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Review of four major distinct types of human phospholipase A_2

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Abstract

The phospholipase A_2 superfamily of enzymes plays a significant role in the development and progression of numerous inflammatory diseases. Through their catalytic action on membrane phospholipids, phospholipases are the upstream regulators of the eicosanoid pathway releasing free fatty acids for cyclooxygenases, lipoxygenases, and cytochrome P450 enzymes which produce various well-known inflammatory mediators including leukotrienes, thromboxanes and prostaglandins. Elucidating the association of phospholipases A_2 with the membrane, the extraction and binding of phospholipid substrates, and their interaction with small-molecule inhibitors is crucial for the development of new anti-inflammatory therapeutics. Studying phospholipases has been challenging because they act on the surface of cellular membranes and micelles. Multidisciplinary approaches including hydrogen/deuterium exchange mass spectrometry, molecular dynamics simulations, and other computer-aided drug design techniques have been successfully employed by our laboratory to study interactions of phospholipases with membranes, phospholipid substrates and inhibitors. This review summarizes the application of these techniques to study four human recombinant phospholipases A_2 *in vitro*.

1. Introduction to phospholipase A_2 superfamily

Phospholipases A_2 were initially purified from the venom of poisonous snakes. As the ability to sequence and purify new proteins became more technologically advanced in the 1970s, it became apparent that these enzymes were abundant with cysteines that formed disulfide bonds (Davidson and Dennis, 1990a, b). At the same time, a separate novel human mammalian enzyme that also maintained this pattern of disulfide bonds and hydrolyzed phospholipid substrates in the body was discovered (Kramer et al., 1989; Seilhamer et al., 1989). Later as intracellular non-disulfide containing PLA_2s were discovered, it was necessary to create a group numbering system that could categorize the rapidly expanding number of PLA_2 enzymes together in a logical manner and make it easy to accurately identify which phospholipase A_2 was being studied (Dennis, 1994). Over the last 50 years, six major types of phospholipase A_2 enzymes have been identified: $sPLA_2$, $cPLA_2$, $iPLA_2$, Lp-PLA_2 (also known as PAF-AH), $LPLA_2$, and $AdPLA_2$ (Table 1.1).

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PLA_2 enzymes utilize either a catalytic dyad or triad to catalyze the hydrolysis of the ester bond at the *sn*-2 position of phospholipid substrates. Hydrolysis of substrates releases free fatty acids and lysophospholipids that can be acted upon in a variety of manners including, when it is arachidonic acid, being converted further to lipid signaling mediators (Figure 1). While features such as structure and the specific activity toward various substrates reveal commonality among the several types of PLA_2s, localization of them varies. Lp-PLA_2 and sPLA_2 are both secreted enzymes and act on membrane lipids extracellularly, while cPLA_2 and iPLA_2, hydrolyze intracellular lipids of bilayer membranes including various sub-cellular organelles, less is understood about the localization of LPLA_2 and AdPLA_2.

It is a particular challenge to study these water-soluble enzymes because they act on phospholipids which typically aggregate in an aqueous environment forming micelles, vesicles, liposomes, and other large structural aggregates (Dennis, 2016). Carrying out kinetics in a mixed micelle system with the non-ionic detergent Triton X-100 using “surface-dilution kinetics” has been a particularly informative model system to study PLA_2 activity since the hydrolysis of the phospholipid substrates occurs at the lipid-water interface (Deems et al., 1975). Studies on phospholipase A_2 enzymes must consider how experimental techniques will operate in this type of cellular environment (substrate aggregation, membrane association, etc.); and since no one technique offers full characterization of PLA_2s, we have had to utilize a wide array of approaches.

Whether it be enzymatic assays or molecular dynamics, each unique method offers advantages and disadvantages to studying PLA_2 enzymes. It has been necessary to utilize a multidisciplinary approach to understand the mechanism of action of these enzymes. Learning about their interactions with membranes (Bucher et al., 2013), phospholipid substrates (Mouchlis et al., 2015) and inhibitors (Mouchlis et al., 2011a) was achieved using a combination of enzyme assays, deuterium exchange mass spectrometry and molecular dynamics (Mouchlis and Dennis, 2016). In this review, results of studies on four human PLA_2s including their structure, function and activity are discussed in detail.

2. Lipoprotein-associated Phospholipase A_2

Platelet activating factor (PAF) is a potent phospholipid mediator that plays a major role in clotting and inflammatory pathways (Prescott et al., 2000). Two groups of phospholipases, Group VII and Group VIII, catalyze the hydrolysis of the *sn*-2 fatty acyl bond in PAF and other lipid substrates (Dennis et al., 2011). Therefore, these enzymes were initially called PAF acetylhydrolases (PAF-AH) (Schaloske and Dennis, 2006). Further studies of the Group VIA enzyme revealed that it associates with both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) leading to the name lipoprotein-associated phospholipase A_2 (Lp-PLA_2).

Lp-PLA_2 is calcium independent, secreted extracellularly and has been proposed to play a significant role in the development of atherosclerosis (Dennis et al., 2011; Tjoelker et al., 1995b). Group VIIA Lp-PLA_2 is a 45 kDa, secreted protein that contains an alpha/beta hydrolase fold and a GXSXG motif that is characteristic of esterases and lipases (Samanta and Bahnson, 2008). The active site contains a catalytic triad composed of Ser/Asp/His

(Tjoelker et al., 1995a) (Figure 2). Group VIIB Lp-PLA_2 is about 40 kDa long and contains the same catalytic triad as GVIIA Lp-PLA_2. Group VIII Lp-PLA_2 consists of two 26 kDa catalytic subunits and one 45 kDa non-catalyitc regulatory subunit. GVII and GVIII Lp-PLA_2 do not show much amino acid sequence identity but both are related to each other since these enzymes are in principle capable of retrieving substrates from the aqueous phase or through direct extraction from membranes (Cao et al., 2011; Min et al., 1999).

Some of the most common substrates for Lp-PLA_2 include PAF, short fatty acyl chain phosphatidylcholines, oxidized phospholipids, and F_2-isoprostanes (Stafforini et al., 1997; Stafforini et al., 2006; Stremler et al., 1991; Stremler et al., 1989). GVIII Lp-PLA_2 utilizes similar substrates but are more restricted in their activity towards the *sn*-2 position by having specificity towards acetyl groups (Manya et al., 1999). Catalytic hydrolysis of these substrates is believed to be stabilized by an oxyanion hole formed by the backbone amides of residues Leu153 and Phe274 (Samanta et al., 2009). H/D exchange studies suggested that residues 360–368 and 113–120 might serve as anchors for the association of the enzyme to the surface of the membrane (Cao, Jian et al., 2013; Cao, J. et al., 2013).

Since Lp-PLA_2 has been found to associate with both HDL and LDL from which it presumably retrieves its oxidized phospholipid substrate, abnormal distribution of this enzyme has been suggested to be linked to cardiovascular diseases (Tellis and Tselepis, 2009). Because the hydrolysis of substrates by Lp-PLA_2 has major upstream implications for inflammatory diseases (such as atherosclerosis and neonatal necrotizing enterocolitis), development of inhibitors and therapeutic interventions has been of great interest for many years. Azetidinones and pyrimidones have demonstrated inhibitory activity for Lp-PLA_2. GlaxoSmithKline has also developed the potent and selective inhibitor Darapladib that reached clinical trials (phase III) (Blackie et al., 2002; Blackie et al., 2003). Since then further investigation has been underway to learn more about the biological relevance of Lp-PLA_2 and its inhibitors.

3. Calcium-independent Phospholipase A_2

Group VIA calcium-independent phospholipase A_2 (iPLA_2), also known as PNPLA9 or i$PLA_2\beta$, is a calcium independent phospholipase A_2 and plays a key role in membrane remodeling (Ackermann et al., 1994; Balsinde et al., 1997). The many isoforms of iPLA_2 differentiate themselves from the many other phospholipase A_2 enzymes that are calcium dependent and they have been thought to be less specific as to the fatty acid at the *sn*-2 position (Lio and Dennis, 1998). These enzymes are the most widespread PLA_2s throughout human tissues. Although the crystal structure of iPLA_2 has not been solved, homology models and sequence alignments revealed that the longest splice variant (806 amino acid residues and 89 kDa) utilizes a catalytic dyad of Ser/Asp and is comprised of seven ankyrin repeats, a linker region, and a patatin-like α/β hydrolase catalytic domain (Larsson Forsell et al., 1999; Ma et al., 1999) (Figure 3). Despite studies demonstrating that iPLA_2 activity is regulated by many different mechanisms, it is still unclear how this enzyme is activated to bind and hydrolyze a specific substrate.

Inference of the structure of $iPLA_2$ from homology models and sequence alignments to previously crystallized structures of patatin lipase and human ankyrin-R d34 repeats has been fundamental to establishing the mechanism of action of $iPLA_2$ (Kienesberger et al., 2009; Michaely et al., 2002; Wilson et al., 2006). Regions of $iPLA_2$ that show the most similarity to patatin are proposed to be the catalytic domain (α/β hydrolase domain) while the rest of the enzyme is considered a regulatory unit (Mouchlis et al., 2015). The ankyrin repeats have been shown by hydrogen/deuterium exchange data to be highly flexible and may be key in regulation of $iPLA_2$ activity.

It has been demonstrated that $iPLA_2$ activity is regulated by ATP binding, caspase cleavage of the ankyrin repeats that results in a hyperactive form of the enzyme, calmodulin inhibition, oligomerization of the enzyme due to aggregation of ankyrin repeats, and membrane allosteric regulation (Mouchlis et al., 2015). ATP activation has been repeatedly demonstrated to be a major regulator of $iPLA_2$ activity. This enzyme is often found to localize in mitochondria, where ATP levels are critical for cellular regulation. $iPLA_2$ is the only PLA_2 believed to be regulated by ATP. ATP does not act as a substrate or cofactor, but rather activates the enzyme, presumably through allosteric regulation (Lio and Dennis, 1998; Ma et al., 1999). It is important to develop $iPLA_2$ inhibitors because the enzyme plays a significant role in many diseases including Barth syndrome and diabetes. Current known inhibitors include trifluoromethyl ketones, tricarbonyls, lactones and polyfluoroketones (Mouchlis, V. D. et al., 2016; Mouchlis, Varnavas D. et al., 2016).

4. Cytosolic Phospholipase A_2

Multiple subgroups of cytosolic phospholipase A_2 ($cPLA_2$) have been discovered in different cell types (Dennis et al., 2011). The first $cPLA_2$ purified and sequenced was Group IVA $cPLA_2$, an 85kDa protein that is regulated by intracellular calcium levels (Clark et al., 1991; Dessen et al., 1999; Kramer et al., 1991). This enzyme is widely distributed in cells throughout most types of human tissue and consists of a C2 domain, linker, and α/β hydrolase domain as determined by x-ray chrystalography (Dessen et al., 1999). Calcium binding to the C2 domain in the N-terminal causes localization of the protein to a phospholipid membrane (Channon and Leslie, 1990). $cPLA_2$ also utilizes a catalytic dyad of Ser/Asp within the C-terminal α/β hydrolase domain (Pickard et al., 1996) as illustrated in Figure 4).

$cPLA_2$ acts on many lipid substrates including but not limited to phosphatidylcholines, phosphatidylethanolamines, and phosphatidylinositols (Leslie et al., 1988). Hydrolysis of phospholipid substrates has shown high specificity towards arachidonic acid at the *sn-2* fatty acyl bond (Clark et al., 1991). In addition to this activity, $cPLA_2$ also displays lysophospholipase and trans-acylase activity (Reynolds et al., 1991). It has been demonstrated that the lipid mediator phosphatidylinositol 4,5-bisphosphate (PIP_2) further increases $cPLA_2$ activity(Mosior et al., 1998; Tamiya-Koizumi et al., 1989) as well as ceramide-1-phosphate (C1P) (Nakamura et al., 2006). Besides calcium, PIP_2, and C1P activation, $cPLA_2$ is regulated by phosphorylation and membrane interactions (Mouchlis et al., 2015). Although no specific disease is associated with regulation of this enzyme, it is known to play a role in inflammation through downstream regulation of COX genes.

Multiple types of inhibitors have been developed including 2-oxoamides and 2-oxoesters (Kokotou et al., 2017; Mouchlis et al., 2012).

5. Secreted Phospholipase A_2

The secreted phospholipase A_2 ($sPLA_2$) was the first phospholipase A_2 enzyme identified and studied in detail from snake venom, mammalian pancreas and mammalian cells (Dennis et al., 2011). $sPLA_2$ is a group of small enzymes (14–18kDa) that use a His/Asp dyad and also required calcium for catalytic activity (Schaloske and Dennis, 2006) (Figure 4). Mechanistic insights were developed from the crystal structure of the cobra venom (Naja naja naja) which was reported (Fremont et al., 1993) by our laboratory as well as crystal structures of other venom and pancreatic PLA_2s by many groups over the years (Seilhamer et al., 1989; Kramer et al., 1989; Guy et al., 2009; Matoba et al., 2002). For mammalian $sPLA_2$s, sequence alignments have been used to interpret the structure of some of these enzymes, such as the Group V $sPLA_2$ based on the crystal structure of the Group IIA $sPLA_2$ (Edwards et al., 2002). Multiple calcium binding sites have been reported according to DXMS studies (Burke et al., 2008). Mechanisms of activation and inhibition of this enzyme have been studied, and it has been shown that the $sPLA_2$ s enzyme do not hydrolyze the sn-1 position of the lysophospholipid products while some of the intracellular enzymes do. Surprisingly, $sPLA_2$ does display an increase in activity when substrate forms aggregates rather than monomers, known as “interfacial activation” (Carman et al., 1995).

Major functions of $sPLA_2$ include antibacterial and antiviral properties, regulating HDL and LDL levels and playing a role in inflammatory diseases such as atherosclerosis. Numerous inhibitors have been developed for $sPLA_2$ including dicarboxylic acids, sulfonamides, amides, indoles, and oxadiazolones, many of which were helped and better understood by *in silico* studies (Mouchlis et al., 2011b; Mouchlis et al., 2010a, b).

6. Conclusion

Understanding the cellular function of phospholipases is a very challenging task. PLA_2 (Dennis and Norris, 2015) and other related enzymes (Grkovich and Dennis, 2009) play a significant role in the development of inflammatory diseases and thus understanding how to regulate their activity is necessary for the advancement of interventional therapeutics and inhibitors. Computational techniques combined with hydrogen-deuterium exchange mass spectrometry experiments have been successfully employed to understand the mechanism of action of phospholipases as well as the structural and physicochemical properties of their active sites (Mouchlis and Dennis, 2016). In conjunction with these approaches, enzymatic assays have also been used to further investigate PLA_2 enzymatic activity. This review summarizes the application of these techniques to study four human phospholipases A_2

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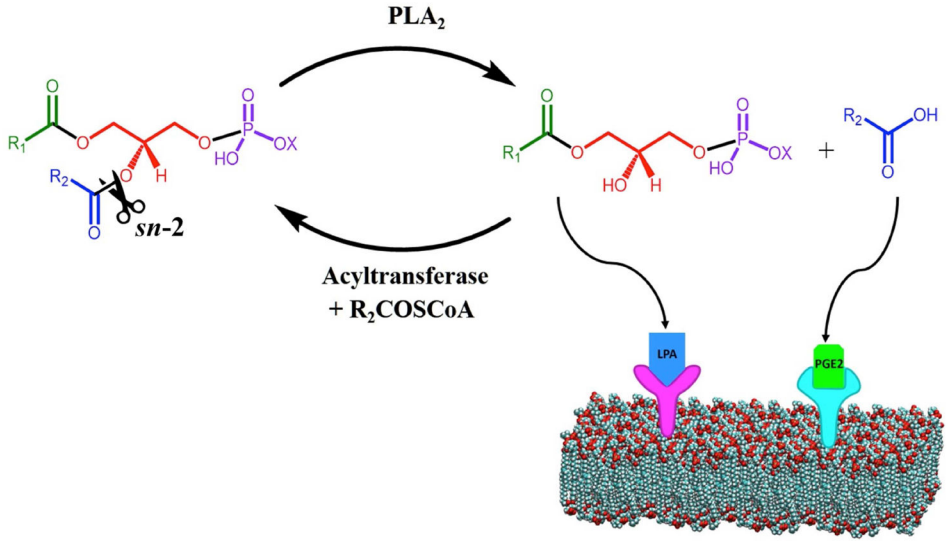

Figure 1. Phospholipases A_2 function as degradative enzymes when they produce lysophospholipids and free fatty acids as products, as biosynthetic enzymes when the lysophospholipid product is reacylated with a PUFA to produce important phospholipids, and as signaling enzymes when the products are converted to agonists of GPCRs
(adapted from (Dennis, 2016)).

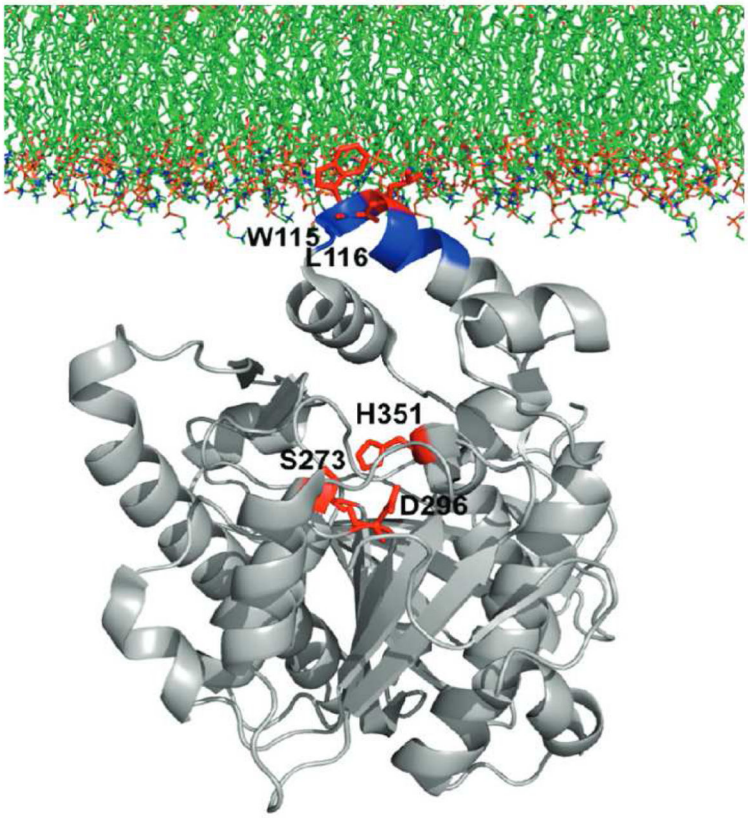

Figure 2. A model of GVIIA Lp-PLA$_2$ binding to the surface of a DMPC membrane

The proposed membrane binding region of Lp-PLA$_2$ is shown in blue (amides 113–120). The proposed region for liposome association (amides 115 and 116) as well as the catalytic triad (Ser273, Asp296, and His351) are shown in red (adapted from (Cao et al., 2011)).

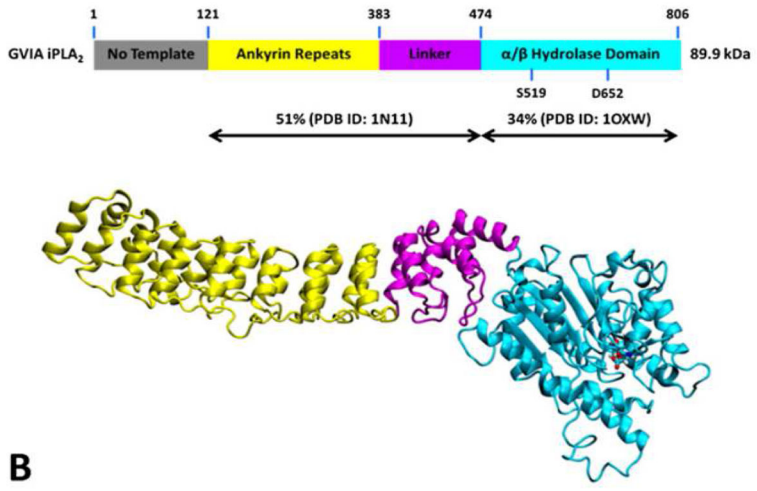

Figure 3. Three-dimensional structure of calcium-independent phospholipase A_2

A homology model of GVIA $iPLA_2$ is shown (adapted from (Mouchlis et al., 2015)).

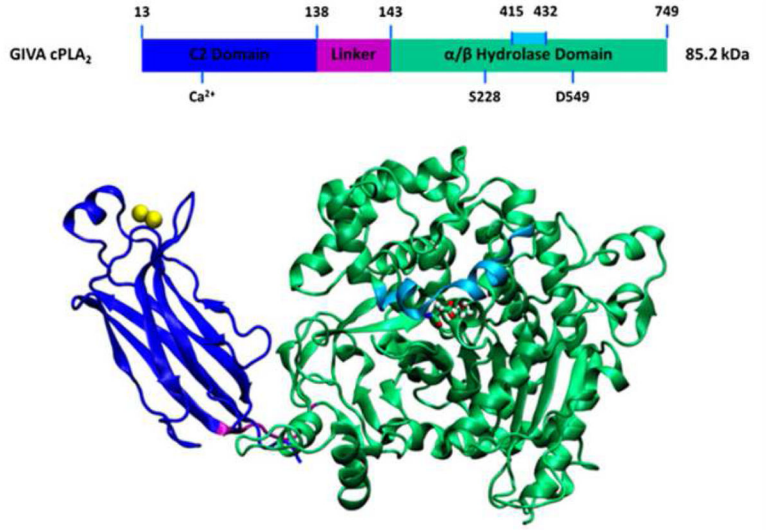

Figure 4. Three-dimensional structure of cytosolic phospholipase A_2

The X-ray crystal structure of GIVA $cPLA_2$ (PDB ID 1CJY) (adapted from (Mouchlis et al., 2015)).

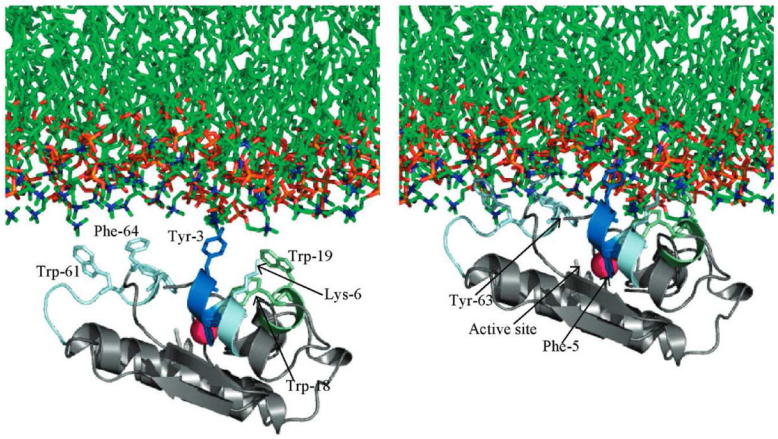

Figure 5. Hypothetical model of GIA sPLA2 before (left) binding to the surface of a DMPC membrane and after (right)

Areas with decreases in deuterium exchange are colored in blue and turquoise (adapted from (Burke et al., 2008)).

Table 1

Phospholipase A_2 superfamily

(adapted from Dennis et al, 2011).

type	group	subgroup	molecular mass (kPa)	catalytic residues
$sPLA_2$	GI	A, B	13–15	His/Asp
	GII	A, B, C, D, E, F	13–17	
	GIII		15–18	
	GV		14	
	GIX		14	
	GX		14	
	GXI		13	
	GXII	A, B	19	
	GXIII	A, B	<10	
	GXIV		13–19	
$cPLA_2$	GIV	A(α), B(β), C(γ), D(δ), E(ε), F(ζ)	60–114	Ser/Asp
$iPLA_2$	GVI	A(β), B(γ), C(δ), D(ε), E(ζ), F(η)	84–90	Ser/Asp
PAF-AH	GVII	A (Lp-PLA_2), B(PAF-AH II)	40–45	Ser/Asp/His
	GVIII	A(α_1), B(α_2), β	26–40	
$LPLA_2$	GXV		45	Ser/Asp/His
$AdPLA_2$	GXVI		18	His/Cys