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CHAPTER 11

Phospholipases

David C. Wilton [1] and Moseley Waite [2]

[1] *Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK, Tel.: +44 (2380) 594308; Fax: +44 (2380) 594459; E-mail: dcw@soton.ac.uk*

[2] *Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*

1. Overview

1.1. Definition of phospholipases

Phospholipases (PLs) are a ubiquitous group of enzymes that share the property of hydrolyzing a common substrate, phospholipid. Nearly all share another property; they are more active on aggregated substrate above the phospholipid's critical micellar concentration (cmc). As shown in Fig. 1, phospholipases have low activity on monomeric substrate but become activated when the substrate concentration exceeds the cmc. The properties of phospholipids that define the aggregation state (micelle, bilayer vesicle, hexagonal array, etc.) are described in Chapter 1.

The phospholipases are diverse in the site of action on the phospholipid molecule, their function and mode of action, and their regulation. The diversity of function suggests that phospholipases are critical to life since the continual remodeling of

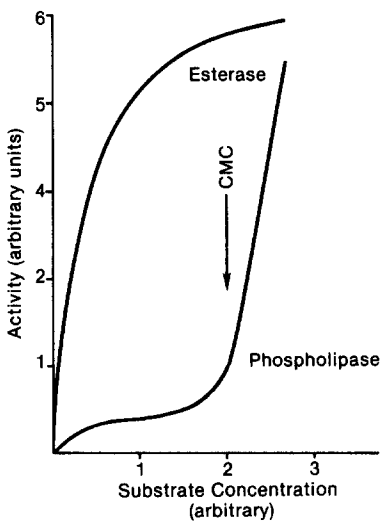

Fig. 1. Dependence of phospholipase and nonspecific esterase activity on substrate concentration. Esterase exhibits Michaelis–Menten kinetics on soluble substrates, whereas a phospholipase becomes fully active above the cmc of the substrate.

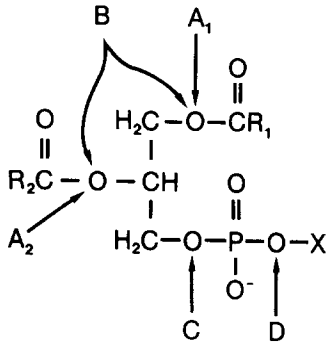

Fig. 2. Sites of hydrolysis by phospholipases.

cellular membranes requires the action of one or more phospholipase. Their functions go beyond their role in membrane homeostasis; they also function in such diverse roles from the digestion of nutrients to the formation of bioactive molecules involved in cell regulation. There are indications that a few phospholipases may carry out a biological function independent of their catalytic activity by binding to a regulatory membrane receptor. Phospholipase-like proteins with toxic properties, yet which lack a functional catalytic site, are found in venoms. It is of interest that most, but not all, phospholipases studied in detail thus far are soluble proteins. The soluble nature of many phospholipases suggests that their interaction with cellular membranes is one of the regulatory mechanisms that exist to prevent membrane degradation or to precisely control the formation of phospholipid-derived signalling molecules.

The classification of the phospholipases, based on their site of attack, is given in Fig. 2. The phospholipases A (PLAs) are acyl hydrolases classified according to their hydrolysis of the 1-acyl ester (PLA_1) or the 2-acyl ester (PLA_2). Some phospholipases will hydrolyze both acyl groups and are called phospholipase B. In addition, lysophospholipases remove the remaining acyl groups from monoacyl(lyso)phospholipids. Cleavage of the glycerophosphate bond is catalyzed by phospholipase C (PLC) while the removal of the base group is catalyzed by phospholipase D (PLD). The phospholipases C and D are therefore phosphodiesterases.

1.2. Assay of phospholipases

The subject of phospholipase assays has been concisely reviewed [1–4]. The simplest procedure is to measure protons released during hydrolysis and is usually made by continuous titration with the aid of a pH Stat. However, this technique lacks the sensitivity and specificity of other types of assays. A second commonly used approach employs synthetic substrates for spectrophotometric or fluorometric assays [3,4]. These substrates permit a continual assay well suited for kinetic studies and are of reasonable sensitivity. The major drawback is that the substrates are not natural substrates and as such, their use should be considered as a model that may or may not reflect the enzyme's kinetic properties in biological systems. With fluorescent substrates the product is

physically removed from the substrate aggregate, resulting in a spectral change in order to achieve kinetic analysis. This separation can be accomplished by the addition of albumin to absorb the product or by partitioning the product of a short-chain substrate into the aqueous phase. The thioacylester analogs of phospholipids provide a sensitive spectrophotometric assay for some PLA_1 or PLA_2 assays based on the reaction of released thiol with Ellmann's reagent.

An alternative fluorescence approach that does not involve synthetic substrates is to use a fluorescent displacement assay where the normal product of hydrolysis such as a long-chain fatty acid displaces a fluorescent probe from a protein that binds long-chain fatty acids. The change in fluorescence is monitored and the assay has the advantage that any source of natural phospholipid substrate can be used including cell membranes and lipoproteins (D.C. Wilton 1990, 1991; G.V. Richieri, 1995).

A third approach employs phospholipids with radioisotopes incorporated into specific positions in the molecule. The products are separated from the substrate by partitioning or chromatographic procedures. By the appropriate choice of labeling, the specificity of the enzymes can readily be established and as little as a few picomoles of product can be detected. The use of isotopes has been helpful in the measurement of phospholipase activity using the membranes of whole cells or isolated subcellular fractions previously labeled with radioactive phospholipid precursors.

A fourth and elegant method of phospholipase assay employs a monomolecular film of phospholipid [5]. With this technique the interfacial properties of the lipid substrate are carefully controlled and zero-order kinetics are obtained. This technique provides important data on the effect of lipid surface pressure and enzyme penetration on catalysis.

1.3. Interaction of phospholipases with interfaces

The catalytic turnover of phospholipases at the interface distinguishes them from the general class of esterases (Fig. 1). Therefore, the study of phospholipases must include an understanding of their interaction with the lipid interface. The increased enzyme activity seen when phospholipids are present above their cmc (Fig. 1) implies that phospholipases have an interfacial binding surface and that interfacial binding will precede normal catalysis. Interfacial binding is crucial because the cmc of normal cellular phospholipids is very low ($\ll 10^{-9}$ M) and hence the concentration of monomeric substrate in the aqueous phase is sub-nanomolar. Moreover, the half-time for desorption of a long-chain phospholipid from the bilayer interface is of the order of hours. What this means in practice is that the phospholipase must first bind to the interface and that the interfacial binding step has a profound effect on overall catalysis. This concept is illustrated in Fig. 3 and interfacial binding ($E \rightarrow E^*$) often provides the basis for the physiological regulation of these types of enzyme. Unless the phospholipase can bind productively to the phospholipid surface, it cannot access substrate and express activity.

The nature of interfacial binding and the rate enhancements that are achieved are controversial areas. However, it is clear that both polar and non-polar interactions are involved and the precise contribution of each must depend on the nature of the

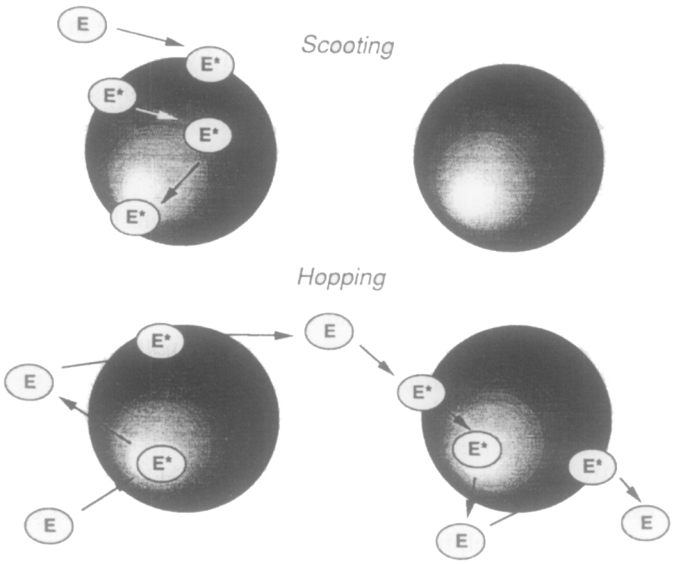

Fig. 3. Schematic illustration of the two modes of interfacial catalysis with vesicles. In the scooting mode (top) the enzyme bound to the interface does not dissociate and exhibits first-order-type kinetics on that vesicle. The excess vesicles which do not contain enzyme are not hydrolyzed. In the hopping mode (bottom) the enzyme desorbs from the interface after each or a few turnover cycles. All vesicles, therefore, are accessible for hydrolysis. E, enzyme in solution; E^* enzyme bound to the vesicle. (Adapted from Berg et al. [7].)

phospholipid interface and the interfacial binding surface of the phospholipase. A number of factors can be considered that could make a major contribution to the enhanced hydrolysis at interfaces and these relate primarily either to the *substrate* or to the *enzyme*.

1.3.1. Substrate effects

Phospholipids in aqueous solution form aggregates the nature of which is determined by the structure of the phospholipid. Long-chain phospholipids normally form structures containing bilayers whereas shorter-chain phospholipids form micelles. Once the phospholipid is present as an aggregate it is the concentration of substrate in the surface of the aggregate that is critical and is defined as mole fraction, being unity with a pure phospholipid. Therefore, provided the enzyme can bind to the interface, the effective concentration of substrate will rise dramatically producing a corresponding rate enhancement.

Several assay systems have been developed to describe kinetic events at the interface. Each system has certain advantages yet have limitations that need recognition. An elegant kinetic analysis of secreted PLA_2s has been developed by Jain and coworkers (reviewed in Refs. [6,7]. This group of PLA_2s in general have a high affinity for anionic phospholipids such as the model system, phosphatidylmethanol. The high dissociation constant for the phosphatidylmethanol interface, $\ll 10^{-10}$ M, allowed the identification of the scooting mode of action of the porcine pancreatic phospholipase (Fig. 3). Under

those conditions the enzyme remains attached to the vesicle interface thus promoting hydrolysis of the outer phospholipid monolayer without loss of vesicle integrity or release of enzyme from the vesicle. Care must be taken, however, to demonstrate that the integrity of the vesicle is maintained (low Ca^{2+} concentrations) and that the number of enzyme molecules is substantially less than the number of vesicles present. Under these 'scooting' conditions, hydrolysis can be measured in the absence of interfacial effects and has provided direct evidence that interfacial binding is separate from catalysis.

Scooting conditions are the preferred assay method the assessment of potential enzyme inhibitors [6,7]. This is because the inhibitor or other factors will not affect interfacial binding of the enzyme and only the effect of the inhibitor on classical catalysis ($E^* + S \rightarrow E^*S \rightarrow E^* + P$) will be measured. By contrast, in the 'hopping' model (Fig. 3) the enzyme is able to leave the vesicle during successive rounds of catalysis and will rebind to different vesicles thus producing a continuum of substrate composition and structure as hydrolysis proceeds, making kinetic analysis more difficult [6,7].

Another useful assay system is the Triton mixed-micelle substrate. This model developed by Reynolds et al. [3] can employ a wide range of substrates and is an effective way of measuring enzyme activity. Since in this system there is an exchange of substrate molecules between the micelles that is more rapid than the catalytic rate (M.J. Thomas, 1999) a large excess of substrate is desirable. As a result, linear kinetics can be obtained until 40–50% of total substrate is degraded. One limitation that needs consideration is the affinity of the enzyme for Triton. This, however, can be determined (R.A. Burns, 1982).

Important factors when considering the enhanced hydrolysis at interfaces are the substrate environment in the monolayer and the need to transfer a substrate molecule from this monolayer to the active site. Interfacial disorder may provide an important parameter that facilitates such transfer of substrate to the active site. Phospholipase activity is enhanced under conditions that affect phospholipid fluidity, packing density of the phospholipids and polymorphism of the aggregate. A highly ordered structure seen with phosphatidylcholine phospholipids either above or below the transition temperature tends to give low rates of hydrolysis. Discontinuities in such ordered structures such as assays at temperatures close to the transition temperatures and the presence of other lipids such as anionic lipids or non-bilayer forming phospholipids, promote catalysis by perturbing the interface. In the case of anionic lipids it may be difficult to distinguish the contribution to catalysis due to enhanced interfacial binding from effects on the structure of the interface that promote phospholipid transfer to the active site.

1.3.2. Enzyme effects

An important question is if binding of the phospholipase to the interface promotes a conformational change in the structure of the enzyme that facilitates catalysis compared with the enzyme structure in free solution. This is clearly the case with many lipases where lid opening is seen on binding to the aggregate. In the case of the porcine pancreatic secreted PLA_2, NMR analysis of the enzyme when bound to anionic micelles

compared to the enzyme in solution has revealed significant differences compared with the X-ray structure. In particular, the N-terminal region of the enzyme is disordered in free solution but in crystal structures is condensed as an α-helix when bound to an anionic micelle (B. van den Berg, 1995).

The effect of specific phospholipid molecules on overall protein conformation and hence activity may be considered as an example of allostericity. Such allosteric behavior has been discussed in terms of a second phospholipid-binding site or as discrete interactions between the head groups of phospholipids and specific residues on the interfacial binding surface of the enzyme. The emerging evidence for secreted PLA_2s would support a model involving multiple interactions between enzyme and interfacial lipid [7].

2. *The phospholipases*

Many types of phospholipases have now been purified and characterized including full crystal structures. Further, the cellular function of many phospholipases has been examined using various techniques of molecular biology including gene transfection, gene knockouts and antisense strategies. Since it is impossible to cover all phospholipases that have been characterized, only examples will be discussed in detail where significant information relating structure to function is available. The roles of phospholipases in signal transduction are dealt with in Chapter 12.

2.1. Phospholipase A_1

The phospholipases A_1 comprise a large group of 1-acyl hydrolases, some of which also degrade neutral lipids (lipases) or remove the acyl group at position 2 in addition to that at position 1 (phospholipase B) and thus must have lysophospholipase activity. Where the enzyme appears to show low selectivity for the *sn*-1 or *sn*-2 positions, the term phospholipase A is used. The term phospholipase B should be restricted to those enzymes where the mechanism involves minimal accumulation of lysophospholipid product. In this section we also consider various enzymes of the PLA type that do not fit a more precise definition in terms of acyl chain selectivity.

2.1.1. Escherichia coli *phospholipases A*

Two phospholipases A have been purified from *Escherichia coli* based on their differential sensitivity to treatment with detergents [2]. A detergent-insensitive enzyme is localized in the outer membrane, whereas a detergent-sensitive enzyme is found on the cytoplasmic membrane and in soluble fractions. The outer membrane enzyme, known as outer membrane phospholipase A has broad substrate specificity, and demonstrates PLA_1, PLA_2, lysophospholipase A_1 and lysophospholipase A_2 activity as well as hydrolyzing mono- and diacylglycerols. The recent crystal structure allows a more detailed discussion of what is an integral membrane phospholipase [8].

The protein is a 12-stranded anti-parallel β-barrel with amphipathic β-strands travers-

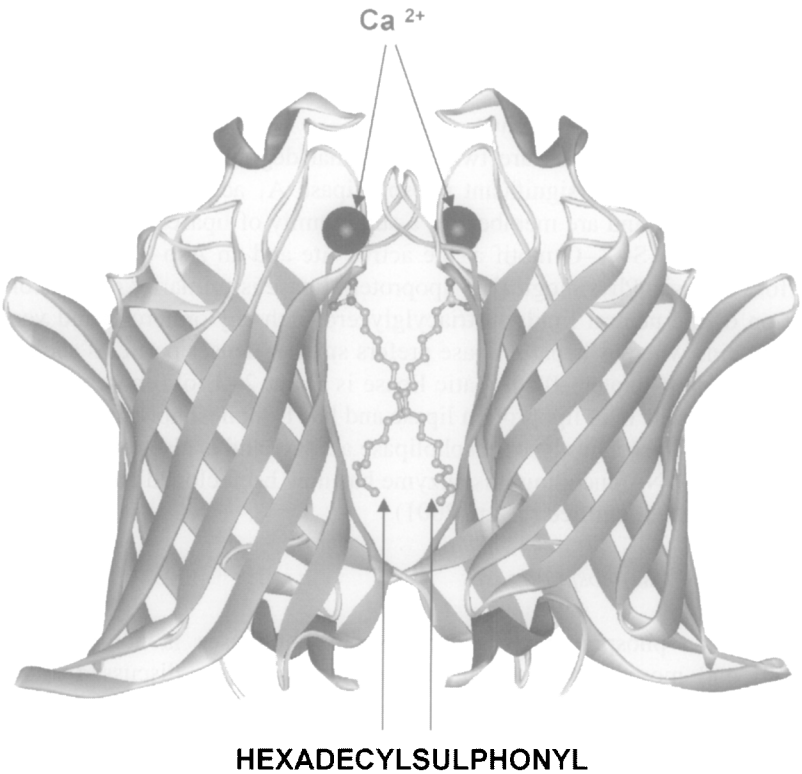

Fig. 4. Crystal structure of the outer membrane phospholipase A dimer from *E. coli* shown in the plane of the membrane. The top half of the molecule is located in the lipopolysaccharide monolayer facing the exterior of the cell. The phospholipid monolayer of the outer cell membrane would be located around the bottom half of the protein. Two calcium ions are shown at the active sites while Ser-144 of each active site is covalently modified with a hexadecylsulphonyl moiety represented in a ball and stick format. Structure is adapted from Snijder and Dijkstra [8] using MSI Weblab Viewer.

ing the membrane (Fig. 4). The active site catalytic residues are similar to a classical serine hydrolase triad except that in addition to the serine (Ser-144) and histidine (His-142) there is an asparagine (Asn-156) in place of the expected aspartic acid. Calcium at the active site is predicted to be involved in polarization of the substrate ester carbonyl group and stabilization of the negatively charged reaction intermediate.

The active site is located at the exterior of the β-barrel at the outer leaflet side of the membrane (Fig. 4). The lipopolysaccharide outer leaflet does not normally contain phospholipid and this structural isolation of active site from substrate present in the inner leaflet is believed to be important in preventing uncontrolled phospholipase activity. It is proposed that appropriate bacterial stimulation results in phospholipid moving to the outer monolayer and enzyme activation, including dimerization (Fig. 4), allows phospholipid hydrolysis. This hydrolysis enhances the permeability of the outer cell membrane producing the appropriate adaptations such as the release of bacteriocins. The fact that *E. coli* mutants deficient in this phospholipase A have normal growth

characteristics and phospholipid turnover is consistent with the specialized role of this enzyme in bacterial adaptation.

2.1.2. Lipases with phospholipase A_1 activity

Lipoprotein lipase and hepatic lipase are two lipases that degrade triacylglycerols in lipoproteins but also demonstrate significant phospholipase A_1 activity. The enzymes have 50% sequence identity and are members of a superfamily of lipases and phospholipases that share the G–X–S–X–G motif at the active site and an Asp–His–Ser triad that is required for catalysis. Physiologically, lipoprotein lipase is primarily responsible for the degradation of the neutral lipids in triacylglycerol-rich chylomicrons and very low density lipoproteins whereas hepatic lipase prefers smaller denser particles such as high density lipoproteins (Chapter 20). Hepatic lipase is about 2–3 fold more efficient at hydrolyzing phospholipids than lipoprotein lipase and has lysophospholipase activity. Another lipase, intestinal lipase, has lysophospholipase activity and is also referred to as a phospholipase B. The cDNA encoding this enzyme has now been cloned and essential catalytic residues have been identified (T. Lu, 2001).

2.2. Phospholipase B and lysophospholipases

The distinction between phospholipase B and lysophospholipases is not clear [2] since both diacyl- and monoacyl-phospholipids are substrates. As discussed above (Section 2.1) a working definition of these enzymes can be based on the extent to which the lysophospholipid accumulates upon hydrolysis of the diacyl substrate. Those phospholipases B for which there is significant structural and functional information are discussed. In addition mammalian lysophospholipases are reviewed.

2.2.1. Phospholipase B from microorganisms

The amino acid sequence of the phospholipase B from *Penicillium notatum* was deduced from its cDNA (N. Masuda, 1991). The details of the overall catalytic mechanism are unknown, but intriguing, as substrate specificity is affected by the presence or absence of detergent as well as the glycosylation state of the enzyme. Phospholipase B activity has subsequently been identified in other fungi including *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Candida albicans*. In the case of *C. albicans*, gene disruption has identified this phospholipase B as a major factor in host cell penetration and hence pathogenicity (S.D. Leidich, 1998). It is likely that this secreted enzyme has a similar role in other fungi. Phospholipase B activity has also been isolated from *Mycobacterium phlei* and *Mycobacterium lepraemurium* where product analysis indicates that the initial activity is that of a PLA_1 (S. Maeda, 1996).

2.2.2. Mammalian lysophospholipases

Lysophospholipids [9] are generally found in very low concentrations (0.5–6% of total lipid membrane weight) in biological membranes. High concentrations of lysophospholipids affect membrane properties and membrane enzymes even leading to cell lysis. Increased lysophospholipid levels are associated with atherosclerosis while lysophosphatidylcholine is highly abundant in atherogenic lipoproteins such as oxidatively

modified low density lipoprotein where it constitutes up to 40% of the total lipid. Other diseases where there are links with lysophospholipid levels include inflammation, hyperlipidemia and lethal dysrhythmias in myocardial ischemia. At low concentrations lysophospholipids such as lysophosphatidylcholine and lysophosphatidic acid have the properties of second messengers. Against this background the role of lysophospholipases in mammalian tissues assumes a considerable significance.

There are two small mammalian lysophospholipases (I and II) which despite their similar size appear to be the products of separate genes rather than splice variants or post-translational modifications [9]. The enzymes, which are calcium-independent, have been purified from a variety of tissues and lack PLA_1, PLA_2 or acyltransferase activity. Their mechanism of action appears to involve the catalytic triad characteristic of serine proteases and lipases. Using an NMR based assay, lysophospholipase I hydrolyzed 1-palmitoyl-lysophosphatidylcholine and 2-palmitoyl-lysophosphatidylcholine at similar rates [9].

In addition to the small lysophospholipases, there are a few high molecular weight enzymes that display lysophospholipase activity. Two such enzymes, the group IV cytosolic PLA_2 and the 85 kDa calcium-independent PLA_2 are discussed in Sections 2.3.2.1 and 2.3.2.2. Similarly, a number of PLA_1s also display significant lysophospholipase activity including enzymes that are specific for phosphatidylserine (Y. Nagai, 1999) and phosphatidic acid (M.H. Han, 2001).

2.3. Phospholipase A_2

The phospholipases A_2 were the first of the phospholipases to be recognized. Over a century ago, Bokay (1877–1878) observed that phosphatidylcholine was degraded by some component in pancreatic fluid that is now known to be the pancreatic phospholipase A_2. At the turn of the century, cobra venom was shown to have hemolytic activity directed toward the membranes of erythrocytes (P. Keyes, 1902). The lytic compound produced by the venom phospholipase was identified a decade later and termed lysocithin (later, lysolecithin). These studies spurred further investigation of this intriguing class of enzymes and their mechanism of attack on water-insoluble substrates.

An increasing number of PLA_2s have now been identified within this expanding super-family and this has required a re-evaluation of classification criteria. Recently, Six and Dennis have categorized these enzymes into two types based on catalytic mechanism [10]. In one group (Table 1) are those enzymes that utilize a catalytic histidine as the primary catalytic residue whereas the other group involves a catalytic serine (Table 2) and normally an acyl-serine intermediate. Within these two categories individual enzymes retain their historic grouping.

2.3.1. The 14 kDa secreted phospholipases A_2

These enzymes now include secreted PLA_2s from such diverse sources as venoms, mammalian and plant tissues (Table 1). They are characterized by a requiring mM concentration of calcium for activity and involving an active site histidine and aspartate pair. They are typically extra-cellular enzymes with a large number of disulfide bonds. The enzymes in groups I–III have provided the foundation for our understanding of

Table 1
Characteristics of secreted PLA_2s using a histidine residue for catalysis [a]

Group		Sources	Size (kDa)	Di-S (no.)	Unique Di-S	C-term extension (no. of residues)	Molecular characteristics
I	A	Cobra/krait venom	13–15	7	11-77	None	
	B	Mammalian pancreas	13–15	7	11-77	None	5 residue pancreatic/elapid loop, propeptide
II	A	Human synovial fluid/platelets, rattlesnake venom	13–15	7	50-137	7	
	B	Gaboon viper venom	13–15	6	50-137	6	Lacks disulfide at C61–C94
	C	Rat/mouse testes	15	8	50-137, 86-92	7	
	D	Human/mouse spleen/pancreas	14–15	7	50-137	7	
	E	Human/mouse brain/heart/ uterus	14–15	7	50-137	7	
	F	Mouse testes/embryo	16–17	7	50-137	30	Extra cysteine in C-terminal extension
III		Bee, lizard, scorpion, human	15–18	5	N/A	N/A	Human form (55 kDa) has novel C- and N-terminal domains
V		Mammalian heart/lung/ macrophage	14	6	None	None	
IX		Marine snail venom	14	6	N/A	N/A	
X		Human spleen/thymus/ leukocyte	14	8	11-77, 50-137	8	
XI	A	Green rice shoots	12.4	6	N/A	N/A	
	B	Green rice shoots	12.9	6	N/A	N/A	

[a] Adapted from Six and Dennis [10]. N/A, not available.

these secreted enzymes and sufficient quantities of natural and mutant enzymes from these groups have been obtained for X-ray crystallographic analysis. Also, the sequences of a very large number of these phospholipases are now known and have been used to demonstrate their structural, functional, and evolutionary relatedness [11].

The conserved active site residues, His-48 and Asp-99 (pancreatic enzyme numbering), provide the catalytic dyad and, with the availability of the crystal structure of the pancreatic enzyme, produced the proton-relay mechanism (Fig. 5) in 1980 [12]. In this mechanism a water molecule directly replaces the serine found in the classical protease/lipase catalytic triad mechanisms. More recently, an alternative mechanism has been proposed (J. Rogers, 1996) that involves two water molecules (W5 and W6) seen at the active site of the crystal structure (Fig. 6). In this mechanism, proposed by Jain and referred to as a 'calcium coordinated oxyanion' mechanism, the attacking nucleophile (W5) is coordinated to the calcium thus enhancing its nucleophilicity. This water is connected to His-48 by a second water molecule (W6). Thus the major formal difference between the two mechanisms is that the latter mechanism involves a second water molecule while the first water molecule is activated by coordination to

Table 2
Characteristics of PLA_2s using a serine residue for catalysis [a]

Group		Alternative name	Sources	Size (kDa)	Ca^{2+} requirement/role	Molecular characteristics
IV	A	$cPLA_2$ α	Human U937 cells/ platelets, rat kidney, RAW 264,7	85	< μM Membrane translocation	C2 domain, α/β-hydrolase, phosphorylation
	B	$cPLA_2$ β	Human, liver/pancreas/ brain/heart	114	< μM Membrane translocation	C2 domain, α/β-hydrolase
	C	$cPLA_2$ γ	Human heart/skeletal muscle	64	None	Prenylated, α/β-hydrolase
VI	A-1	$iPLA_2$-A	$P388D_1$ macrophages, CHO cells	84–85	N/A	8 ankyrin repeats
	A-2	$iPLA_2$-B	Human B-lymphocytes	88–90	N/A	7 ankyrin repeats
	B	$iPLA_2$-γ/2	Human heart/skeletal muscle	88	N/A	Membrane-bound
VII	A	PAF-AH	Mammalian plasma	45	N/A	Secreted, α/β-hydrolase, Ser/His/Asp triad
	B	PAF-AH (II)	Human/bovine liver/kidney	40	N/A	Myristoylated, Ser/His/ Asp triad
VIII	A	PAF-AH Ib α_1 (subunit of trimer)	Human brain	26	N/A	G-protein fold, Ser/His/ Asp triad, dimeric
	B	PAF-AH Ib α_2 (subunit of trimer)	Human brain	26	N/A	G-protein fold, Ser/His/ Asp triad, dimeric as hetero- or homodimer

[a] Adapted from Six and Dennis [10]. N/A, Not available; c, cytosolic; i, (calcium) independent; PAF-AH, platelet-activating factor acetylhydrolase.

the calcium. It is argued that the rate limiting step lies during the decomposition of the tetrahedral intermediate whereas in the originally proposed mechanism the formation of this tetrahedral intermediate is rate limiting [7].

2.3.1.1. Group I secreted PLA_2s. This group historically contains group IA enzymes such as those from cobra and krait venom while the IB enzymes are the mammalian pancreatic enzymes. Both the cobra and pancreatic enzymes were early models for structure–function analysis [14] including crystal structures of both apo-enzyme and enzyme with bound phospholipid inhibitors. However, at this time there are no crystal structures of enzymes bound to a lipid interface or with a phospholipid substrate at the active site.

A space-fitting model of substrate bound to the active site of the cobra venom enzyme (Fig. 7) gives insight into how the enzyme functions even though bulk interactions with the lipid interface are missing. The most obvious feature is existence of the active-site tunnel into which the substrate enters. However, the enzyme interacts loosely with the first 9–10 carbons of the acyl group at position 2 of the glycerol that may account for the enzyme's lack of acyl specificity. This model also suggests that the substrate molecule is not completely withdrawn from the bilayer and significant

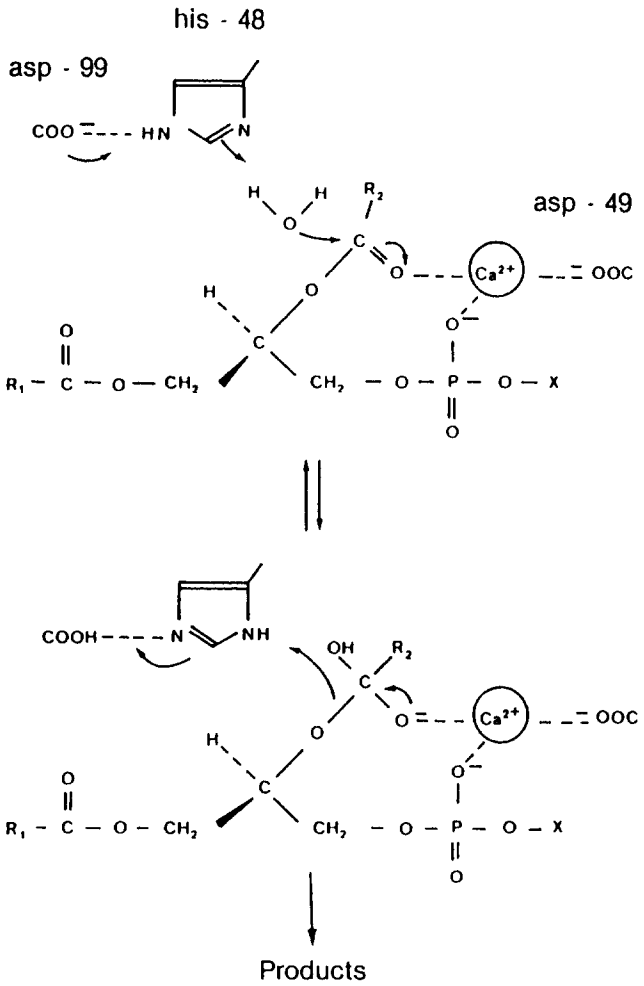

Fig. 5. Proton-relay mechanism of hydrolysis proposed by Verheij et al. [12] for secreted phospholipases A_2.

hydrophobic interactions of the molecule undergoing hydrolysis and the interface are maintained. A particular feature is the ability of the cobra venom enzyme to hydrolyze phosphatidylcholine in vesicles and cell membranes, a feature that may in part reflect the presence of tryptophans and other aromatic residues on the interfacial surface. Such residues, particularly tryptophan, are able to partition into the interfacial region of a phosphatidylcholine interface (W.M. Yau, 1998) promoting interfacial binding and catalysis [15]. The presence of such residues in the presumptive interface region can be clearly seen in Fig. 7.

The mammalian pancreatic enzymes (group IB) have primarily a digestive role and are secreted as the pro-enzyme (zymogen) that requires subsequent proteolytic cleavage to remove a hexapeptide at the N-terminus. The pro-enzyme is unable to bind to the phospholipid interface. Unlike the cobra venom enzyme, the pancreatic

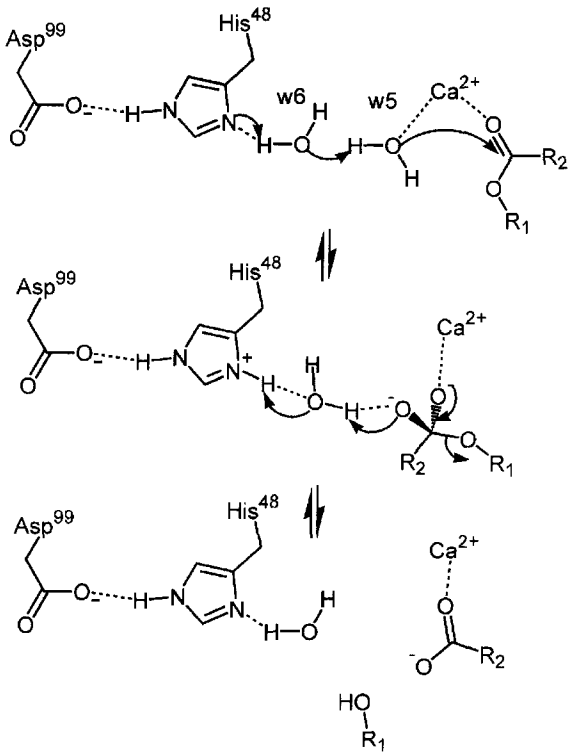

Fig. 6. Calcium coordinated oxyanion mechanism of hydrolysis proposed by Jain for secreted phospholipases A_2 (J. Rogers, 1996). The two water molecules at the active site that are implicated in catalysis are shown as W5 and W6.

enzyme expresses low activity with a zwitterionic interface such as that provided by phosphatidylcholine. The enzyme has a considerable preference for anionic interface that can be provided by anionic phospholipid per se or by the inclusion of other anionic lipids in the phosphatidylcholine interface. Presumably the anionic bile salts provide such negative charge in the mixed micelles produced in the intestine to allow lipid digestion. The expression of the pancreatic enzyme in other tissues and the presence of cell surface receptors for this enzyme (as well as other secreted phospholipases A_2) suggest additional physiological roles [11,16].

2.3.1.2. Group IIA–F secreted PLA_2s. Historically, the group IIA secreted PLA_2s included venom enzymes from rattlesnakes and vipers. They are characterized by a C-terminal extension while they lack a surface loop region (elapid loop) present in group I enzymes. The most interesting member of this group IIA is the mammalian enzyme that was isolated from the synovial fluid of patients with rheumatoid arthritis and from platelets. This was the first mammalian non-pancreatic enzyme to be identified and was implicated as a key enzyme in arachidonic acid release from phospholipids, the first step in the production of the inflammatory eicosanoids. However, it has subsequently been

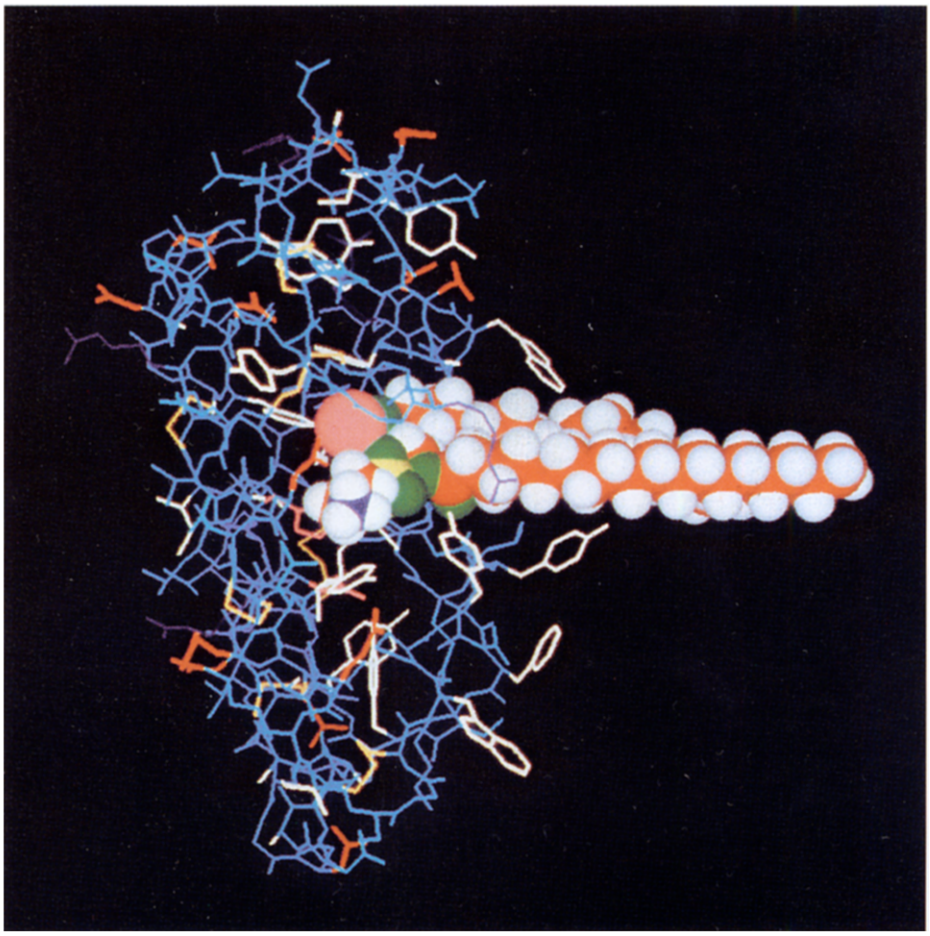

Fig. 7. X-ray crystal structure of cobra venom (*Naja naja naja*) phospholipase A_2 with bound Ca^{2+} showing a space-filling model of dimyristoyl phosphatidylethanolamine bound in the catalytic site. The ends of the fatty acid chains stick out of the enzyme and are presumably associated with the micelle or membrane [13].

established that it is the intracellular group IV enzyme that plays the dominant role in eicosanoid production (see Section 2.3.2.1) and hence the precise physiological role of this IIA enzyme in the inflammatory response remains to be clarified.

It is clear that the human IIA enzyme behaves as an acute phase protein (R.M. Crowl, 1991) and extra-cellular levels increase dramatically in acute inflammatory conditions such as septicemia where blood levels can rise over 100-fold. The detailed structural properties of the enzyme are unusual and appear to reflect at least some of its physiological roles. The enzyme is highly cationic ($pI > 10$) and has a net positive charge of +19 due mostly to cationic residues distributed across the surface of the protein, a feature that is linked to the high affinity of the protein for heparin. An unusual characteristic of the enzyme is its marked preference for anionic phospholipid interfaces and very low affinity for a zwitterionic interface as provided by phosphatidylcholine.

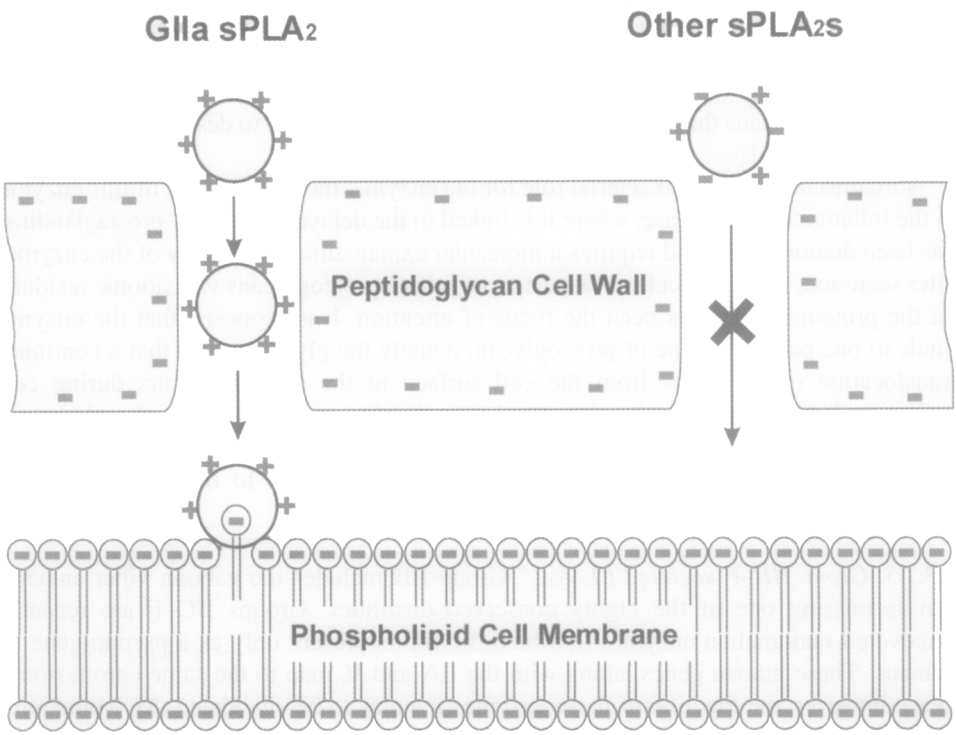

Fig. 8. A model of bacterial cell wall penetration and phospholipid hydrolysis by human group IIA secreted PLA_2 (gIIA $sPLA_2$). The extreme and global nature of the positive charge of this secreted PLA_2 has been proposed to allow the enzyme to move through the negatively charged cell wall mediated by the continual making and breaking of electrostatic bonds. For simplicity bacterial phospholipids are shown as being negatively charged, although the bacterial membrane will contain some zwitterionic phospholipids, normally phosphatidylethanolamine.

This preference is expressed at the stage of interfacial binding while active site substrate preference is modest and favors phospholipids such as phosphatidylglycerol over phosphatidylcholine. These surface properties are consistent with one particular physiological role for the enzyme, namely as an antibacterial protein that shows specificity towards gram-positive bacteria [17]. The highly cationic nature of the protein allows the enzyme (but not other more neutral secreted PLA_2s) to penetrate the highly anionic bacterial cell wall. Moreover, the anionic bacterial cell membrane that is rich in phosphatidylglycerol provides an optimum substrate for this enzyme (Fig. 8). In contrast, this enzyme is essentially inactive against the host cell membranes that are normally zwitterionic being rich in phosphatidylcholine and sphingomyelin. This lack of activity against host membranes (which is partly due to lack of an interfacial tryptophan residue (S.F. Baker, 1998)) is important as the serum levels of the enzyme can rise to above 1 μg/ml.

The enzyme is released from cells with known antibacterial activity (macrophages and Paneth cells) and is present at very high concentrations ($\sim$10 μg/ml) in human tears

(X.D. Qu, 1998) along with lysozyme. The increased sensitivity of mice lacking the group IIA enzyme to gram-positive bacteria provides further support for the antibacterial role of the enzyme (V.J.O. Laine, 1999). The preference of this enzyme for anionic interfaces also means that this enzyme may be active in helping to destroy apoptotic and damaged cells [16].

Notwithstanding an antibacterial role for the enzyme, the involvement of this enzyme in the inflammatory response, where it is linked to the delayed release of prostaglandins, has been demonstrated and requires a molecular explanation. The ability of the enzyme, after secretion, to bind to cell surface heparan sulfate proteoglycans via cationic residues on the protein surface has been the focus of attention. It is proposed that the enzyme binds to one particular type of proteoglycan, namely the glypicans and that a continual translocation of glypicans from the cell surface to the nucleus occurs during cell activation [16]. This is proposed to be the result of a caveolae-mediated endocytotic event called potocytosis which transfers the group IIA secreted PLA_2 within calcium-rich vesicles to the nuclear region of the cell in proximity to the cyclooxygenase (Chapter 13) [16].

2.3.1.3. Group IIB–F secreted PLA_2s. Group IIB includes the gaboon viper enzyme that is missing one of the highly conserved disulfides. Groups IIC–F are recently discovered mammalian enzymes of which the IIC is present only as a pseudo-gene in humans. These human genes, along with the IIA and V, map to the same chromosome locus; however, the function of these enzymes remains to be elucidated (M. Murakami, 2001).

2.3.1.4. Group III secreted PLA_2. The bee venom enzyme was historically placed in group III and is the primary allergen in bee venom. The enzyme is significantly larger than other secreted PLA_2s with less structural homology. The availability of a crystal structure has allowed extensive mutagenesis. In particular, the charge reversal mutagenesis of 5 of the 6 cationic residues on the interfacial binding surface produced a mutant with minimal effects on binding to anionic interfaces. This result highlighted the importance of non-electrostatic interaction in interfacial binding (F. Ghomashchi, 1998). The incorporation of spin labels on the protein surface following cysteine mutagenesis provided a method for defining the topological relationship between the protein and the membrane interface (Y. Lin, 1998). The results demonstrated the interaction of hydrophobic residues but not cationic residues with the interfacial region of the membrane.

A group III human enzyme has recently been identified that possesses long N- and C-terminal extensions but as yet no function has been ascribed to this enzyme.

2.3.1.5. Group V secreted PLA_2. This enzyme is the most studied human secreted PLA_2 after the group IIA enzyme. The enzyme has a tryptophan on the interfacial binding surface, Trp-31, which is partly responsible for its ability to hydrolyze cell membranes. Exogenously added group V enzyme can catalyze phosphatidylcholine hydrolysis in the cell surface with subsequent activation of group IV cytosolic PLA_2 and enhanced leukotriene biosynthesis. Removal of Trp-31 by mutagenesis greatly reduces

the effectiveness of added enzyme [18]. Like the IIA enzyme, the group V enzyme binds to heparan sulfate proteoglycans on the cell surface and is internalized. However, the dynamics of this process and also mutagenic studies (K.P. Kim, 2001) indicate that internalization is linked to degradation of the enzyme and not to eicosanoid production in human neutrophils and mast cells. In contrast, internalization results in eicosanoid production in human embryonic kidney 293 cells [16,21] (Y.J. Kim, 2002). Thus, the apparent differences between the cellular fate of the group IIA and V enzymes may reflect the types of cells being used for these studies.

2.3.1.6. Group X secreted PLA_2. This enzyme is effective in the hydrolysis of phosphatidylcholine vesicles and the plasma membrane when it is added exogenously to adherent mammalian cells. Hydrolysis is accompanied by enhanced prostaglandin E2 production. The enzyme does not bind to heparin so heparan sulfate proteoglycan-associated internalization and degradation seen with the group IIA and V enzymes, respectively, cannot occur with this enzyme (S. Bezzine, 2000). The crystal structure of the group X enzyme is now available (Y.H. Pan, 2002).

2.3.1.7. Group IX, group XI and other secreted PLA_2s. At this time these groups are represented by proteins from a snail venom (group IX) (conodipine-M) and green rice shoots (group XI) respectively (Table 1) while an increasing number of secreted PLA_2s are being identified from analysis of genomic data bases in both the plant and animal kingdoms [11]. The cloning and expression of these enzymes and related proteins will be the first step in understanding their physiological functions.

2.3.2. Phospholipases A_2 that involve a catalytic serine residue

2.3.2.1. Group IV cytosolic PLA_2. Over the past decade or more the group IV cytosolic PLA_2 has taken center stage as the phospholipase that is primarily involved in the regulation of prostaglandin and leukotriene biosynthesis (Chapter 13) as part of the inflammatory response [19]. Even though this enzyme translocates to a membranous fraction, it is recovered from the cytosolic fraction of the cell and hence it is termed cytosolic. This enzyme is distinct from the mammalian secreted phospholipases A_2 in its size (85 versus 14 kDa), stimulation by Ca^{2+} (at micromolar versus millimolar concentration), specificity at the *sn*-2 position of the substrate (arachidonate versus no specificity) and catalytic mechanism. This enzyme shows minimal head-group specificity and phosphatidylcholine is the normal substrate. The discovery of the cytosolic phospholipase A_2 has provided new insights into the cell signalling events that initiate the 'arachidonate cascade' described in Chapter 13.

The sequence of the cytosolic PLA_2 was deduced from the cDNA isolated from a number of species and is now referred to as cytosolic PLA_2 α as the result of the more recent discovery of further isozymes (β and γ). The human enzyme, mapped to chromosome 1, is highly conserved amongst mammalian species but the sequence can differ up to 20–30% between mammals and non-mammalian vertebrates. Distinct regions of the enzyme have been identified including two domains, an N-terminal C2 domain and a larger C-terminal catalytic domain (Fig. 9). The γ-isozyme lacks the C2 domain and is prenylated at the C-terminus. The complete crystal structure of human

cytosolic PLA_2 α (A. Dessen, 1999) has been reviewed [20] and provides a logical basis for discussion of the function and regulation of this important enzyme.

The catalytic domain. The enzyme shows lysophospholipase and transacylase activities that are consistent with the formation of an acyl-serine intermediate characteristic of lipases, and Ser-228 has been identified as the involved residue. In addition Asp-549 has been demonstrated to be the second member of the predicted catalytic triad. However, histidine has not been identified as the third member and none of the 19 histidine residues in the protein has been shown to play any catalytic role. At present a dyad mechanism must be invoked while Arg-200 is in a position to stabilize the oxyanion intermediate. Interestingly, 1-palmitoyl lysophosphatidylcholine is degraded at a rate comparable to 2-arachidonoyl phosphatidylcholine which raises the possibility that the enzyme serves multiple functions in the cell.

The C2 domain. The enzyme has a Ca^{2+}-dependent phospholipid binding domain (CaLB or C2 domain) common to many proteins that translocate to membranes from the cytosol in the presence of Ca^{2+}. The activity of the cytosolic PLA_2 increases several fold as the Ca^{2+} concentration is increased to concentrations found in activated cells (300 nM). Since Ca^{2+} is not involved in the catalytic event, Ca^{2+} promotion of enzyme–membrane interaction probably accounts for Ca^{2+}'s stimulatory effect. The molecular mechanism by which Ca^{2+} binding promotes membrane interactions has been the subject of intensive investigations. The C2 domain consists of an anti-parallel β-sandwich composed of two four-stranded sheets (Fig. 9) while the structure is capped

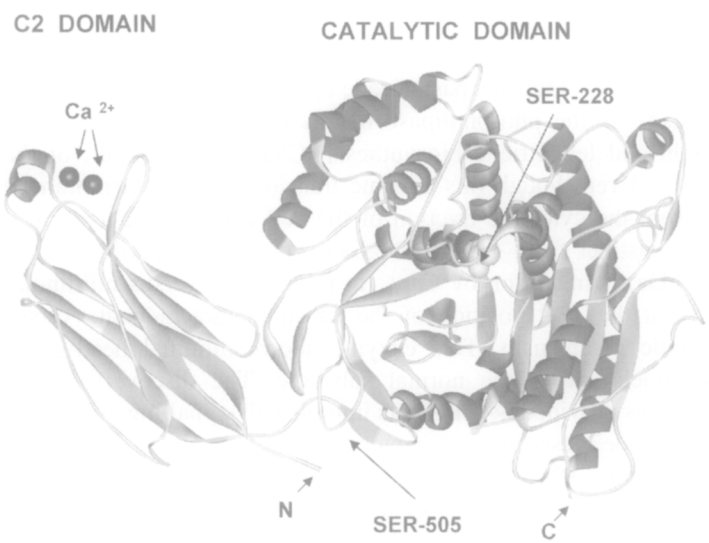

Fig. 9. Crystal structure of group IV cytosolic PLA_2 showing the C2 and catalytic domain. The two calcium ions bond to the C2 domain and the active site Ser-228 are highlighted. The phospholipid interface would be parallel to the top surface of the molecule as shown. A flexible region between residues 499 and 538 is not seen in the crystal structure but contains Ser-505, the approximate position of which is indicated by the arrow. The N- and C-terminals of the protein are indicated. Adapted from A. Dessen (1999) and Dessen [20] using MSI Weblab Viewer.

by three Ca^{2+} binding loops known as the calcium-binding regions. It would appear that the binding of Ca^{2+}, allows penetration of two of the loops into the membrane providing a stable membrane–protein interaction (O. Perisic, 1999; L. Bittova, 1999). The net result is that the enzyme translocates to the membrane surface to allow catalysis. Immunofluorescence studies have revealed that the enzyme preferentially translocates to the nuclear envelope region, a location that also contains the cyclooxygenase and 5-lipooxygenase, presumably allowing facile transfer of the released arachidonic acid to these first enzymes in the prostaglandin and leukotriene pathways. The mechanism by which cytosolic PLA_2 selectively translocates to this specific nuclear region is unclear in view of the lack of obvious phospholipid head-group selectivity of the enzyme. It has been proposed that the 60 kDa protein vimentin acts as an adaptor for membrane targeting and interacts directly with the C2 domain in a Ca^{2+}-dependent manner [21].

Phosphorylation of cytosolic PLA_2 α. There are multiple sites for phosphorylation on the $cPLA_2$ and a number of protein kinases appear to use the enzyme as substrate. A critical site is Ser-505, the site phosphorylated by mitogen-activated protein kinase. Phosphorylation at this site increases the activity of the enzyme both in vitro and in vivo. However, other phosphorylation sites may be involved; in particular, Ser-727 may also have an important role in some cell systems (M.A. Gijon, 2000). The Ser-505 is located in a flexible loop that connects the C2 and catalytic domains (Fig. 9). It is possible that phosphorylation of this residue produces the optimum orientation of the two domains with respect to the membrane interface or affects the interaction of these two domains with another membrane protein such as vimentin [20].

Gene knockouts. The very large number of enzymes that have now been discovered with PLA_2 activity increases the difficulty of being able to unambiguously define the role of a regulator of the inflammatory response involving arachidonic acid to one particular enzyme. Gene knockout studies in mice involving cytosolic PLA_2 α have been particularly successful in both defining the primary importance of cytosolic PLA_2 in inflammation and identifying specific physiological roles [22]. Thus cytosolic PLA_2 knockout mice have revealed important roles in normal fertility, generation of eicosanoids from inflammatory cells, brain injuries and allergenic responses. Other forms of PLA_2 cannot replace these functions and hence the enzyme becomes a prime pharmacological target.

2.3.2.2. Group VI phospholipase A_2. This group consists of the intracellular calcium-independent PLA_2s and at this time it is the group VIA enzyme for which most information is available [23]. An additional calcium-independent PLA_2 has recently been described (D.J. Mancuso, 2000) and has been categorized as group VIB.

The group VIA enzyme is 85–88 kDa and consists of multiple splice variants that contain 7 or 8 ankyrin repeats. The enzymes are widely distributed and have both a cytosolic and membrane location within the cell. They exhibit lysophospholipase and transacylase activity while their ability to be inactivated by hydrophobic serine-reactive inhibitors is indicative of a catalytic serine and acyl-serine intermediates. A variety of evidence indicates that these enzymes are involved in basal phospholipid fatty acid remodeling as a general housekeeping function within most cell types. It is possible that these enzymes also have roles in signal transduction and other physiological functions [23].

2.3.2.3. Group VII and group VIII PLA_2s. The group VII and VIII enzymes (Table 2) are better known as platelet-activating factor acetylhydrolases [24]. These enzymes catalyze hydrolysis of the *sn*-2 ester bond of platelet-activating factor (see Chapter 9) and related pro-inflammatory phospholipids, and thus attenuate their bioactivity. They exist as both secreted (plasma) and intracellular forms and the plasma form has attracted most attention. The plasma form not only hydrolyzes platelet-activating factor but also a range of oxidatively damaged phosphatidylcholines from cell membranes and lipoproteins that originally contained arachidonic acid at the *sn*-2 position. Such oxidative damage can be significant in reperfusion injury, cigarette smoking and inflammation and as such their formation is essentially uncontrolled. Therefore, the sole mechanism for regulating the biological impact of these compounds lies in their degradation by the acetylhydrolases.

The clinical importance of this enzyme is highlighted by the lack of platelet-activating factor acetylhydrolase activity in a significant proportion of the Japanese population and in the majority of cases this is due to a mutation, V279F. This mutation is associated with asthma, stroke, myocardial infarction, brain hemorrhage and non-familial cardiomyopathy. It is possible that treatment with recombinant platelet-activating factor acetylhydrolase may be beneficial [24].

Intracellular forms of the enzyme all hydrolyze platelet-activating factor acetyl hydrolase but some are more selective and do not hydrolyze oxidized phospholipids [24].

2.4. Phospholipase C

2.4.1. Bacterial phospholipases C

Phospholipases C have been known to be associated with bacteria since the classic demonstration by Macfarlane and Knight (1941) that α-toxin in *Clostridium perfringens* was a phospholipase C (reviewed in Waite [2]). The most extensively studied phospholipases C are those from *Bacillus cereus* and provided the first crystal structure for a PLC (E. Hough, 1989). Another PLC that is specific for phosphatidylinositol is also secreted in large amounts by *B. cereus* and has provided a crystal structure (D.W. Heinz, 1995).

2.4.2. Mammalian phospholipases C

These phospholipases are primarily involved in signal transduction and are reviewed in detail in Chapter 12. The structure and mechanism of action of phosphatidylinositol-specific PLCs from both mammalian and bacterial sources has been presented [25,26]. The structure of mammalian phosphatidylinositol-PLC δ is shown in Fig. 10 and highlights the domain structure. This protein was crystallized in the absence of its PH domain that is at the N-terminal. A 'tether and fix' model of membrane association is suggested (L.-O. Essen, 1996) whereby initial specific interaction is via the PH domain that will target membrane phosphoinositides followed by the binding of the C2 and catalytic domains. A two-step catalytic mechanism is proposed for both mammalian and bacterial enzymes [26] involving a cyclic phosphodiester intermediate which in the case of the bacterial enzyme can be the major product of the reaction.

The major pathway of sphingomyelin degradation involves a special phospholipase

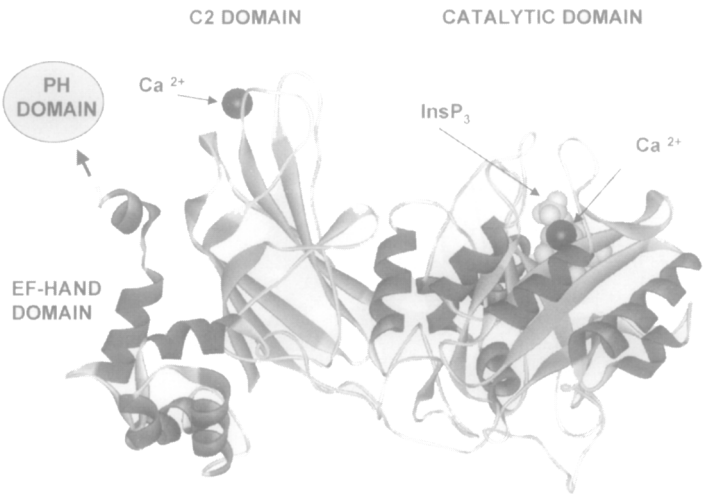

Fig. 10. Crystal structure of phosphatidylinositol-PLC δ showing the C2 and catalytic domains. The position of the PH domain that would be attached to the EF-hand domain is indicated. The membrane surface would be parallel to the top surface of the molecule as shown. Calcium ions are shown bound to the active site and to the C2 domain. The position of inositol trisphosphate in space-filling format at the active site is indicated. Adapted from L.-O. Essen (1996) and Heinz et al. [26] using MSI Weblab Viewer.

C, a sphingomyelinase. The enzyme is secreted by *B. cereus* while several mammalian sphingomyelinases are achieving a prominent role in signal transduction in mammalian systems. The description of sphingolipids and their metabolism is covered in Chapter 14.

2.5. Phospholipase D

Classically, plants and bacteria have been the major sources for the purification of phospholipases D. The function of phospholipase D in plants is not known although it may be involved in cell turnover and energy utilization during different cycles in plant life. Bacterial phospholipases D in some cases are toxins and can lead to severe cellular damage either alone or in combination with other proteins secreted from bacteria. These bacterial enzymes may also serve to help provide nutrients for the cell such as inorganic phosphate, as do the bacterial phospholipases C [2].

All phospholipases D characterized thus far act by a phosphatidate exchange reaction that has a covalent phosphatidyl-enzyme as an intermediate [27]. For this reason, the enzyme can catalyze a 'base-exchange' reaction in which alcohols can substitute for water as the phosphatidate acceptor. In fact, alcohols are better than water as phosphatidate acceptors; about 1% of an alcohol (e.g., ethanol) in water yields phosphatidylethanol almost exclusively and this principle is the basis of some PLD assays.

In the case of the member of the PLD family, Nuc, when the enzyme was incubated with ^{32}P-labeled inorganic phosphate, a phospho-enzyme intermediate was produced and identified as phospho-histidine (E.B. Gottlin, 1998). Recently, the first crystal structure of a PLD from *Streptomyces* sp. has been published (I. Leiros, 2000) and

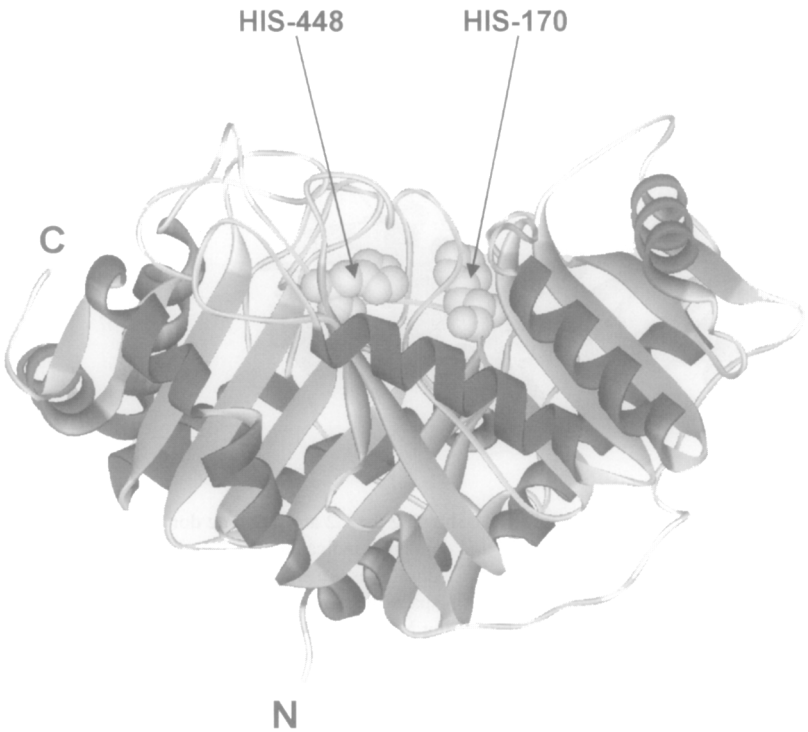

Fig. 11. Crystal structure of the PLD from *Streptomyces* sp. showing two active site histidines in space-filling format together with the N- and C-termini of the protein. Adapted from I. Leiros (2000) using MSA Weblab Viewer.

is shown in Fig. 11. The positions of two active site histidines, His-170 and His-448, are indicated and it is proposed that His-170 is the more likely residue to act as the nucleophile and thus be covalently modified during catalysis.

Mammalian PLDs are now the subject of intense interest as they appear to be intimately involved in signal transduction. Two mammalian PLDs have been identified and these contain several domains often associated with signal transduction proteins including a PH domain and a separate phosphatidylinositol-*bis*-phosphate binding domain. These proteins appear to be controlled by a variety of regulatory molecules [28]. The potential roles of the enzymes in cell signaling [29] and membrane traffic [30] are discussed in detail in Chapter 12.

Phospholipases C and D that act on glycosylphosphatidylinositol-anchored proteins on the cell surface are of growing importance and are discussed in Chapter 2.

3. *Future directions*

Since the last edition of 'Biochemistry of Lipids, Lipoproteins and Membranes' there has been remarkable progress in our knowledge of phospholipases. This progress

has included the crystal structures of many more phospholipases and the use of the techniques of molecular genetics in order to define the precise physiological function of individual enzymes. However, at this time the rate of elucidation of the structure and function of known enzymes is being matched by the discovery of new phospholipases in a very diverse array of organisms. This is particularly the case with the secreted PLA_2s and a major challenge is to understand the role or roles of many of these enzymes in their respective organisms. A major factor that has evolved is the important role of binding to the phospholipid interface in determining and regulating physiological function. Our knowledge of the molecular interactions at membrane interfaces is limited and will require advances in both X-ray crystallography and NMR to elucidate such details. There are exciting times ahead!

Abbreviations

cmc	critical micellar concentration
PLs	phospholipases
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D

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