 69–76, and 113–121 in the precursor by limited proteolysis generated an inactive crotapotin (Fig. 3). The two subunits (crotoxin A and B) form a strong complex and free subunits could hardly be found in the venom (Chen et al., 2004). Binding of crotoxin A increases the toxicity of crotoxin B by up to 36-fold, depending on the neuromuscular system assayed (Hermandez-Oliveira et al., 2005), but it inhibits enzymatic activity of crotoxin B *in vitro* (Chen et al., 2004). Also, crotoxin A itself may have some specific functions on some target cells, e.g. T-lymphocytes (Garcia et al., 2003).

D. Multiple Mutations of Interfacial Binding Sites in the Acidic PLA-Subunit of Heterodimeric Viperinae Toxins

Some Viperinae venom species (e.g., those under the genera *Daboia* and *Vipera*) contain heterodimeric PLA-toxins (Wang et al., 1992; Francis et al., 1995; Komori et al., 1996; Tsai et al.,

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                          .        20         .        40         .        60
1.RV-7                    NLFQFGEMILEKTG-KEVVHSYAIYGCYCGWGGQGRAQDATDRCCFVHDCCYGTVN---DC
2. Vipoxin Inh.           ......D...Q...-..A.............................AQ.....R..---..
3. Vaspin-A               ......D...Q...-..A.............................AQ.....R..---..
4. P. fiedi Cb-Ia         .........F....-..A...................M..............R..---G.
5. V. pales. VPa          H.T...D..NK...-TFGLL..VY......L..K.KP.....................---G.
6. Am-I1a                 H.S...D..NK...-IFGIM..IY.........K.KPL..............R..---G.

                  .        80         .       100         .       120         .
1.    ----NPKTATYSYSFENGDIVCGD-NDLCLRTVCECDRAAAICLGQNVNTYDKNY-EYYSISHCTEESEQC
2.    ----.......T............-......A............E.........-..............
3.    ----...M...T............-......A............E.........-..............
4.    ----...M....T..Q........-......A..................N...-..H......M......
5.    ----D..LS......Q........-D.P...A......V....F.E.M.....K.-ML..FFD.M....K.
6.    ----D..LS..............G-D.P...A......V....F.E.M.....K.-ML..LLD.K......
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FIGURE 4 Sequence alignments for the acidic subunits of Viperinae heterodimeric PLA toxins (1–4) and two monomeric PLAs (5 and 6). Single-letter codes of amino acids are used, and residues identical to those in the top line are denoted with dots. The numbering system follows that of Renetseder et al. (1985). GenBank or Swiss-prot accession numbers and venom species for the PLAs are: *Daboia russelii* RV-7, X68386; *Vipera ammodytes meridionalis*, Vipoxin Inhibitor, P04084; *Vipera a. aspis* Vaspin-A, AF548351, Am-I1a, AY159807; *Pseudocerastes fieldi* Cb-Ia (Francis et al., 1995); *Vipera palaestinae* VPa (Krizag et al., 1996).

1996; Jan et al., 2002). Two PLA subunits designated as RV-4 (pI 10) and RV-7 (pI 4.3) form heterodimers in the venom of Taiwanese *Daboia siamensis* (Wang et al., 1992; Tsai et al., 1996). RV-7 had very low enzymatic activity (100–300 fold lower than that of RV-4), but it enhanced the neurotoxic and hypotensive effects of RV-4. The amino acid sequences of RV-7 like subunits of different venom species were aligned as in Fig. 4 along with two acidic Viperinae PLAs, which apparently are not a chaperon of basic subunits (Krizaj et al., 1996; Guillemin et al., 2003). Vipoxin and its inhibitor (from Bulgarian *V. a. meridionalis* venom) share 92% sequence identities with RV-4 and RV-7, respectively, but vipoxin inhibitor contains His48Gln (Fig. 4) and thus is totally inactivated (Perbandt et al., 2003).

RV-7 also acts like a competitive inhibitor of RV-4 catalyzed hydrolysis *in vitro* (data not shown). RV-7 had very low enzymatic

activity (100–300 fold lower than that of RV-4), but it enhanced the neurotoxic and hypotensive effects of RV-4. Its low activities may be attributed to less basic or less hydrophobic substitutions at the interfacial binding sites, i.e. G6, E/D7, E11, E17, Q34, D60, D114, E119, and H124 (Fig. 4). Crystallographic study of the RV-4/RV-7 complex showed that residues N1, Q34, N56, D60, D49 and K69 of RV-7 are involved in binding to the basicRV-4 subunit (Georgiera et al., 2004). Recently it was found that intravenous injection of RV-7 solution into anesthetized experimental animals (0.1 μg/g body weight) depressed blood pressure. RV-7 also elicited relaxation in a phenylephrine-precontracted aortic model *in vitro*, and it was shown that RV-7 might activate eNOS in endothelial cells, and thus relax smooth muscle (Chih, 2001). In addition, oligomeric or complex type presynaptic neurotoxins of Elapid venom species, e.g. taipoxin and textilotoxin, also contain nontruncated, but inactivated PLA chaperon subunits with substitutions at both interfacial binding sites and catalytic sites (Fig. 1). Strong association among subunits of the toxins may reduce non-specific binding and enzyme activity of the only active PLA subunit (Chang, 1985; Tzeng, 1993).

Conclusions

Besides being hydrolases, secreted PLAs of vertebrates are well known to induce degranulation of mast cells and promote cytokine and chemokine production by direct binding to specific receptors on target cells (Triggiani et al., 2005). So far there is no convincing evidence to support the direct association of venom PLAs with prey-digestion. Many venom PLAs are toxins with specific pharmacological targets but low enzyme activities. During venom evolution, group II basic PLA-cytotoxins may be mutated at the active site Asp49 and loose their essential Ca^{+2} but retain binding to cell membranes. In contrast, certain Elapid venom basic PLAs are catalytically inactivated by Pro31 substitution. Venom PLAs bind strongly to coagulation factor may alter their interfacial binding sites and no longer hydrolyze the aggregated lipid substrate. To become the chaperon subunits of presynaptic neurotoxins, the acidic PLA may be truncated at the interfacial binding regions or have I-face mutations that allow a strong binding to the basic catalytic active subunits. This

type of acidic PLAs subunit (e.g., RV7 of *Daboia siamensis*) itself appears to show other special pharmacological effects. The above examples demonstrate that venom PLAs may play the role of protein scaffold, like the nonenzymatic three-fingered toxins of Elapid venoms and other polypeptide toxins from invertebrate venoms.

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