Snake Venom Phospholipase A_2: Evolution and Diversity

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Abstract

Snake venom phospholipases A_2 (vPLA_2s) most likely originated from more than one body gene and have undergone multiple convergent and divergent evolutionary events in the snakes' adaptation and survival. The evolution of vPLA_2s is inextricably linked to snake phylogeography, ecology, and natural history. It has been shown that both mammalian secretory PLA_2s and snake vPLA_2s exist as multiple isoforms. The vPLA_2 genes undergo duplication, accelerated evolution, and positive Darwinian selection, and so the structures, functions, and expression levels of the isoforms vary greatly. The successful applications of advanced liquid chromatography and mass spectrometry have promoted vPLA_2 isolation, characterization, and related research significantly. Major pharmacological effects of vPLA_2 isoforms have been found to be either antiplatelet, anticoagulant, myotoxic, neurotoxic, edematous, hypotensive, or any combination of the above. Some vPLA_2s have reduced or lost their catalytic activities to exert special target-binding, chaperoning, or membrane-disrupting functions. Structural changes at their interface recognition sites and specific mutations at the active sites affect both catalytic-dependent and non-catalytic-dependent toxicities

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of vPLA$_2$s. The *N*-terminal and the *C*-terminal regions of vPLA$_2$ usually contain special features highlighting the molecular evolution of each vPLA$_2$ subtype. Moreover, synergisms between vPLA$_2$s and other venom components may result in prominent hemorrhagic, neurotoxic, hypotensive, edematous, or antibacterial effects after envenoming. How vPLA$_2$s and snake venom have diversified and evolved to fulfill the role of an efficient and powerful arsenal toward potential preys and enemies is demonstrated through the ample updated examples.

Introduction

Both venom and front fangs are key evolutionary innovations in the diversification of advanced snakes (Colubroidea). The evolution of the venom toxins predates the evolution of Viperidae and Elapidae, and snake venom appears to emerge at the base of the phylogenetic tree of advanced snakes and probably developed over a period of more than 60 million years (Fry et al. 2008, 2012). The 14-kDa secretory phospholipases A$_2$ (sPLA$_2$s; EC 3.1.1.4) are present in the body fluid of all vertebrates and are one of the major toxin families in snake venoms. These enzymes catalyze the hydrolysis of the sn-2 fatty acyl bond of glycerophospholipids and release lysophospholipids and free fatty acids. The reaction products may further participate in a diversity of toxic effects or affect the local physiological conditions (Neidlinger et al. 2006; Rigoni et al. 2007). Like several other venom toxin families, the molecular evolution of the venom phospholipase A$_2$ (vPLA$_2$) families evolve via a process by which several genes encoding for body secretory proteins are duplicated, recombined, mutated, and selectively expressed in the venom glands. Functionally important vPLA$_2$ subtypes are reinforced through accelerated evolution and selection (Ikeda et al. 2010; Lynch 2007; Ogawa et al. 1996). The vPLA$_2$ genes of Elapidae are derived from snake body group I (GI-A/B) sPLA$_2$ genes, while those of Viperidae are from snake body group II (GII-A/E) sPLA$_2$ genes. Some of the vPLA$_2$ genes may be replaced by others and become useless pseudogenes. The expression of vPLA$_2$s can be regulated dynamically and affected by the prey-ecology of the snakes (Gibbs and Rossiter 2008).

Because of their abundance, small molecular weight, and ease of isolation, many vPLA$_2$s have been purified and studied extensively. The complementary DNAs (cDNAs) encoding vPLA$_2$s usually share highly conserved untranslated regions and signal peptides, so their cDNAs can be sequenced and amplified based on the sequences of known homologous vPLA$_2$ genes; the amino acid sequences can then be deduced from sequencing the cDNA clones or obtained from transcriptome analyses. Notably, vPLA$_2$s containing six or seven disulfide bridges are rather thermally stable and soluble under acidic conditions and in the presence of water-miscible organic solvent at room temperature, so it is not difficult to purify them using reversed-phase high-performance liquid chromatography without denaturation after the solvent removal. Liquid chromatography combined with mass spectrometry has greatly facilitated the proteomic analyses of many vPLA$_2$s from a wide array of venom species. Ample sequence data of the vPLA$_2$s have

demonstrated great intrageneric and intraspecific diversities and geographic variations of this toxin family. Recent discovery on the molecular diversities of $sPLA_2$s in humans and other mammals (Dennis et al. 2011) also shed light on the evolutionary variations of $vPLA_2$s. A comprehensive book related to the molecular biology, biochemistry, and mechanism of $vPLA_2$s has been published about two decades ago (Kini 1997). The present review aimed to update and discuss some interesting discoveries and insights related with the evolution, variations, and functional subtypes of $vPLA_2$s from front-fanged venomous snakes.

Snake Venom Phospholipase A_2, Evolution and Diversity

The $vPLA_2$s are Derived from Body Secretory PLA_2s

Ten members or groups of the $sPLA_2$ family (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII) have been identified in mammals and other vertebrates; these are numbered and grouped according to their pattern of disulfide bonds and in order of their discovery (Dennis et al. 2011). In humans and mice, the genes for groups GIIA, GIIC, GIID, GIIE, and GIIF are linked within the same chromosome and probably derived from gene duplication, while genes encoding GIB and GX map to another two chromosomes. Some mammalian GII-$sPLA_2$ genes are prone to undergo duplications. For example, five duplications of bovine GIID genes lead to various isoforms while a single gene copy of GIID is present in the human and rodent genomes (Golik et al. 2006). The bovine GIID isoforms are expressed in the mammary gland and possibly participate in the innate immune response. The vertebrate $sPLA_2$s have been found to be implicated in an array of physiological and pathological activities with specific tissue distribution and under dynamic regulations. For example, human and mouse GIIA-PLA_2s are highly basic and efficient bactericidal enzymes, whereas GIB-PLA_2 is a digestive enzyme for lipid micelles.

All $sPLA_2$s in groups GI, GII, and GX have very similar tertiary structures that contain three long α-helices, two β-sheets referred to as β-wings, and a conserved Ca^{2+}-binding loop. Although their sequence identity level is low and *C*-terminal region extension may be varied, they share a common protein fold and feature the same catalytic His48/Asp93 dyad. GIA and GIB $sPLA_2$s with 118–124 residues are found to be selectively expressed in pancreas and venom glands of elapid snakes and some non-front-fanged snakes, while GII-$sPLA_2$s with 121–123 residues are expressed in venom glands of viperid and crotalid snakes. Most of the $vPLA_2$s contain six or seven conserved disulfide bridges; however, GI-$vPLA_2$s differ from GII-$vPLA_2$s by substituting the Cys11-Cys77 for another disulfide bond and lacking the 5–7 amino acid residues C-terminal extension in GII-$vPLA_2$s. GIA-$vPLA_2$s are in general more reactive toward membrane substrates than GIB-$vPLA_2$s; an extra “pancreatic loop” at positions 62–66 of GIB distinguishes it from GIA. Remarkably, the mammalian GIIA and GIIE are structurally similar to each other, and it was suggested that the GIIA-$sPLA_2$s might be derived from duplication of GIIE-$sPLA_2$s (Yamaguchi et al. 2014). Interestingly, the amino acid

sequences of some $vPLA_2$s are more similar to that of human GIIE than to that of human GIIA. Recently, GIIE-PLA_2 messengers have been detected in the maxillary venom glands of *Dispholidus typus* and *Leioheterodon madagascarensis* (Fry et al. 2012) and in the venom glands of *Protobothrops flavoviridis* (Yamaguchi et al. 2014). However, the corresponding proteins have not been detected in any snake venom.

Catalytic Mechanism and Active Site of $vPLA_2$s

The 3D structures of many $vPLA_2$s are available in protein data banks, and the reaction mechanisms of not only $vPLA_2$s but also the model pancreatic GIB $sPLA_2$s have been studied extensively (Dennis et al. 2011; Kini 1997). Most $sPLA_2$ enzymes act on cell membranes or a water-lipid interface. They display a characteristic increase in activity when a substrate is switched from monomeric to higher-ordered lipid aggregates, which is known as interfacial activation (Cho et al. 1988). The catalytic site of $sPLA_2$s resembles that of serine proteases but with Ca^{2+}-linked water molecules playing the nucleophilic role, and both the hydrolase families use imidazole of His residue (linked with an Asp carboxyl group) as general base to facilitate the proton transfer and tetrahedron formation during the transition state of the hydrolysis. Thus, three conserve active-site residues or regions (His48, Asp49, and Ca^{2+}-binding loop), and the conserved Cys residues highlight the sequence conservation of the $vPLA_2$ superfamily. The *N*-terminal regions 1–13 of $vPLA_2$s form an alpha-helix, and residues 2, 6, and 7 and other regions (e.g., residues 19–22, 31, 63–68, and 106–112) are involved in the water-lipid interface interactions. The catalytic Ca^{2+} is coordinated by the β-carboxyl group of Asp^{49} and backbone carbonyl groups of Tyr28, Gly30, and Gly32 in the Ca^{2+}-binding loop ($Y^{25}GCYCGXGG^{33}$). Mutations at this loop in a $vPLA_2$ usually signify inactivation or reduction of its catalytic activity. It was also shown that micelle binding was accompanied by the conformational change of the *N*-terminal region of GII-PLA_2 (Qin et al. 2005), while the *N*-terminal region of GI-PLA_2s contained the disulfide 11–77 and adopted more fixed conformations upon binding the phospholipid micelles.

Additionally, the mutations at key catalytic residues (His48 and Asp49) or the charge and hydrophobicity of the substrate and interface recognition sites (e.g., Leu2, Phe5, Trp31) serve as clues to explain variations of the catalytic power and specificity of $vPLA_2$s. As long as the catalytic activities are not required for some of the $vPLA_2$ functions, their catalytic sites or interfacial binding residues could be mutated without affecting the protein folding or gross conformation, e.g., the Gln48, Asn49, or Pro31 substitutions may reduce the $vPLA_2$ catalytic activities (Tsai 2007). It is interesting to note that some vertebrate GIID-$sPLA_2$s also contain similar inactivating mutations in their Ca^{2+}-binding loops; e.g., the GIID-PLA_2s of *Alligator mississippiensis* (accession No. XP_006275522) contains the Asn49 mutation, while those from elephant (*Loxodonta africana*), polar bear (*Ursus maritimus*), monkey (*Macaca fascicularis* XP_005544618), and baboon (*Papio anubis* XP_003891301) contain Pro31 substitutions.

Phylogenetic Analyses of Snake Species and their $vPLA_2s$

The evolutions of $vPLA_2s$ are inextricably linked to snake phylogeography, natural history, ecology, and diets (Gibbs and Rossiter 2008; Gubenšek and Kordiš 1997; Lynch 2007). Using the intron nucleotide sequence of the GII-$vPLA_2s$ as the dataset generated a molecular phylogeny tree according to viperid taxonomy, but using the protein sequences of GII-$vPLA_2s$ resulted in the clustering of different paralogous subtypes in the tree, supporting that the accelerated evolution has occurred in the protein-coding regions of the GII-$vPLA_2s$ genes to generate different functional subtypes (Gubenšek and Kordiš 1997; Ogawa et al. 1996). In contrast, the GI-$vPLA_2s$ tree was consistent with the phylogeographic relationships of the elapid species (Tsai 1997). Thus far, the amino acid sequences of about 300 $vPLA_2s$ have been solved and deposited in databanks. Comprehensive phylogeny trees based on all the GII-$vPLA_2$ sequences further confirmed that there are two Viperinae clusters and four or five Crotalinae clusters of $vPLA_2s$; each of the clusters appears to represent a distinct functional or structural subtype (Lynch 2007; Malhotra et al. 2013; Mebs et al. 2006). Three-dimensional structural models of the four subtypes of crotalid $vPLA_2s$ have been built to show different functional epitopes and variations at the surface and interface residues of each subtype (Malhotra et al. 2013). Moreover, molecular phylogenetic analyses on GII-$vPLA_2$ could be very useful for function prediction or subtyping of novel $vPLA_2s$, e.g., phylogenetic analysis and sequence alignment have aided in the identification and functional assay of a new PLA_2 neurotoxin (Yang et al. 2015).

Like human GI- and GIIA/B-$sPLA_2s$, most of the $vPLA_2s$ have Asn, Ser as the *N*-terminal residue, while a few Crotalid GII-$vPLA_2s$ have His^1, Asp^1, and Gly^1 (Tsai et al. 2004b). It was noticed that the four Ser^1-PLA_2s of *Vipera a. ammodytes* venoms contain a transposon in one of the introns in contrast to the corresponding intron of the Asn^1-PLA_2s in the same venom (Gubenšek and Kordiš 1997), suggesting possibly different evolutionary history between the Viperinae Ser^1 and the Asn^1 $vPLA_2$ subtypes. Ammodytoxins from *V. a. ammodytes* venom contain Ser^1 while caudoxin from *Bitis caudalis* venom contains Asn^1; the low sequence homology between both Viperinae neurotoxins perhaps implies a convergent evolution of their neurotoxicity. Among the crotalid $vPLA_2$ isoforms, the amino acid residues 6, 49, and the pI value empirically serve as convenient references for the classification of functional subtypes or paralogues. Five or six subtypes have been proposed for the pit viper $vPLA_2s$, i.e., the K49, N6D49, acidic R6, basic R6, and E6D49-PLA_2 types (Tsai et al. 2004a). The separated evolution of these subtypes was supported by phylogenetic analyses based on more than 60 selected pit viper $vPLA_2$ sequences, and the E6-type was found to be rooted within the basic R6-type (Mebs et al. 2006). The crotalid $vPLA_2s$ with Glu, Asn, Arg, or Gly/Trp at position 6 (i.e., the E6, N6, R6, or K49 subtypes) represent the acidic antiplatelet, the neurtotoxic or myotoxic, the basic anticoagulant, and the noncatalytic membrane-disrupting subtypes of the $vPLA_2$ toxins, respectively (Tsai 1997; Tsai et al. 2004a). This simplified rule possibly is not surprising given the fact that residue 6 of GII-$vPLA_2$ is one of the most important interface recognition sites and the residues

1–7 and 19–22 contain important structural determinants for their functions (Qin et al. 2005; Tsai et al. 2011b). However, exceptions to this rule of relating residues 6 to the functional subtypes do exist; e.g., the monomeric E6-vPLA$_2$s isolated from North American *Agkistrodon* venoms are myotoxic and basic (Komori et al. 2002) in contrast to the dimeric and acidic E6-vPLA$_2$s in other pit viper venom; while most of the basic Arg6-vPLA$_2$s of Asian pit vipers are anticoagulants with low enzymatic activities (Tsai et al. 2004b; Wang et al. 2005), one of the Arg6-vPLA$_2$s isolated from *Protobothrops mucrosquamatus* and *Protobothrops jerdonii* venom is acidic, catalytically active, and plays antiplatelet roles (Tsai et al. 2004a).

Expression Level and Diversity of Isoforms

The expression level of vPLA$_2$ isoforms varied greatly according to the species, population and ecology, and age of the snake, among other factors. High vPLA$_2$ contents (48–73 % of total venom proteins) have been reported for a wide variety of species including *Bungarus fasciatus* (Tsai et al. 2007a), *Pseudachis australis* (Takasaki et al. 1990), *Micrurus nigrocinctus* (Fernández et al. 2011), *Daboia russelii* and *Daboia siamensis* (Tsai et al. 2007b), *Zhaoermia mangshanensis* (Mebs et al. 2006), and southern population of *Crotalus duressis terrificus* (Boldrini-França et al. 2010). In contrast, vPLA$_2$ enzymes could be below 3 ~ 5 % of the total venom proteins, or absent in the venom of *Dendroaspis, Causus*, certain Australian elapids (Jackson et al. 2013), and some *Crotalus horridus horridus* populations (Wang et al. 2010). The numbers of vPLA$_2$ isoforms that could be isolated from most venom species are usually 1–4 but could be 8 or more in the venom of certain elapids, e.g., *B. fasciatus* (Tsai et al. 2007a) and *P. australis* (Takasaki et al. 1990). Moreover, contents and proportions of the vPLA$_2$ isoforms usually vary intragenerically and intraspecifically, according to the age, natural environment, or prey ecology of the snake (Gibbs and Rossiter 2008; Tsai et al. 2004b). A strong relationship between venom composition and snake feeding adaptations may explain for the low vPLA$_2$ isoform number of sea snake venom, and the evolution trend appears to be adopted by different taxa in response to either loss or gain of neurotoxicity (Calvete et al. 2012).

The sequences of vPLA$_2$s from the same snake genus could be more similar than those from a different genus, as usually expected. However, species under the same genus can be rather diversified genetically, e.g., the less toxic vPLA$_2$s in *Bungarus fasciatus* venom are very distinct from the vPLA$_2$s in *Bungarus multicinctus* (with high content of potent β-bungarotoxins) (Tsai et al. 2007a). Furthermore, venoms of the same or closely related species could show dichotomy or bimorphism in their venom profiles and pharmacological effects. For example, the European *Vipera* and the Asian *Daboia* are phylogenetically related genera, and their venoms contain either of the two vPLA$_2$s subtypes (Gao et al. 2009; Jan et al. 2007). The neurotoxic vPLA$_2$s in the venom of *D. siamensis*, Pakistan *D. russelii* (Tsai et al. 2007b), *Vipera a. montandoni*, *Vipera aspis,* and *Vipera nikolskii* (Gao et al. 2009) are 1:1-heterodimeric complexes, while those in *D. russelii* (Sri Lanka and southern India),

V. a. ammodytes, *Vipera berus,* and *V. u. renardi* (Tsai et al. 2011a) are two or three similar and monomeric isoforms. Paradoxical molecular evolution of $vPLA_2$s has also been observed in some rattlesnake species. The entire $vPLA_2$ family was completely replaced in the *C. h. horridus* neurotoxic-type venom as compared to the hemorrhagic-type venom of the same species, possibly through intergeneric hybridization of rattlesnakes (Rokyta et al. 2015). Moreover, the venoms of neonates or juveniles of some *Crotalus* are more neurotoxic than those of their adults and contain distinct $vPLA_2$s; this ontological variation possibly reflects a phenomenon of paedomorphism (Mackessy et al. 2003). Remarkably, the numbers of $vPLA_2$ isoforms in the transcriptome (including those encoded by the pseudogenes) can be higher than those detected in the proteome or isolated from the venom, and their expression can be regulated epigenetically by special venom gland microRNA (Durban et al. 2013) and specific transcriptional factors (Nakamura et al. 2014).

The $vPLA_2$ isoform profile appears to be characteristic for each venom species, in spite of the fact that variations in the proportion of $vPLA_2$s are frequently observed. Important geographic variations in $vPLA_2$ isoforms have been unveiled by proteomic analyses of the venom from individual specimens or different populations of the same species, e.g., king cobra (Chang et al. 2013), Taipan (Chaisakul et al. 2014), *D. russelii* (Tsai et al. 2007b), *Trimeresurus stejnegeri* (Tsai et al. 2004b), and rattlesnakes (Boldrini-França et al. 2010; Tsai et al. 2003). It is essential to know the locality and number of snakes from which the venom has been collected when studying the geographic variations of a venom species. Occasionally, controversial or contradictory results of venom characterization were published in referred journals using unreliable venom collected from unidentified localities and even contaminated or mixed with venom from other species. Researchers should be responsible and cautious not to use unreliable venom samples or publish the results. Under such circumstances, possible venom contamination could be detected or verified by analyzing the accurate mass and *N*-terminal sequence or peptide mass fingerprint of each purified $vPLA_2$ in the sample since most snake venoms contain specific $vPLA_2$s. Because mRNAs encoding the expressed $vPLA_2$ isoforms are more abundant and their cDNAs are more frequently cloned or sequenced than those encoding the nonexpressed $vPLA_2$s, the $vPLA_2$ proteomic results should match the transcriptomic results, i.e., all the $vPLA_2$s that are present in a venom species should have the corresponding cDNAs cloned from its venom glands. This could serve as a further criterion to check whether the venom used is authentic and not contaminated.

Evolution of Different Pharmacological Effects

The lipolytic activities of $vPLA_2$s are difficult to assay in vivo, and only the in vitro models have been used to test or examine their pharmacological effects. Most of the acidic $vPLA_2$s induce or inhibit platelet aggregation, but their action mechanisms have not been well elucidated. Not only the $vPLA_2$ "effective doses" varied greatly, the effects could be either dependent or independent of the enzymatic

activities (Kini 1997). The antiplatelet responsible residues of pit viper acidic $vPLA_2$s were found to contain acidic and aromatic residues at positions 6, 20–22, and 114–116 by mutagenesis study (Liu et al. 2001). Recently, it was reported that an acidic $vPLA_2$ from *Naja naja* ($NnPLA_2$-I) could inhibit thrombin with a K_i value of 9.3 nM while $NnPLA_2$-I also dose-dependently inhibits the aggregation of platelet-rich plasma (Dutta et al. 2015). Earlier, an African cobra $vPLA_2$ was found to inhibit thrombin (Osipov et al. 2010). Therefore, the acidic $vPLA_2$s in general may contribute to thrombocytopenia and hemorrhage effects.

Although most $vPLA_2$s with catalytic activity could elicit weak anticoagulant and transient hypotensive effects, strongly anticoagulant $vPLA_2$s usually have high pI values and bind to coagulation factor Xa (Kini 2005), and they may show low or no lipolytic activities. The responsible residues and mechanism of several potent anticoagulant $vPLA_2$s have been explored by site-directed mutagenesis (Kini 2005; Prijatelj et al. 2006; Tsai et al. 2011b; Zhong et al. 2002). Remarkably, the anticoagulant responsible sites of the GI-$vPLA_2$ GII-$vPLA_2$ could be different, suggesting convergent evolution of the functions. In addition, some basic $vPLA_2$s can insert into the membrane outer layer and change the conformation and permeability of cell membranes (Qin et al. 2005). These highly basic $vPLA_2$s (pI >8.7) usually show antibacterial activities by penetration of the cell membrane, which could be independent of the catalytic activities (Mora-Obando et al. 2014).

Some moderately basic $vPLA_2$s with catalytic activities have evolved into neurotoxins or myotoxins, and high contents of neurotoxic $vPLA_2$ usually signify a high lethal potency of the envenoming. The difference and similarity in the reaction mechanism between the myotoxic and neurotoxic $vPLA_2$s have been reviewed (Montecucco et al. 2008; Šribar et al. 2014). Monomeric PLA_2 neurotoxins are present in the venom of highly poisonous Australian *Notechis* and *Pseudechis* and a few sea snakes. More potent and multimeric neurotoxic PLA_2s are present in the venom of kraits (e.g., β-bungarotoxins) and Australian elapids (e.g., taipoxin and texilotoxin). Recently, PLA_2 subunits with sequences homologous to all the three chains of taipoxin were identified in *Austrelaps superbus* venom (Marcon et al. 2013) and in the transcriptome of *Acanthophis wellsi* and *Suta fasciata*; supporting that the oligomeric PLA_2 neurotoxins were likely widespread in Australian elapid venoms (Jackson et al. 2013). Monomeric PLA_2 neurotoxins are present in many Viperidae, including *B. caudalis*, *Vipera, Daboia* (Tsai et al. 2007b), *Protobothrops,* and *Gloydius* (Chen et al. 2004; Yang et al. 2015). The heterodimeric PLA_2 neurotoxins (e.g., crotoxin and vipoxin) are only present in the venom of several genera of pit vipers (Chen et al. 2004; Yang et al. 2015) and true vipers (Šribar et al. 2014). Usually, a basic PLA_2 subunit that is enzymatically active is the key subunit in the multimeric toxins while other subunits are chaperons to increase the presynaptic specificity of the key subunit.

Monomeric and heterodimeric myo/neurotoxic Asn6-PLA_2s appear to be selectively expressed in the venom of Asian and American pit vipers. Crotoxin-like toxins have been identified in some rattlesnakes (*Crotalus* and *Sistrurus*) and *Bothriechis nigroviridis* (Lomonte et al. 2015) but not in most other rattlesnakes and *Bothriechis*. The recent discovery of Gintexin (a crotoxin-like neurotoxin) from

the venom of Chinese *Gloydius intermedius* revealed that the neurotoxic rattlesnakes are probably the descendants of an ancient sister species of *G. intermedius* that migrated to the New World about 30 million years ago (Wüster et al. 2008; Yang et al. 2015). In the absence of acidic subunits, the isolated basic subunits of crotoxin or similar neurotoxins showed higher anticoagulant and myotoxic effects than the native crotoxin (Yang et al. 2015). In contrast, monomeric and less potent neutotoxic PLA_2s, structurally similar to the crotoxin-B2 subunit, are present in the venom of *Gloydius brevicaudus and Gloydius saxilis* (Chen et al. 2004). Other monomeric and myotoxic Asn6-PLA_2s have been isolated from the venom of *Protobothrops* species and *Crotalus v. viridis* (Tsai et al. 2003) and *Bothriechis schlegelii* (Chen et al. 2004) and were also cloned from *Ovophis monticola* (Malhotra et al. 2013) and *Deinagkistrodon acutus* venom glands (Wang et al. 1996).

Increasing examples reveal that $vPLA_2$s are the most versatile enzyme in snake venom and the same enzyme molecule may play multiple roles (Gutiérrez and Lomonte 2013). At least in vitro or in animal models, certain $vPLA_2$ toxins show dual or multiple toxicities, e.g., ammodytoxin (Prijatelj et al. 2006) and trimucrotoxin (Tsai et al. 2011b) are both neurotoxic and strongly anticoagulant, and the major $vPLA_2$s of *Daboia and Vipera* are both neurotoxic and hypotensive (Chi 2001). Likewise, the venoms of Australian taipans and eastern brown snakes also adopt the evolutionary strategy of paralysis and hypotension, and their $vPLA_2$s are responsible for the vascular muscle relaxation and cardiovascular collapse in the victim (Chaisakul et al. 2014). Interestingly, a special $vPLA_2$s was recently reported to interact with nicotinic acetylcholine receptors rather than binding to presynaptic sites (Vulfius et al. 2014).

Synergism Between $vPLA_2$ and Other Venom Toxins

As expected, the venom components evolved as a whole and cooperated with one another in certain ways. Many examples demonstrate the synergism between different $vPLA_2$ isoforms or between $vPLA_2$s and other toxin families in the same venom, and more details remain to be explored. The potent presynaptic neurotoxins, β-bungarotoxins, synergize with the postsynaptic α-bungarotoxin and κ-bungarotoxin (i.e., three-finger toxins) and result in respiratory failure for lethal envenoming of kraits. Likewise, taipoxin (Chaisakul et al. 2014), texilotoxin, and the presynaptic neurotoxins of *A. superbus* venom (Marcon et al. 2013) may synergize with the postsynaptic α-neurotoxins (three-finger toxins) present in the venoms. The acidic $vPLA_2$s of cobra venom synergize with the abundant cardiotoxins (i.e., direct lytic factors) to damage cell membranes and result in hyperkalacemia (high plasma $[K^{+1}]$) and heart failure. The synergism between crotoxin and crotamine, a myotoxic and permeability-enhancing peptide (Rádis-Baptista and Kerkis 2011), may facilitate the internalization (Šribar et al. 2014) of crotoxin-B subunits and thus their toxicity to the neurons. It would be interesting to investigate whether the abundant crotamine in *C. v. viridis* venom also synergizes with its myotoxic Asn6-$vPLA_2$s (Tsai et al. 2003) for the envenoming. Additionally, it has been noted that kallikrein-like serine proteases

and bradykinin potentiation peptides and natriuretic peptides are more abundant in viperid venoms that contain neurotoxic $vPLA_2$s (e.g., *P. mucrosquamatus, G. intermedius, G. brevicaudus*, and some *Daboia* and *Vipera* venom) than in those containing mainly hemorrhagic toxins, suggesting possible coevolution of hypotensive and paralyzing effects of the venom components.

For hemorrhagic venoms, antiplatelet acidic $vPLA_2$s may synergize with disintegrins (that bind to the fibrinogen receptor or other platelet receptors) and various C-lectin-like binding proteins to induce thrombocytopenia. The highly catalytic $vPLA_2$s may also release lysophospholipids; this product could affect platelet aggregation and membrane permeability. In various yellowish snake venoms, *L*-amino acid oxidase can generate H_2O_2 and thus elicit strong antiplatelet effects and thus may also synergize with acidic $vPLA_2$s in the same venom. *Bothrops asper* venom is rich in myotoxic $vPLA_2$s as well as potent nucleotidase so the adenosine triphosphate (ATP) released from damaged muscle cells is converted into adenosine diphosphate (ADP) and monophosphate (AMP) and adenosine; the in situ generated adenosine could result in hypotension and anticoagulant effects (Caccin et al. 2013). It was recently confirmed by in vitro and in vivo study that the *B. asper* venom Asp49-myotoxin synergized with its Lys49-PLA_2 to ameliorate myotoxicity upon envenoming (Mora-Obando et al. 2014). This synergism may also occur in the envenoming by the American *Agkistrodon* since their venoms are abundant in both myotoxic Asp49-PLA_2 and Lys49-PLA_2 (Komori et al. 2002). Remarkably, some isolated acidic $vPLA_2$ isoforms showed rather low or ambiguous toxicities; whether they exert special effects in the presence of other venom components remains to be clarified.

Reduction or Loss of Lipolytic Activity as an Evolutionary Strategy

When a $vPLA_2$ does not depend on reaction products for its function, its catalytic activities could be lost through mutation at key residues at the active site, such as Gln4, Phe5, Tyr28, Lys31 or Trp31, His48, and Asp49. Many GI-$vPLA_2$s with much reduced catalytic activities have been found to contain Pro31 substitution, e.g., the major $vPLA_2$ isoforms of *B. fasciatus* and certain $vPLA_2$ isoforms of *Laticauda* and *P. australis* (Tsai 2007). These Pro31 mutants have reduced Ca^{2+}-binding ability and catalytic activity (Tsai et al. 2007a) and can be present in a higher amount than other $vPLA_2$s of the same venom and contribute to cytotoxic, myotoxic, and cardiotoxic effects (Liu et al. 1992). Many Viperidae and Crotalinae venoms also contain nonenzymatic $vPLA_2$s with distorted Ca^{2+}-binding loop or active site conformation. Besides the more commonly present Lys49-PLA_2 homologues in both Old World and New World pit vipers, basic GII-$vPLA_2$s with Arg49 or Asn49 substitutions have been found in some venom species of *Protobothrops* and *Trimeresurus* pit vipers (Mebs et al. 2006; Tsai et al. 2004b; Wei et al. 2010), respectively. Ser49-PLA_2s have been isolated from the venoms of *Echis* species, *V. a. ammodytes*, and *V. u. renardi* (Tsai et al. 2011a). Among the elapid venoms studied, a *B. fasciatus* $vPLA_2$ with unique Ala49 substitution (Liu et al. 1992) and a

Laticauda colubrina $vPLA_2$ isoform with His48Asn mutation (Takasaki et al. 1988) have been characterized. These basic proteins could be abundant in the venom and damage cell membranes through a noncatalytic mechanism and result in local edema, inflammation, and myonecrosis. It has been noted that the myotoxic effects of enzymatically active Asp49 $vPLA_2$s are systematic and that their mechanisms for sarcolemma damage differ from those of the locally effective Lys49 $vPLA_2$s, while both myotoxin types induce Ca^{2+} entry and release of ATP (Fernández et al. 2013).

Post-Translational Modifications of $vPLA_2$s

The compact and well-soluble $vPLA_2$ molecules in general do not undergo post-translational modification, except the disulfide bond formation that occurs during their biosynthesis and folding in endoplasmic reticulum. This fact allows a convenient and direct match of the protein mass of an isolated $vPLA_2$ to that calculated from its deduced protein sequence assuming complete disulfide pairing. Accurate pairing of the 12–14 Cys residues results in a compact 3D structure and the high thermal stability of $vPLA_2$s in an acidic buffer or in the presence of 40–60 % of acetonitrile or alcohol. The absence of Cys61-Cys99 in the Asp49-$vPLA_2$s of *Bitis gabonica* and *Bitis nacicornis* venoms and in the Lys49-$vPLA_2$s of *Trimeresurus puniceus* and *Trimeresurus borneensis* venoms does not affect protein folding but reduces the $vPLA_2$ thermal stability (Wang et al. 2005). Additionally, some exposed methionine residues of $vPLA_2$ can be partially oxidized in air to less hydrophobic Met-sulfoxide, especially when the venom contains abundant *L*-amino acid oxidase that may produce H_2O_2 by oxidizing free amino acids. The oxidation of methionine residue in a $vPLA_2$ usually results in its reduced toxicity and enzymatic activity (Tsai et al. 2000).

Tropidolaemus wagleri holds a unique taxonomic position among pit viper species (Wüster et al. 2008), and its acidic E6-PLA_2 was found to be fully glycosylated at residue 13 with a small complex type of *N*-glycan (Tsai et al. 2012). The bee venom $sPLA_2$, taipoxin γ-subunit, and the texilotoxin C-subunit are also glycosylated but at different positions of Asn residues. However, deglycosylation of the bee and the *T. wagleri* E6-$vPLA_2$s by specific glycosidase did not affect their enzymatic activities significantly (Tsai et al. 2012). The truncated acidic subunits of Crotoxin A and Gintexin A are derived from unique acidic PLA_2 precursors by endopeptidases to remove three interface recognition regions of the PLA_2s (Yang et al. 2015), and modification of the *N*-terminal Gln of one of the crotoxin-A fragment to a pyroglutamine residue is achieved by a Gln-cyclase in the venom.

Fatty acylation of the side chains of Lys7 and Lys10 in the basic Asp49-PLA_2 of *Agkistrodon p. piscivorus* occurred in vitro when the enzyme was incubated with a high concentration of activated esters or pseudosubstrates (Cho et al. 1988; Shen et al. 1994). The acylation of $vPLA_2$ at specific Lys side chains was based on an autocatalytic mechanism and could promote enzyme dimerization and increase

their activities toward densely packed liquid-crystalline phospholipid bilayers. However, there is no evidence to support that vPLA$_2$ can be acylated in vivo as part of its reaction mechanism and regulation.

Conclusion and Future Direction

In recent decades, toxinologists have made significant contributions to deciphering the structure, toxicity and selectivity, and biodiversity of venom proteins and aid in the treatment of envenomed patients. The present work highlights some of the most relevant contributions in the study of vPLA$_2$ evolution and diversity. Apparently, a vast amount of sequence data for vPLA$_2$s has been generated by the omic technology, and the information helps to clarify how venomous snakes and their venom evolved. Hopefully, the fascinating molecular diversity of vPLA$_2$ family can raise interest and concern in the study of new snake venom species and their ecology as well as the conservation of venomous snakes.

There is more to be discovered about the specificity, structure, and action mechanisms of vPLA$_2$s at molecular and cellular level. The genomics and expression regulation of vPLA$_2$s, the impact of vPLA$_2$s in overall pathophysiology of envenoming, and how their variations correlated with prey-ecology and snake adaptation remain to be elucidated. Receptors and binding proteins for vPLA$_2$s have been identified in mammals, suggesting that functions and specificities of vPLA$_2$s could be regulated through specific protein-protein interactions and membrane interactions. The potential for vPLA$_2$s in therapeutic and diagnostic applications, alone or combined with other molecules, also remains to be tested and formulated using assays and model systems related to humans.

Cross-References

► Squamate Reptile Genomics and Evolution
► Structure-Function Relationship in Heterodimeric Neurotoxin PLA2s from Viperidae Snakes Inhabiting Europe, South America, and Asia

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