Review

Phospholipases A_2 From *Viperidae* Snake Venoms: How do They Induce Skeletal Muscle Damage?

Bruno Lomonte* and José María Gutiérrez

Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, 11501 Costa Rica, San José 11501

** Corresponding author: E-mail: bruno.lomonte@ucr.ac.cr*

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Dedicated to the memory of Professor Franc Gubenšek

Abstract

Phospholipases A_2 (PLA_2s) are abundant components in snake venoms, which play important toxic roles. This review focuses on group II PLA_2s endowed with myotoxic effects, present in Viperidae venoms. These PLA_2s are subdivided into catalytically-active (Asp49) PLA_2s, and catalytically-inactive PLA_2 homologues, the latter most frequently presenting the Lys49 substitution. Both protein subgroups induce skeletal muscle necrosis, although by different mechanisms. Current evidence indicates that phospholipid hydrolysis plays a central role in the necrotizing action of Asp49 myotoxins, whereas PLA_2 homologues rely on the direct membrane-destabilizing actions of their cationic C-terminal region to achieve such effect, in the absence of catalysis. Both mechanisms converge in sarcolemmal permeabilization, triggering a series of intracellular events that lead to necrosis. Most viperid PLA_2 myotoxins act only locally, but those forming heterodimeric complexes such as crotoxin spread to distant muscles and induce rhabdomyolysis. This divergence between local and systemic myotoxicity might be related to differences in binding specificity to cell targets. Nevertheless, the identity of molecular targets recognized by viperid PLA_2 myotoxins remains elusive. Identification of their membrane target(s), and a deeper understanding of the catalytic-dependent and -independent mechanisms that result in membrane destabilization, are two crucial, but still unclarified aspects of their myotoxic action.

Keywords: Phospholipase A_2, myotoxin, snake venom, myonecrosis

1. Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) comprise a large and diverse group of interfacial enzymes which hydrolyze the *sn*-2 ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides to produce lysophospholipids and fatty acids.[1–4] In snake venom secretions, PLA_2s are particularly common and abundant components which play important roles in toxicity.[5,6] Among the wide array of activities exerted by these proteins, neurotoxicity and myotoxicity are two potent actions of major clinical relevance in snakebite envenomings.[7–9] In spite of the large number of venom PLA_2s isolated, and of the considerable body of work on their biochemical and structural characterization, as well as on their pathological effects and other bioactivities, a thorough understanding of their mechanisms of toxicity has not yet been achieved. The present review focuses on PLA_2s endowed with myotoxic effects, present in Viperidae snake venoms. Recent advances towards understanding their mechanisms of action, as well as key issues that remain to be solved, are discussed. In order to provide a broader context to this discussion, some general aspects of snake venom PLA_2s are briefly reviewed.

2. Snake Venom PLA_2s Belong to Two Related, But Structurally Distinguishable Groups

PLA_2s constitute a large superfamily of enzymes which are widely distributed in living organisms. On the basis of their structural and biochemical properties, these enzymes have been classified within at least 15 groups

(I–XV), including several subgroups.[10] All of the PLA_2s found in snake venoms belong to the small (12–15 kDa), secreted-type of enzymes, composed of 119–134 amino acids. Those present in the venoms of Elapidae are classified within group I, whereas those found in Viperidae correspond to group II. Both groups share a conserved structural fold and identical catalytic machinery, but differ in the position of one of their seven disulfide bonds, and in their C-terminal regions.[11] Group I PLA_2s present a Cys11-Cys77 bond (numbering of Renetseder[12]), absent in group II enzymes, which instead possess a Cys51-Cys133 bond. Additionally, group I PLA_2s display in their sequence an insertion of two or three amino acids within the region 52–65, the so-called "elapid loop", whereas group II enzymes present a C-terminal extension of 5–7 amino acids, absent in group I PLA_2s (Fig.1).

these water-soluble enzymes to adsorb to water-insoluble lipid interfaces in order to access their substrates.[4] After enzyme binding, phospholipid substrates need to reach the His48-Asp99 pair at the active site slot, and consequently move from their original position at the interface, traveling about 15 Å through the "hydrophobic channel" of these enzymes. The interfacial recognition surface of PLA_2s, or "i-face", is of utmost importance to the specificity of their actions. The final catalytic efficiency of a given PLA_2 upon a particular substrate is determined not only by the phospholipid head group specificity of its active site, but also by the binding properties of its interfacial recognition surface. In the case of venom PLA_2s endowed with toxic activities, such "dual recognition" becomes essential to understand the diversity and specificity of their pharmacological effects, as will be further discussed

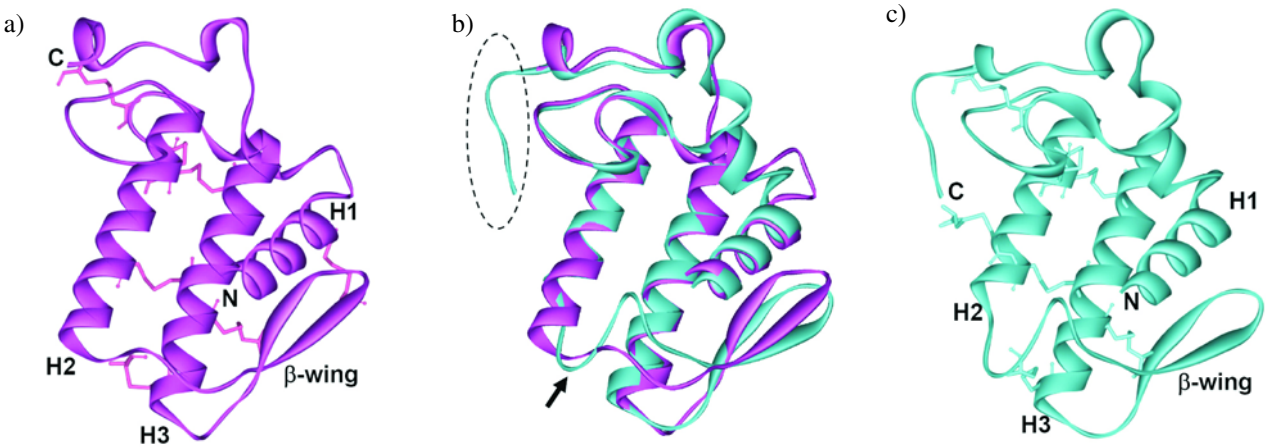

Figure 1: Comparison of the three-dimensional structure of group I and group II snake venom phospholipases A_2. Ribbon representations of **(a)** notexin (PDB: 1AE7), a group I phospholipase A_2 from the elapid snake *Notechis s. scutatus* and **(c)** D49 basic PLA_2 (PDB: 1VAP), a group II enzyme from the viperid snake *Agkistrodon p. piscivorus*. Disulfide bonds are shown in stick representation. Superposition of both structures in **(b)** illustrates their overall three-dimensional structure conservation. N- and C-termini, as well as helices (H1, H2, H3) and the β-wing region are labeled. Distinctive features as the extended C-terminal of the group II enzymes, and the "elapid loop" region of group I enzymes, are indicated with a dotted oval and an arrow, respectively, in **(b)**. Adapted from Ref. [111]

A growing number of three-dimensional structures of snake venom PLA_2s have been solved and made available through the Protein Data Bank (www.rcsb.org). Their conserved structural fold is formed by a short N-terminal α-helix, followed by the "calcium-binding loop"; a second, long α-helix, connecting to the "β-wing"; and a third, long α-helix, ending with the C-terminal loop.[11] The catalytic network of both group I and II enzymes, formed by residues His48, Tyr52, Tyr73, andAsp99, is also highly conserved.[3] These residues, together with those coordinating the essential calcium ion cofactor (Asp49, Tyr28, Gly30, Gly32) drive the hydrolysis of the *sn*-2 ester bond of phospholipids at the water-lipid interface of micelles, monolayers, vesicles, or membranes. The catalytic activity of PLA_2s upon such aggregated forms of substrates is markedly higher than upon monomeric, dispersed substrates. Such preference is explained by the specialization of

below. Indeed, this concept has been pivotal to clarify the long-standing paradox on the lack of correlation between the toxic potency of venom PLA_2s, and their enzymatic activity as measured using general substrates *in vitro*.[13,14]

3. Evolutionary Convergence of Toxicity in Elapidae and Viperidae PLA_2s

The structural properties shared between group I PLA_2s from elapids and group II PLA_2s from viperids, namely the conservation of their three-dimensional fold and catalytic machinery, obviously imply a common ancestor in evolution. However, the existence of differences regarding their distinctive disulfide bonding patterns and the

alternative presence of a C-terminal extension, or an "elapid loop", have relevant evolutionary implications when viewed within the context of the non-toxic, group I and group II secreted PLA_2s of mammalian origin.

Snake venom toxins arose by the recruitment of few ancestral genes coding for non-toxic proteins, which gradually evolved under natural selection by duplication and divergence, most notably in surface amino acid residues, to acquire the ability to interfere with vital physiological processes of prey.[15–24] The evident structural similarity between PLA_2s from elapid venoms and the mammalian pancreatic PLA_2s (group I), on one hand, and between the PLA_2s from viperid venoms and mammalian synovial or inflammatory PLA_2s (group II), on the other hand, indicates that at least two independent gene recruitment events for venom gland PLA_2s occurred during the evolution and diversification of snakes.[20–24] Clearly, the PLA_2 genes that were recruited into the elapid and viperid snake lineages, respectively, had already diverged from their common ancestor at the time of recruitment, as they already possessed the distinctive features of group I and group II enzymes (Fig.2). Notwithstanding these distinct structural characteristics, both group I and II ancestral PLA_2 genes proved to be versatile enough to evolve into potent toxins, selectively expressed in venom gland secretions. Since the group I and group II structural scaffolds were already different when recruited into the venom proteomes of elapids and viperids, respectively, and since both gene lineages evolved from non-toxic enzymes into neurotoxic and/or myotoxic enzymes, the acquisition of these novel toxic activities by snake venom PLA_2s should be regarded as a case of convergent evolution (Fig.2).

A consequence of this view, in terms of understanding the structure-function relationships of venom PLA_2s

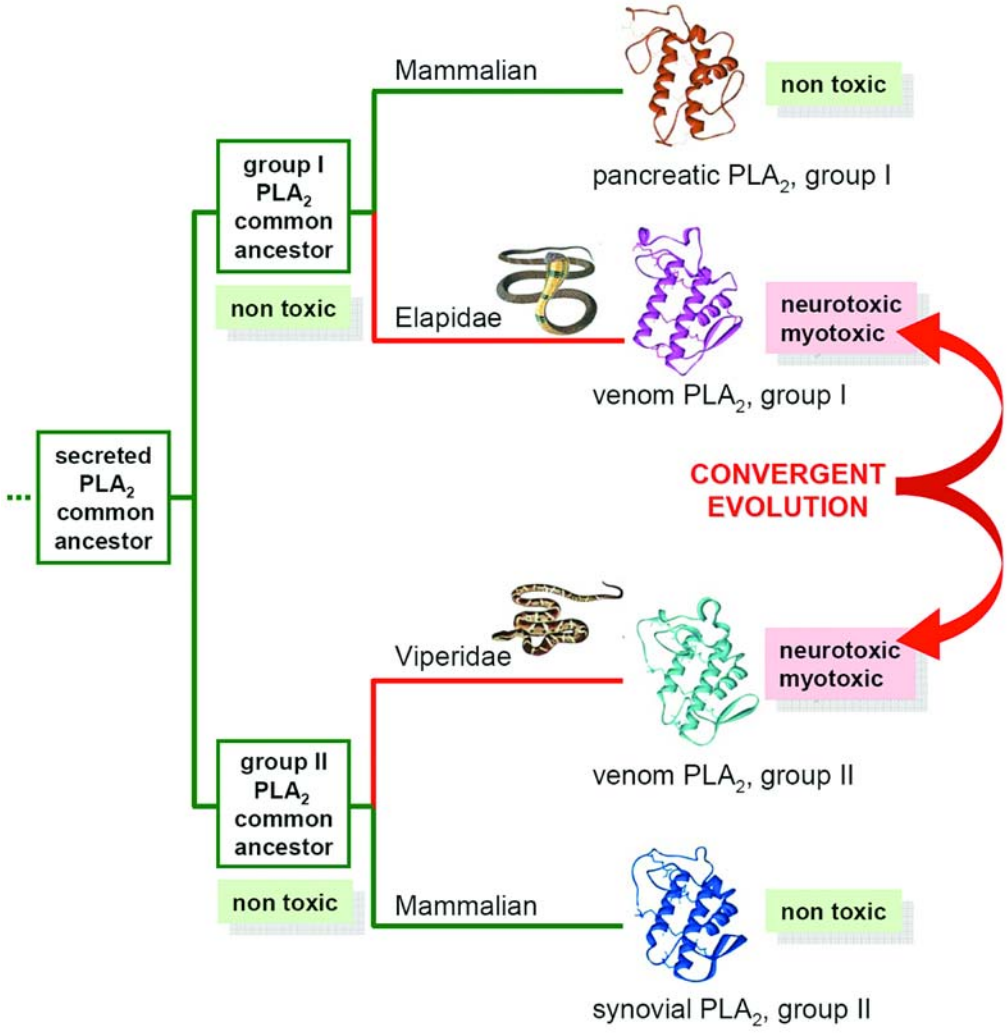

Figure 2: Convergent evolution of the toxic activities of snake venom phospholipases A_2 (PLA_2) in Elapidae and Viperidae. All PLA_2s found in elapid venoms belong to group I, sharing structural features with the non toxic pancreatic enzymes of mammals. Viperid PLA_2s, on the other hand, belong to group II, and share structural features with the non toxic "inflammatory" or "synovial" PLA_2s. Therefore, different ancestors were recruited in elapids and viperids during the evolution and diversification of snakes. Both types of non toxic ancestors underwent a process of gene duplication and accelerated evolution which generated neurotoxic and/or myotoxic PLA_2s, exemplifying a case of convergent evolution.

and their molecular mechanisms of toxicity, is that significant differences may exist in the mode of action of elapid and viperid toxins that exert the same toxic effect, i.e. they do not necessarily act by using the same structural determinants and mechanisms of toxicity, even if the final outcome may be indistinguishable. Therefore, the reciprocal extrapolation of findings between elapid and viperid PLA_2s is not warranted, and should be analyzed cautiously in every case.

4. Viperidae Venom PLA_2s: Group II Enzymes Further Diverged into two Main Subgroups

Virtually all Viperidae venoms studied contain PLA_2s, which in some species may reach proportions as high as 70–80% of their total proteins.[25,26] Often, a single species, or even a single individual, can express a multiplicity of PLA_2 isoforms in its venom, a strong indication of the duplication and divergence process that these genes underwent during their accelerated evolution,[15,16] and of the biological relevance of their new adaptive roles.[27,28] Remarkably, a further major structural subdivison exists within the group II PLA_2s of Viperidae, represented by the classical "Asp49 PLA_2s" and the "PLA_2 homologues". The latter proteins, in contrast to the enzymatically active PLA_2s which present an invariant Asp49 in their catalytic center, possess a number of amino acid substitutions, including the notable replacement of Asp49 by Lys49 (or less frequently by Ser, Arg, Gln, or Asn).[29–37] As a major consequence, these proteins cannot hydrolyze phospholipids,[38,39] and therefore are not true PLA_2s *sensu stricto*. Nevertheless, due to their high structural similarity with classical Asp49 PLA_2s, these proteins have been referred to as PLA_2 homologues.[28,40]

Both the Asp49 PLA_2s and the PLA_2 homologue subgroups of viperids conserve the distinctive features of group II enzymes, i.e. disulfide bonding pattern and C-terminal extension. Group II enzymes may present a variety of states of oligomerization, either remaining as monomers, or forming in some cases homodimers, heterodimeric complexes, trimers, or larger oligomers. The variable oligomerization of these proteins may have relevant implications for their toxic potencies and mechanisms of action.[40–47] In addition to their variable quaternary structural organization, viperid venom PLA_2s having a wide range of isoelectric points can be found, from acidic to highly basic proteins. As a general trend, the toxicity of PLA_2s is associated with a more basic character of the protein, i.e. a higher content of Lys and Arg residues in their primary structure. Many acidic PLA_2s found in these venoms do not display evident toxic properties *in vivo*, and for this reason may be considered to play a merely digestive role.[48,49] However, exceptions to this general trend exist, since some acidic PLA_2 have been shown to induce important toxic effects *in vivo*, including myotoxicity, albeit with a weaker potency than their basic counterparts.[50–53]

The toxic group II venom PLA_2s are targeted mainly toward the neuromuscular system *in vivo*, displaying either myotoxicity alone, or a combined presynaptic neurotoxic and myotoxic action.[7,32,54,55] Proinflammatory and other effects of these PLA_2s have also been demonstrated *in vivo*, for example, hypotensive activity.[56–59] On the other hand, a number of activities of these proteins, such as anticoagulant and platelet-modulating effects, have been characterized *in vitro*, but evidence to support similar roles *in vivo* is generally more scarce.

5. Myotoxicity of Viperid PLA_2s: Different Pathways Lead to a Common Outcome

5. 1. Catalytically-active (Asp49) PLA_2 Myotoxins.

Early studies on the toxic activities of snake venom PLA_2s attributed their effects to their ability to hydrolyze phospholipids, releasing lysophospholipids and fatty acids, even though it was soon appreciated that toxicity and catalytic efficiency were poorly correlated. Some highly toxic PLA_2s have very low enzymatic activity, and conversely, a number of highly active enzymes display low toxicity, or even are non-toxic. As mentioned before, such puzzling observations became more clear when the interfacial binding properties of PLA_2s toward their biologically relevant substrates were considered as a key element in their mechanisms of toxicity.[13,60] The catalytic activity of a PLA_2 toxin upon "general" substrates *in vitro* does not necessarily reflect the type and magnitude of the catalysis that may occur when acting upon their biologically relevant targets *in vivo*. Yet, characterizing the latter phenomenon has proven to be a challenging task, with many inherent technical difficulties to overcome.[13,14] Nevertheless, as a general principle, it has been shown that catalytically-active viperid PLA_2s which present myotoxic activity rely upon phospholipid hydrolysis to induce their necrotizing effect on skeletal muscle tissue. When the catalytic activity of these enzymes is abrogated, for example by chemical modifications (most notably alkylation of His48 by *p*-bromophenacylbromide,[61] by the use of some inhibitors, or by calcium-ion chelation), myotoxicity is drastically impaired, if not abolished.[50,62–64] Therefore, it has been reasonably concluded that enzymatic activity is necessary for myotoxicity, either because phospholipid hydrolysis promotes membrane instability or because the lysophospholipid and fatty acid products, generated by the phospholipid hydrolysis taking place at the surface of

skeletal muscle fibers, play a role in the mechanism exerted by the myotoxic Asp49 PLA_2s from viperids. Both of these products are well known to induce important biophysical alterations in membranes, which affect their stability.[65–67] Furthermore, some studies have demonstrated that the myotoxic action of particular PLA_2s can be mimicked solely by their products of hydrolysis,[51,68] a phenomenon also demonstrated in studies with presynaptically-acting neurotoxic PLA_2s.[69,70] Thus, hydrolysis of membrane phospholipids by Asp49 PLA_2s clearly constitutes at least one mechanism that leads to myotoxicity. In addition to this catalytic-dependent pathway, it has been proposed that Asp49 PLA_2 myotoxins might have the ability to induce muscle damage by a catalytic-independent action, perhaps in similarity to PLA_2 homologues. Support to this proposal has relied on the observation that a complete enzymatic inactivation of these proteins, in some cases, does not completely abrogate their myotoxic activity, but instead a variable residual effect remains.[62,71–74] While plausible, this hypothesis needs to be re-evaluated in the light of more recent findings which indicate that Asp49 PLA_2 myotoxins can be easily contaminated by Lys49 variants co-existing in the same venom (our unpublished data). Contamination of Lys49 myotoxins with Asp49 isoforms can be detected by evaluating the enzymatic activity of the preparations, but the reciprocal situation poses a more difficult challenge, as there is no specific functional assay to detect the presence of Lys49 proteins in Asp49 PLA_2 preparations. More sensitive analytical techniques available nowadays would allow to re-evaluate experiments performed during the 1990's and early 2000's, to clarify this relevant issue, for a more precise understanding of the structure-function relationships in Asp49 PLA_2 myotoxins.

5. 2. Catalytically-inactive PLA_2 Homologue Myotoxins

The discovery of PLA_2 homologues,[29] which exert potent myotoxicity despite lacking the ability to hydrolyze phospholipids,[38,39] required a new explanation for their mode of action. A particular region near the C-terminus of these proteins, rich in cationic and hydrophobic residues, was first identified to have a role in membrane damage and cytolysis, when a short synthetic peptide representing such region was found to mimic these toxic activities.[75] Subsequent studies confirmed the key role of this "toxic site" located on the surface of PLA_2 homologues,[76–88] which therefore represents a new mechanism leading to myotoxicity, completely independent of phospholipid hydrolysis. The final delineation of this particular "toxic site" of PLA_2 homologues is still under investigation, and has been reviewed elsewhere.[34,46,47,80,81] PLA_2 homologues occur in a large number of viperid taxa, with more than 60 proteins isolated to date. However, they are not ubiquitous in all viperids, and, intriguingly, appear to be absent in the venoms of some species that are phylogenetically very close to others that contain them.[28] The evolutionary emergence of PLA_2 homologues is enigmatic, considering that they originated from catalytically-active Asp49 PLA_2s, before the separation of Viperinae and Crotalinae lineages.[28,36,37,52,89–91] However, it is clear that myotoxicity must have been a relevant driving force for their evolution, as all PLA_2 homologues studied to date share this activity.

5. 3. Heterodimeric (Asp49) PLA_2 Myotoxins

A third pathway towards myotoxicity is represented by the case of heterodimeric PLA_2s from viperids, such as crotoxin and Mojave toxin, found in several species of *Crotalus*,[92–94] and possibly in few other viperid genera, as recently discovered for the arboreal pitviper *Bothriechis nigroviridis*.[95] These strongly myotoxic and neurotoxic molecules are composed of a non-covalently linked basic PLA_2 subunit, which is a catalytically-active Asp49 enzyme (subunit B), and an acidic, non-enzymatic subunit, also called a "chaperone" (subunit A). Crotoxin, the paradigmatic molecule of this group, is quite distinct from other viperid PLA_2 myotoxins, since it is able to spread systemically from its site of injection, and after reaching its target, to dissociate the chaperone subunit from the PLA_2 subunit, which, in turn, is responsible for toxicity.[59,96] The mode of action of the B subunit might be similar to that of other catalytically-active, Asp49 PLA_2s, but the fact that crotoxin has evolved a distinct, more elaborate mechanism of action allows to classify this myotoxic and neurotoxic protein as a group of its own. In contrast to all other myotoxic PLA_2s from viperids. which induce skeletal muscle damage only nearby their site of injection, crotoxin and crotoxin-like proteins induce also systemic myotoxicity.[97–99] The dichotomic presentation of myotoxicity in viperid envenomings as either local or systemic has been explained on the basis of the specificity that different myotoxic PLA_2s have for acceptors in muscle and other cell types.[74] Some myotoxic PLA_2s bind to various types of cells upon injection in the tissues. Consequently these toxins are likely to remain at the site of venom injection, provoking local myotoxicity, but being unable to distribute to distant muscles. In contrast, myotoxic PLA_2s that present higher selectivity to targets in muscle cells, such as crotoxin and Mojave toxin, provoke local myotoxicity at the site of injection, but are also capable of distributing to distant muscle compartments, where they bind and affect muscle fibers, thus inducing systemic myotoxicity which, clinically, manifests as rhabdomyolysis.[74,100]

In summary, myotoxic PLA_2s from viperids have evolved at least three different strategies that lead to a rapid skeletal muscle necrosis as the final outcome

(Fig.3): **(a)** an ability to hydrolyze phospholipids and generate lysophospholipid and fatty acid products within or nearby the muscle cell membrane, either by acting as monomers or homo-oligomers, **(b)** by acting as heterodimeric complexes that dissociate after binding to their target; phospholipid hydrolysis *per se*, together with the released hydrolysis products, in turn, alter the integrity of the plasma membrane; or **(c)** an ability to directly affect the integrity of the muscle cell membrane using a non-enzymatic mechanism. All three strategies displayed by viperid PLA_2 myotoxins eventually converge in the permeabilization of the plasma membrane, with catastrophic consequences for the affected muscle fibers (Fig.3).

6. Molecular Pathogenesis of Skeletal Muscle Damage: What Happens When Viperid PLA_2 Myotoxins Encounter Muscle Fibers?

6. 1. Initial Plasma Membrane Damage

The mechanisms outlined in the preceding section all converge to a common target: the plasma cell membrane of skeletal muscle fibers, or sarcolemma. Although the direct binding of viperid PLA_2s myotoxins has been difficult to document experimentally, due to the particulatly strong non-specific binding properties of these toxins, the wealth of ex-

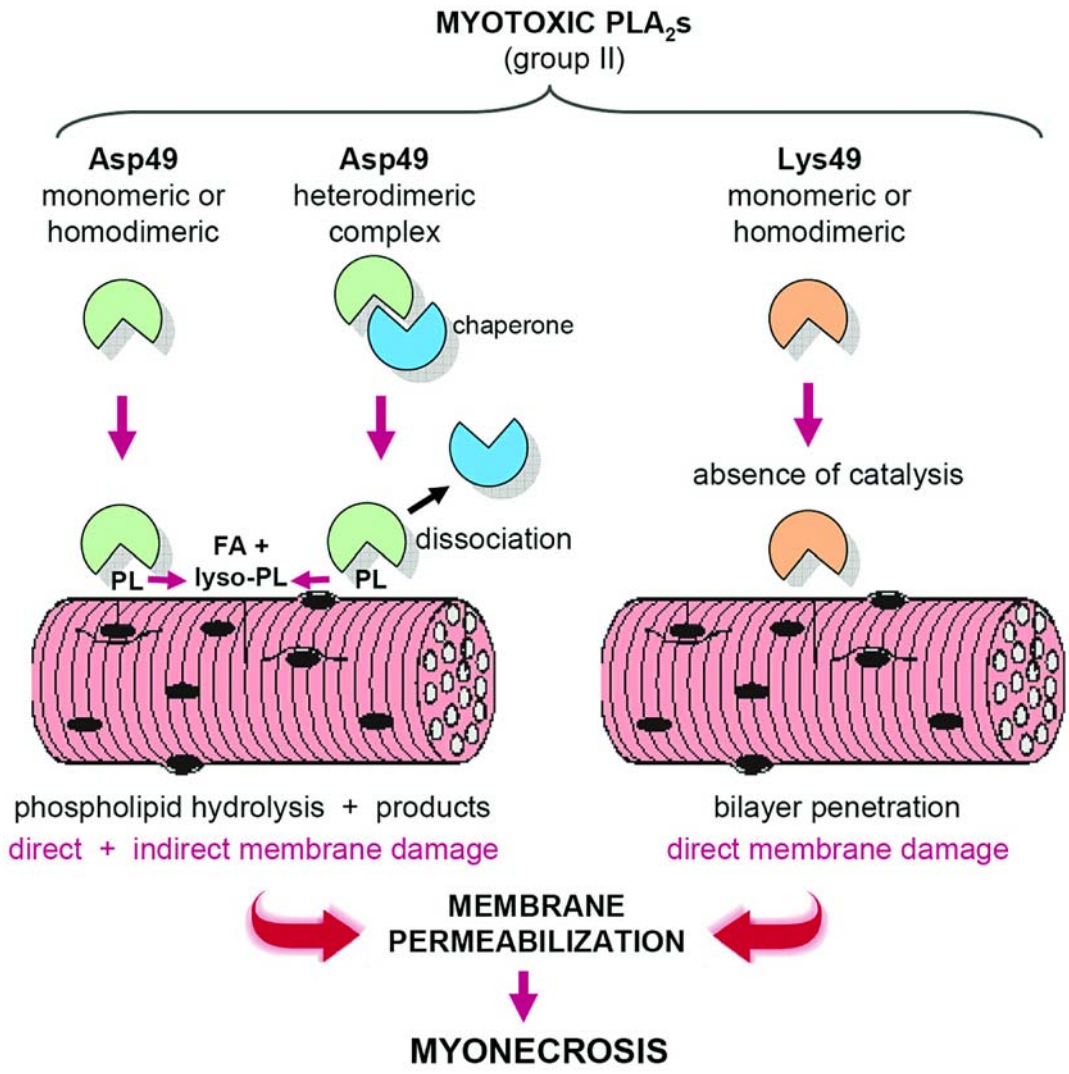

Figure 3: Different mechanisms for the induction of myonecrosis by viperid (group II) venom phospholipases A_2 (PLA_2s). The catalytically-active Asp49 PLA_2s (left) mainly rely on their enzymatic activity to hydrolyze membrane phospholipids (PL) and release fatty acids (FA) and lyso-PL products, destabilizing membrane integrity. Some of these Asp49 PLA_2s, such as crotoxin, act as a heterodimeric complex which dissociates upon binding and releases the "chaperone" subunit, leaving the catalytic subunit free to exert its membrane-damaging action. On the other hand, the catalytically-inactive PLA_2 homologues (right) such as the Lys49 myotoxins, are able to interact with membranes and directly destabilize their integrity, in the absence of catalysis. All of these strategies converge in the permeabilization of the sarcolemma, which initiates a stereotyped series of common events that lead to a rapid cell death by necrosis.

perimental evidence clearly supports the view that sarcolemma is their first site of action, and that the rest of degenerative events occurring in skeletal muscle fibers are secondary to this primary event. However, the precise targets of PLA_2s in sarcolemma have not been identified (see section 7). Regardless of the nature of sarcolemmal binding sites, the interaction of myotoxic PLA_2s with this membrane provokes rapid and drastic alterations in structure and function, revealed by ultrastructural evidence of membrane disruption,[101–103] by rapid depolarization,[94,105] by the efflux of cytosolic markers such as myoglobin, creatine kinase and lactate dehydrogenase,[101,106–108] and by an influx of Ca^{2+} ions.[68,109,110] The possibility of internalization of group II PLA_2 myotoxins to cause additional effects upon intracellular targets, further extending the consequences of the initial membrane damage, has not yet been experimentally addressed.

As discussed before, the mechanism of membrane damage is likely to involve catalytically-independent events in the case of PLA_2 homologues, and, in enzymatically-active PLA_2s, the hydrolysis of membrane phospholipids. Catalytically-inactive PLA_2 homologues, such as Lys49 variants, are able to disrupt the integrity of membranes by a process that involves the interaction of a cationic-hydrophobic stretch of residues, located near the C-terminus, with membrane phospholipids, resulting in penetration and/or disorganization of the bilayer structure.[75,79,111] Whether these toxins affect the membrane by penetration[112,113] or whether they "extract" membrane constituents by a "micellar nucleation" event,[114,115] or combine both mechanisms, warrants further investigation using biophysical experimental approaches. Membrane characteristics, such as the presence of anionic sites and cholesterol, as well as fluidity, influence the ability of Lys49 PLA_2s homologues to affect membrane structure.[62,107,113] In the case of Asp49 myotoxic PLA_2s, plasma membrane damage is associated with phospholipid hydrolysis.[71,73] Hydrolysis *per se* may cause membrane alterations, and may also affect membrane integrity through the detergent action of the hydrolytic products of phospholipids, i.e. lysophospholipids and fatty acids.[111,116] In addition, catalytically-active myotoxic PLA_2s may also present molecular determinants, such as cationic-hydrophobic surfaces, which could allow them to interact and disorganize bilayers independently of enzymatic hydrolysis.[62,117] Interestingly, a synergism between Asp49 PLA_2s and PLA_2 homologues regarding their membrane-permeabilizing actions has been demonstrated *in vitro*, using liposomes[118] or C2C12 cultures.[68] This synergism might provide an adaptive explanation for the presence of both types of myotoxic PLA_2s within a single venom, as frequently found in viperids.

6. 2. Intracellular Degenerative Events that Follow Initial Sarcolemmal Damage

One of the main consequences of plasma membrane disruption is the rapid influx of Ca^{2+} ions following a steep electrochemical gradient across the sarcolemma. This phenomenon has been documented *in vivo*[101] and, in greater detail, in cell culture studies.[68,87,110] The consequent increment in cytosolic Ca^{2+} concentration induces a series of degenerative events which, very rapidly, can bring the cell beyond the "point of no return", i.e. to irreversible cell damage. The most important consequences of this Ca^{2+} increment are: **(a)** Hypercontraction of myofilaments, evidenced by light and electron microscopic observations;[102,119–121] such hypercontraction, in turn, may induce further mechanical damage to the integrity of plasma membrane.[121] **(b)** Mitochondrial damage, characterized at the ultrastructural level by the appearance of high amplitude swelling, flocculent densities, hydroxyapatite crystals, and membrane disruption.[102,103,119] It is likely that mitochondria undergo a process of Ca^{2+} accumulation, through the Ca^{2+} uniporter, followed by hydroxyapatite precipitation and by the formation of flocculent densities and the permeability transition pore.[111] Such events have drastic consequences for cell viability, owing to the key role that this organelle has for ATP generation and cell survival. **(c)** Activation of Ca^{2+}-dependent proteinases, such as m- and μ-calpains, which contribute to the degradation of cytoskeletal components, such as desmin, titin and α-actinin.[122,123] **(d)** Activation of cytosolic PLA_2s, which could contribute to phospholipid hydrolysis in the plasma membrane, as well as in the membranes of organelles such as mitochondria and sarcoplasmic reticulum, further compromising cell viability. Interestingly, these series of cellular alterations are common to many pathological processes associated with plasma membrane damage. Thus, regardless of the initial mechanism of sarcolemmal lesion, the consequent influx of Ca^{2+} provokes a stereotyped series of degenerative events which are shared in many pathological models, including those induced by snake venom myotoxic PLA_2s.[111]

6. 3. A New Insight: the Expansion of Myocyte Damage by Released Intracellular Components

The scenario of PLA_2-induced muscle damage involves an additional aspect just recently described: the deleterious actions of ions and molecules released from damaged cells. The disruption of the integrity of plasma membrane provokes the efflux of cytosolic components. Among them, K^+ and ATP diffuse in the vicinity of damaged muscle fibers, reaching other fibers not yet directly affected by the myotoxins. K^+ is known to induce pain, by acting on afferent neurons, and has been proposed to play a role in the acute pain associated with the action of myotoxic PLA_2s.[124] ATP, in turn, is likely to amplify the effect of myotoxins by acting as a "danger signal", binding to ATP-gated purinergic muscle channels in the plasma membrane, and provoking a Ca^{+2} influx which may cause deleterious cell events in the neighborhood of myotoxin-

damaged cells. The reduction of increments in cytosolic Ca^{2+} concentration and of cell damage observed when myotubes in culture are exposed to myotoxins in the presence of apyrase, which quenches ATP, or of oxidized ATP, which blocks the purinergic receptors, supports the contention that ATP released from myotoxin-damaged myotubes diffuses and provokes Ca^{2+} entry and damage in myotubes not directly affected by the toxin.[124] These findings introduce a novel perspective to understand the mechanism of action of myotoxic PLA_2s whereby the direct cytotoxic action of myotoxins in the plasma membrane occurs concomitantly with an indirect action based on the effect of released ATP in neighboring muscle cells. The possible therapeutic implications of these findings are obvious, since it can be hypothesized that the blockade of purinergic channels may reduce the extent of muscle damage induced by myotoxic PLA_2s.

7. Acceptors: Key Missing Elements in Our Understanding of the Mechanisms of Myotoxicity in Viperid PLA_2s

In order to induce myotoxicity, viperid PLA_2s must interact with particular components on the surface of muscle fibers whose identities, until now, have remained elusive. Such molecular targets or "acceptor" sites should be more abundant on skeletal muscle fibers than on other cell types, as suggested by several lines of evidence: **(a)** Histological and ultrastructural evaluations of muscles where viperid PLA_2s have been injected reveal widespread myonecrosis, whereas other cell types in the vicinity of the destroyed fibers appear to be spared from damage, as judged by morphological criteria;[102] **(b)** *In vitro*, myotoxic PLA_2s are able to cause a rapid cytolysis of all types of cells tested so far, excluding erythrocytes, although showing a moderately higher activity upon myogenic cell lines;[107,125,126] **(c)** During the *in vitro* differentiation of myogenic cell lines, a specific increment occurs in their susceptibility to viperid PLA_2s, i.e. myotubes become more sensitive than their precursor myoblasts.[68,127] Notwithstanding, efforts made so far to identify the relevant molecular target(s) of these myotoxins have been inconclusive.

Myotoxic PLA_2s from viperids are proteins with highly basic isoelectric points, which *a priori* would suggest that they might interact with negatively charged membrane targets. Experiments using different types of liposomes evidenced the ability of these toxins to disrupt their integrity causing release of entrapped markers, and this effect was demonstrated to be significantly higher when negatively charged phospholipids are present in the liposome composition.[62,112,128,129] Liposome disruption by viperid PLA_2s demonstrates that these myotoxins are capable of drastically altering phospholipid membrane bilayers without the requirement of proteins, but this does not exclude the possibility that membrane proteins on the sarcolemma may be important for the specificity and efficiency of their myotoxic effect, especially considering that negatively charged phospholipids are very scarce on the outer layer of cell membranes. Moreover, it is also possible that molecules other than proteins or phospholipids could play the role of membrane acceptors for these viperid myotoxins. Only few attempts to explore such possibilities have been reported in the literature. *In vitro* treatment of cells with tunicamycin to inhibit overall N-linked glycosylations, or with neuraminidase to hydrolyze the negatively charged N-acetylneuraminic acid on their surface, did not affect the ability of a Lys49 myotoxin to exert its cytolytic action.[107] Target cells have also been treated *in vitro* to decrease their heparan sulfate proteoglycan, another negatively-charged surface component, either by enzymatic hydrolysis, or by inhibiting its sulfation step during synthesis using sodium chlorate. Neither of these treatments, nor the use of a cell mutant devoid of heparan sulfate, had an influence on the cytolytic action of a Lys49 myotoxin.[130]

Protein acceptors have been identified for secreted PLA_2s, both of mammalian[131] and snake venom[132–140] origins. In the case of viperid PLA_2 myotoxins, only crotoxin has been found to interact with a membrane protein of 45–48 kDa, albeit in relation to its neurotoxic actions using membrane preparations from guinea pig brain or *Torpedo marmorata* electric organ.[132,138] However, thus far no evidence has been reported to support the involvement of specific protein acceptors in the mechanism of membrane damage induced by viperid PLA_2 myotoxins. An exciting prospect emerged by the finding that the PLA_2 homologue myotoxins bind with high affinity to the vascular endothelial growth factor (VEGF) receptor-2, or KDR,[141] and by the subsequent mapping of their interaction site at the same region involved in toxicity, that is, the cationic C-terminal region 115–129.[142] However, it has not been established whether such interaction is functionally relevant to the myotoxic mechanism exerted by Lys49 PLA_2 homologues. Our preliminary findings suggest that VEGF receptor-2 is not required for the functional expression of toxic activity by these proteins (unpublished data). Asp49 myotoxic PLA_2s, on the other hand, do not bind to this particular receptor.[142]

Some experimental observations suggest the possibility that a protein acceptor may not be required for the toxic mechanism of viperid PLA_2s. For example, a synthetic peptide representing the C-terminal region of a Lys49 myotoxin reproduced its toxic effects *in vitro* as well as *in vivo*, not only in its normal L-configuration, but also when synthesized with all-D amino acids,[83] a finding that would argue against an interaction with a configuration-dependent molecular acceptor such as a protein. Other lines of evidence suggest that membrane anionic

phospholipids could constitute relevant binding sites for viperid PLA_2 myotoxins. Enrichment of the outer membrane leaflet of erythrocytes with phosphatidylserine or phosphatidic acid rendered these cells susceptible to lysis, whilst they are normally insensitive to the cytolytic action of both Asp49 and Lys49 myotoxins.[143]

Disruption of membrane lipid rafts by the use of cyclodextrin in cultured myogenic cells did not prevent the cytolytic activity exerted by these two types of PLA_2s, ruling out their possible role as acceptors.[113] Whatever the identity of the unknown acceptor(s) for viperid PLA_2s, a recent study provided evidence that it is likely to be coupled to a rapid signaling mechanism for intracellular calcium release, a phenomenon that precedes the massive influx of this ion from the extracellular medium.[68] The fact that group II PLA_2 myotoxins have such a broad range of cytolytic specificity *in vitro*, including not only many eukaryotic cell types but also a variety of bacteria,[84,85] suggests that their requirements for binding sites on membranes might not be too stringent, and perhaps do not involve a highly specific protein acceptor. In support of this speculation, the doses at which group II PLA_2 toxins induce myonecrosis in experimental animals are relatively high, when compared to some highly potent group I PLA_2s endowed with both myotoxic and neurotoxic effects,[121] or to the systemically acting heterodimeric PLA_2s such as the crotoxin complex.[99] The observed selectivity of viperid PLA_2s toward skeletal muscle fibers, when injected locally, might simply be a reflection of the highly disparate proportion of sarcolemma available, in comparison to membranes of other cell types.

8. The Action of Viperid Myotoxic PLA2s in the Clinical Context

The clinical presentation of myotoxicity in the vast majority of viperid snakebite envenomings is characterized by prominent local myonecrosis.[144–146] In these cases, the deleterious action of myotoxic PLA_2s occurs concomitantly with drastic alterations induced by hemorrhagic metalloproteinases in the microvasculature, generating an ischemic scenario which further contributes to muscle damage.[147] Moreover, alterations in intramuscular nerves[148,149] add to this complex picture. As a consequence of these alterations, the process of muscle regeneration is impaired,[149] leaving the patients with permanent sequelae.[145] Thus, in these cases, viperid myotoxic PLA_2s contribute to local pathological damage, but do not generate systemic myotoxicity. In contrast, envenomings by the rattlesnakes *C. d. terrificus* and some populations of *C. scutulatus* are characterized by local and systemic myotoxicity, evidenced by very high increments in plasma CK activity.[150] This is likely to be a consequence of the systemic action of the dimeric PLA_2s crotoxin and Mojave toxin on skeletal muscles. In these cases, rhabdomyolysis results in myoglobin accumulation in the kidneys, which may result in acute renal failure, a severe complication in these envenomings.[150]

9. Future Directions

A growing body of information, gathered after the isolation and characterization of a number of viperid snake venom PLA_2s endowed with myotoxic activity, has provided significant insights into their structure and its relationships to the functional mechanisms leading to skeletal muscle necrosis. It has been clearly established that these toxins affect muscle cells by catalytically-dependent and -independent mechanisms, and that disruption of the integrity of skeletal muscle sarcolemma constitutes the first step in their mode of action, followed by a stereotyped series of intracellular degenerative events mostly associated with increments in cytosolyc Ca^{2+} concentration. However, a thorough understanding of their modes of action has been hampered by the lack of knowledge on their target molecules on the surface of skeletal muscle fibers. Further efforts are needed to identify such targets, to characterize their molecular interactions with the toxins, and to address the details of the mechanisms that lead to sarcolemma permeabilization, the primary event leading to myonecrosis.

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Povzetek

Fosfolipaze A_2 (PLA_2) so bogato zastopane komponente kačjih strupov, ki igrajo pomembno vlogo pri izražanju toksičnosti le-teh. Pregledni članek se osredotoča na PLA_2 iz skupine II, ki jih najdemo v strupih viperid in, ki izražajo miotoksične učinke. Te PLA_2 razdelimo na katalitsko aktivne (Asp49) PLA_2 in na katalitsko neaktivne PLA_2 homologe. Slednji imajo najpogosteje na položaju 49 Lys. Obe skupini PLA_2 izzoveta nekrozo skeletnih mišic, a po različnih mehanizmih. Rezultati kažejo, da hidroliza fosfolipidov igra osrednjo vlogo pri nekroznem delovanju Asp49 miotoksinov, medtem ko miotoksičnost PLA_2 homologov temelji na neposredni destabilizaciji membrane s kationskim predelom na C-koncu teh molekul. Oba načina delovanja rezultirata v permeabilizaciji sarkoleme, kar sproži vrsto znotraj celičnih dogodkov, ki vodijo v nekrozo celice. Večina viperidnih PLA_2 miotoksinov deluje lokalno, a tisti, ki tvorijo heterodimerne komplekse, na primer krotoksin, se razširijo do oddaljenih mišic in izzovejo rabdomiolizo. Razlika med lokalno in sistemsko miotoksičnostjo PLA_2 bi lahko izhajala iz razlik v vezavni specifičnosti teh molekul za celične tarče. Identiteta celičnih tarč, na katere bi se vezali viperidni PLA_2 miotoksini je še neznana. Opis membranskih receptorjev za miotoksične PLA_2 in natančno razumevanje od encimske aktivnosti-odvisnega in neodvisnega mehanizma delovanja PLA_2, ki vodi do destabilizacije membrane, sta dva pomembna, a še vedno nepojasnjena vidika miotoksičnega delovanja teh molekul.