Minireview

Structure, function and regulation of Ca^{2+}-sensitive cytosolic phospholipase A2 (cPLA2)

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Abstract The 85-kDa cytosolic PLA2 (cPLA2) is present in many cells and tissues and its unusual functional properties and catalytic mechanism are being elucidated. Notably, cPLA2 becomes catalytically active in the presence of free Ca^{2+} concentrations as present in stimulated cells and preferentially cleaves arachidonic acid-containing phospholipids. A variety of agonists, growth factors and cytokines, as well as stressful stimuli activate cPLA2 to hydrolyze cellular phospholipids thereby liberating fatty acids and lysophospholipids and providing the precursor substrates for the biosynthesis of eicosanoids and platelet-activating factor. These products of cPLA2 contribute to inflammatory and degenerative disease states and cPLA2 is therefore an attractive target for the development of novel therapies.

Key words: Cytosolic phospholipase A2; Structure; Function; Regulation

1. Introduction

Phospholipases A2 (PLA2s) are ubiquitous enzymes that are present in most cells and tissues. Hydrolysis of cellular phospholipids by PLA2s causes release of free fatty acids with concomitant formation of lysophospholipids. The newly released free fatty acids may affect cellular functions, since they activate protein kinase C [1] and MAP kinases [2,3] and the concomitantly generated lysophospholipids may cause damage to cellular membranes [4]. The products of PLA2 catalyzed phospholipid hydrolysis include arachidonic acid and lyso-PAF, which are the precursors of a wide spectrum of pro-inflammatory mediators (including prostaglandins, thromboxanes, leukotrienes and PAF) [5,6]. Once formed these lipid mediators may act as intracellular messengers acting on protein targets in the cell where they have been formed or, alternatively, they may leave the cell and interact with specific receptors on the cell surface of the parent and/or neighboring cells. The release of arachidonic acid, formation of the intermediate metabolite (endoperoxide or hydroperoxide) and conversion to the final eicosanoid can take place in two separate cell types [7]. Many of the newly generated lipids derived from products of PLA2 action have profound biological properties. They promote inflammatory reactions and participate in processes that lead to tissue injury. PLA2s are therefore attractive targets for the development of inhibitors that may be useful novel therapeutic agents for the treatment of inflammatory and degenerative diseases.

2. The PLA2 subfamilies

Using functional criteria for classification, one can distinguish at least four different subfamilies of PLA2 enzymes. First, secretory PLA2s (sPLA2s) that have a molecular mass of 14 kDa and are characterized by a catalytic requirement for Ca^{2+} and a rigid three-dimensional structure maintained by disulfide bridges. Two mammalian sPLA2s (referred to as group I and II) have been purified, cloned and extensively characterized over the last two decades [8] and two genes encoding two additional mammalian sPLA2s have recently been identified [9]. Second, an 85 kDa Ca^{2+}-sensitive cytosolic PLA2 (cPLA2) has been purified, cloned and biochemically characterized [10,11]. The structural and biochemical properties of cPLA2 are very different from those of the other well-known PLA2s and will be discussed in detail below. Third, several distinct Ca^{2+}-independent cytosolic PLA2 activities (iPLA2s) with molecular masses ranging from 29 to 85 kDa have been purified from different tissues and cellular sources. Although the biochemical properties of these iPLA2s have been extensively characterized [12], to date only the primary structure of the CHO cell-derived iPLA2 has been delineated and it is noteworthy that it has no structural relationship to cPLA2 [13]. Fourth, the important features that differentiate the PAF acetylhydrolases (PAF-AHs) from the above described PLA2s is their remarkable specificity for short and/or oxidized acyl groups at the *sn*-2 position of phospholipids and their Ca^{2+} independence [14]. A PAF-AH present in plasma where it is bound to lipoproteins (and therefore also referred to as lipoprotein-associated PLA2) has been purified and cloned. Several intracellular PAF-AHs have been purified from brain and the cloned cDNAs of three isoforms encode two catalytic subunits (β and γ) with molecular masses of 30 and 29 kDa, respectively, and a regulatory (α) subunit of 45 kDa. There is no sequence homology between the extracellular and intracellular PAF-AH [14].

3. The cPLA2 gene

The Ca^{2+}-sensitive cytosolic PLA2 (cPLA2) was first characterized in platelets and macrophage cells [10,11]. The cDNA of cPLA2 was subsequently cloned from macrophage cell libraries [10,11,15] and the promoter of the cPLA2 gene was isolated [16–18]. Collectively, the data showed that the cPLA2 cDNA comprised a total of 2880 nucleotides, including about 200 nucleotides for the 5′-untranslated region and about 500

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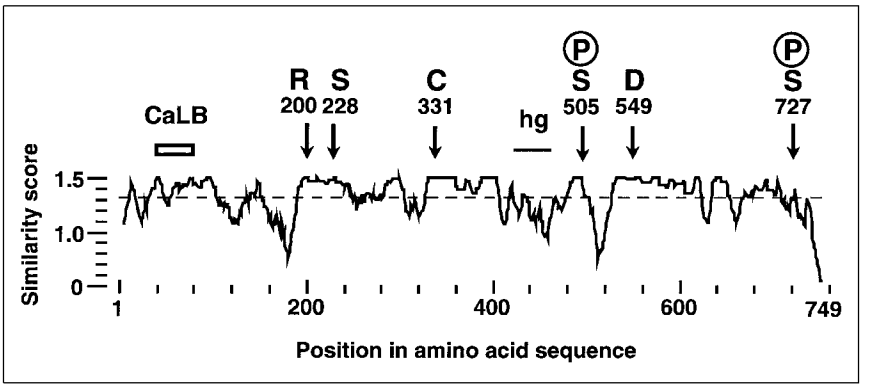

Fig. 1. Protein sequence similarity between cPLA2 from six species. The cPLA2 protein sequences deduced from the DNA sequences of six different species [11,25] are compared using the GCG program *plotsimilarity*. A similarity score of 1.5 indicates perfect conservation within a local region. The locations of the CaLB domain, the 'hinge' region, the catalytically essential residues (R-200, S-228 and D-549), the critical cysteine residue (C-331) and the two conserved phosphorylation sites (S-505 and S-727) are indicated.

nucleotides for the 3′-untranslated region. While the complete chromosomal DNA for cPLA2 has not yet been sequenced, there appear to be at least 7 introns, and some features of the genetic control mechanisms of the cPLA2 gene were revealed by analysis of its 5′ flanking sequence [16–18]. Additional regulation appears to be mediated post-transcriptionally [19]. The genomic location of cPLA2 and useful associated variable markers have been identified [17,20]. A CA repeat appears 160 bases upstream from the transcriptional start site, in both the rat and human 5′ flanking regions [17,18,20]. The study of this dinucleotide repeat allowed for the mapping of the cPLA2 gene to rat chromosome 13 [20]. Another heterozygosity in the human 5′ flanking region, the poly-A segment of a partial Alu sequence, was utilized to map cPLA2 to human chromosome 1q25. Since this region also contains the functionally related prostaglandin synthase 2 gene, it was suggested that there may be coordinate regulation of cPLA2 and prostaglandin synthase 2. Upstream of the 5′-untranslated segment of the cDNA cloned earlier, a 5′ exon of 136 nucleotides is separated by an intron of about 6 kb [16–18]. The 5′ flanking region or the first exon contains exact or modified copies of sequence elements that may contribute to transcriptional regulation. There are no perfect TATA or CCAAT elements, but close homologs have been noted, along with a homolog of the Sp1 element [18]. In addition, there are consensus sites for NFκB, NF-IL-6, AP-1, AP-2, PEA3, OCT, C/EBP and GRE. While none of these elements has been shown experimentally to regulate cPLA2 expression, one detailed study has revealed an important 27 bp poly-pyrimidine region immediately preceding the transcription start site [17]. This small region alone appears to support a significant amount of the total transcriptional activity of the cPLA2 gene, and may be responsible for the observed tight control that does not allow dramatic variation of the activity of this promoter [17]. The 3′-untranslated region of the cPLA2 mRNA contains regions with increased AU content and multiple conserved AUUUA sequences that appear to regulate mRNA stability enhancing expression in mitogen-stimulated mesangial cells [19]. Although Southern blot analysis of human genomic DNA originally suggested the absence of genes closely related to cPLA2, it is quite likely that cPLA2 is a member of a larger family of structurally related enzymes.

4. The cPLA2 protein

The cPLA2 cDNA encodes a 749 amino acid protein with a predicted molecular mass of 85.2 kDa. The inferred sequence of murine cPLA2 is more than 95% homologous to the human cPLA2 sequence indicating great structural similarity between cPLA2 from different species. However, as shown in Fig. 1, the similarity between cPLA2 sequences from more evolutionarily distant species is reduced and reveals the less conserved regions that may serve as linkers between domains [11]. Three stretches of lesser identity are noteworthy: the region between Glu-163 and Ala-191, Leu-507 and Leu-521, as well as the C-terminal portion from Ala-711 to Ala-749. As further depicted in Fig. 1, the deduced cPLA2 protein sequences have several interesting structural features in common. First, the cPLA2 sequence contains a 68 amino acid stretch in the N-terminal portion termed CaLB domain that shows sequence homology with the C2 region of protein kinase C. This CaLB domain was also noted in the GTPase activating protein (GAP), phospholipase C and the synaptic vesicle protein p65. This domain was found to be responsible for the Ca^{2+}-dependent binding of cPLA2 to membranes or phospholipid substrate [21] and was recently crystallized [22]. Second, several essential amino acid residues have been identified that may be important for the catalytic mechanism of cPLA2. The cPLA2 sequence contains the segment Gly-Leu-Ser^{228}-Gly-Ser that closely resembles the amino acid motif Gly-X-Ser-X-Gly present in many serine esterases and neutral lipases. The central serine of this motif is known to serve as the active site nucleophile in these enzymes. Replacing Ser-228 with alanine (threonine or cysteine) by site-directed mutagenesis yielded correctly folded, but catalytically inactive cPLA2 thus demonstrating that Ser-228 is required for the catalytic function of cPLA2 [23]. The role of Ser-228 as the active-site nucleophile was confirmed with novel substrates of cPLA2 and active-site directed inhibitors [24]. The Asp-549 of cPLA2 resides within a sequence motif (Ile-X-Val-Val-Asp-Ser-Gly-Leu-X-X-X-Asn) that perfectly matches the specifications for catalytic aspartic acid residues in the subtilisin family of proteases. On mutation of Asp-549 to alanine (or asparagine or glutamic acid) the enzymatic activity of cPLA2 was abolished suggesting that Asp-549 participates in the catalytic mechanism [25].

Unlike other serine esterases and lipases, cPLA2 does not appear to have a histidine requirement for its catalytic function. Thus, all 19 His-to-Ala mutants of cPLA2 were found to exhibit significant enzymatic activity. The cPLA2 sequence contains nine free cysteine residues. Although the enzyme is readily inactivated by preincubation with the sulfhydryl reagent *N*-ethylmaleimide (NEM), none of these cysteines was found to be essential for catalytic activity. However, only one of the cysteines, Cys-331, is responsible for the NEM sensitivity and its substitution with alanine renders cPLA2 resistant to NEM. This indicates that Cys-331 may be located close to the active site. Third, the human cPLA2 sequence contains numerous diverse consensus phosphorylation sites for both serine/threonine and tyrosine protein kinases [15]. To date four serine residues were found to be phosphorylated in human cPLA2 overexpressed in cultured cells [26]. However, only two such sites, Ser-505 and Ser-727, are conserved in cPLA2 from other species. Ser-505 resides within the sequence Pro-Leu-Ser-Pro typically recognized by proline-directed kinases, such as MAP kinases [27], and Ser-727 is flanked by arginines (Arg-Arg-$(X)_4$-Arg-X-Ser-$(X)_8$-Arg-Arg) typical of sites preferred by basotrophic kinases, such as protein kinase C and protein kinase A. Finally, other domains of interest include a 33 amino acid segment (residues 431–463) in the middle of the sequence that is deficient in hydrophobic amino acids and may represent a flexible 'hinge' region. Moreover, the cPLA2 sequence contains a domain towards the C-terminus (residues 520–680) modestly enriched in proline ($\sim$12% of total residues) that may be responsible for the abnormal electrophoretic mobility of cPLA2 on SDS polyacrylamide gels.

5. Biochemical and catalytic properties of cPLA2

Using in vitro enzyme assay systems cPLA2 was found to become catalytically active in the presence of 0.3–2 μM Ca^{2+} as present in the cytosol of stimulated cells [10,11]. However, enzymatic activity of cPLA2 could be promoted by high salt in the absence of Ca^{2+} indicating that Ca^{2+} is not required for the catalytic activity of cPLA2 [28]. The salt effect is likely due to stimulation of hydrophobic interactions between cPLA2 and phospholipids leading to its association with the substrate in the absence of Ca^{2+}. cPLA2 preferentially cleaves arachidonic acid-containing phospholipids and hydrolyzes different 1-stearoyl-2-arachidonoyl phospholipids with the order of preference phosphatidylcholine (PC) = phosphatidylinositol > phosphatidylethanolamine (PE) > phosphatidic acid = phosphatidylserine. With PC as substrate the fatty acid preference of cPLA2 was found to be arachidonic (20:4) > linolenic (18:3) > linoleic (18:2) > oleic (18:1) ≥ palmitoleic (16:1) [29]. The order of preference among PCs containing 20-carbon *sn*-2 acyl chains was arachidonic (20:4) > homogammalinoleic (20:3) > eicosadienoic (20:2) > eicosenoic (20:1) > eicosanoic (20:0) and there appeared to be a preference for positional isomers with double bonds closest to the *sn*-2 ester bond (eicosatrienoic 5,8,11 > 5,8,14 > 5,11,14 > 8,11,14). Although cPLA2 shows a marked preference for poly-unsaturated long fatty acyl chains, its activity towards PE substrates decreased dramatically with chains longer and more unsaturated than arachidonic acid (20:4) the order being arachidonic (20:4) > pentaenoic (20:5) > docosahexaenoic (C22:6) = O [30]. Interestingly, cPLA2 displays other catalytic activities and exhibited lysophospholipase and transacylase activities when incubated with lyso-LPC micelles [28]. However, incubation of cPLA2 with PC containing dual-labeled fatty acids (1-palmitoyl-2-arachidonoyl-PC) revealed that the lysophospholipase activity on newly formed 1-palmitoyl-lyso-PC was low.

6. Activation of cPLA2

cPLA2 is the only known PLA2 that exhibits functional properties indicative of a receptor-regulated PLA2 and is thus likely to be involved in receptor-mediated eicosanoid production and intracellular signal transduction processes. Studies from many different laboratories using different cellular systems have revealed that a wide spectrum of extracellular stimuli activate cPLA2 to mobilize PLA2 from cellular phospholipids. These stimuli include growth factors, mitogens, vasoactive peptides, crosslinking of Fc receptors, integrin engagement, cytokines and interferons. However, non-receptor-mediated stimulation of cells by stressful stimuli, including oxidation, hyperglycemia, UV light and shear stress, may also promote cPLA2 activation. It is noteworthy that Ca^{2+} ionophores induce maximal release of arachidonic acid from cellular phospholipids suggesting that the increase in cytosolic free [Ca^{2+}] is a predominant factor causing cPLA2 activation. Since cPLA2 binds to membranes in a Ca^{2+}-dependent fashion, increased cytosolic free Ca^{2+} mobilized from intracellular stores and/or derived from increased influx of extracellular Ca^{2+} is likely to induce the association of cPLA2 with cellular membranes. A wide variety of extracellular stimuli were found to cause rapid phosphorylation of cPLA2 suggesting that phosphorylation may also play an important role in the regulation of cPLA2 activation. In platelets, thrombin induced a time- and dose-dependent phosphorylation of cPLA2 that resulted in enhanced catalytic activity, as well as a change in the electrophoretic and chromatographic properties of cPLA2 [31]. By comparing the functional properties of cPLA2 from control and thrombin-stimulated platelets, it was found that while phosphorylated cPLA2 exhibited the same Ca^{2+} requirement and apparent substrate affinity (K_m), its catalytic activity (V_{max}) was increased compared to control cPLA2. The importance of this phosphorylation for the activation of cPLA2 as well as the identity of the involved kinase(s) have not been fully elucidated. An involvement of protein kinase C in the regulation of PLA2 has been demonstrated in some cells. However, in other cells down-regulation of protein kinase C did not affect the activation of cPLA2. In human platelets phosphorylation of cPLA2 induced by thrombin and collagen was not affected by specific inhibitors of protein kinase C demonstrating that platelet cPLA2 is phosphorylated by pathways independent of protein kinase C [32]. Early studies demonstrated that cultured cells transfected with cPLA2 lacking the MAP kinase phosphorylation site (Ser^{505}-Ala cPLA2) exhibited diminished ability to release PLA2 in response to agonists compared to control cells expressing comparable levels of unmutated cPLA2 [33]. Furthermore, studies with cultured cells and in vitro kinase assays showed that cPLA2 is a substrate for the p42 MAP kinase (now referred to as ERK2). Many subsequent reports have proposed that phosphorylation of cPLA2 by ERK kinases is a critical step in the sequence of events leading to the mobilization of arachidonic acid in stimulated cells, but direct evidence to support this hypothesis

has not been provided. Unexpectedly, in human platelets stimulated with the thrombin receptor agonist peptide SFLLRN phosphorylation of cPLA2 occurred in the absence of ERK activation [34]. Furthermore, under conditions where ERK activation was completely suppressed, cPLA2 phosphorylation was unaffected [32]. Lastly, PD 098059, a specific inhibitor of the activation of ERKs did not block thrombin-induced cPLA2 phosphorylation [35]. Taken together, these findings showed that in human platelets proline-directed kinases distinct from the ERKs may be involved in the phosphorylation of cPLA2. It was indeed observed that a recently discovered stress-activated kinase, referred to as p38, is responsible for the phosphorylation of the Ser-505 site of platelet cPLA2 [36]. Surprisingly, however, prevention of the proline-directed phosphorylation of cPLA2 by specific inhibitors of p38 kinase did not affect its ability to rapidly mobilize PLA2 in stimulated platelets. These findings indicate that, at least in platelets, proline-directed phosphorylation is not a prerequisite for receptor-mediated activation of cPLA2. It is still possible that phosphorylation sites distinct from Ser-505 of cPLA2 may be targeted by as yet unidentified kinases and it will be of great interest to further study their potential involvement in the regulation of cPLA2.

7. Cellular localization of cPLA2

Many different cell types contain cPLA2, including platelets, macrophages, neutrophils, endothelial cells, vascular smooth muscle cells, alveolar epithelial cells, renal mesangial cells and keratinocytes. The cPLA2 gene is widely expressed and mRNA levels were most prominent in brain, lung, kidney, heart and spleen [37]. Furthermore, using anti-cPLA2 antibodies, the presence of cPLA2 protein was demonstrated in a variety of tissues from the guinea pig, in particular lung, spleen, brain and kidney [10]. Analysis of the subcellular distribution of cPLA2 in resting and stimulated cells indicated that upon cell activation cPLA2 redistributes from the cytosolic to the membrane fraction. Examination of the subcellular localization of cPLA2 in a variety of cells using immunofluorescence microscopy showed that upon cell activation cPLA2 translocates to the endoplasmic reticulum and the nuclear membranes [38,39]. In subconfluent endothelial cells a significant portion of cPLA2 appears to reside within the nucleus [40]. There is recent evidence demonstrating that prostaglandin synthase 1 and 2 are localized in the endoplasmic reticulum and in the nuclear envelope, respectively [41]. Moreover, 5-lipoxygenase translocates to a perinuclear site in stimulated cells [42]. It thus appears that in activated cells cPLA2 may be ideally positioned to provide free arachidonic acid to the enzymes of the eicosanoid cascade. Furthermore, eicosanoids newly generated in close proximity to the nucleus can not only serve as agonists for cell surface receptors, but may also target intracellular (nuclear) recognition sites.

8. cPLA2 and disease

Phospholipid breakdown and concomitant release of free fatty acids and lysophospholipids, as well as production of eicosanoids and PAF are amongst the early events in the brain after global cerebral ischemia. Agents considered hallmarks of ischemia and reperfusion, in particular the high intracellular levels of free Ca^{2+} resulting from ischemic membrane depolarization and opening of Ca^{2+} channels, are likely to trigger cPLA2 activation. Furthermore, pro-inflammatory cytokines such as IL-1 and TNF produced during ischemia are known to induce de novo synthesis of cPLA2 and may thus further potentiate the mobilization of PLA2 and subsequent production of eicosanoids and PAF. During global cerebral ischemia (as typically observed during cardiac arrest) and mimicked in the four vessel occlusion model of ischemia in the rat, neurons of the hippocampal CA1 region of the brain are particularly sensitive to the ischemic insult and undergo a process of delayed cell death (also referred to as apoptosis) over a period of days after the ischemia. Biochemical analyses, including immunoblotting and enzymatic assay, showed elevated levels of cPLA2 in ischemic compared to control hippocampal tissues [43]. Furthermore, immunohistochemical analysis of brain sections from rats subjected to ischemia demonstrated a marked induction of cPLA2 in the CA1 hippocampal region. Notably, this immunoreactivity was confined to astrocytes and microglia in areas of neurodegeneration. Recent studies with cellular systems have documented that the increase in cellular eicosanoid production promoted by cytokines and agents causing cell damage is, at least in part, due to activation of cPLA2 and elevation of its cellular levels [44]. It was also found that cPLA2 is a target of anti-inflammatory glucocorticoids well-known for their ability to attenuate eicosanoid synthesis in a number of different cell types. Thus, it has become clear that cPLA2 may play an important role in both the rapid and the prolonged cellular responses occurring during inflammatory and degenerative processes.

9. Conclusions

The Ca^{2+}-sensitive 85 kDa cytosolic PLA2 (cPLA2) is activated by receptor-mediated events and stressful stimuli and initiates the cascade of events leading to the production of free fatty acids, lysophospholipids, eicosanoids and PAF. Activation of cellular cPLA2 is clearly dependent on an increase in cytosolic free $[Ca^{2+}]$ to allow for association of cPLA2 with the membrane phospholipid substrate and, in some cellular systems, may also require phosphorylation of cPLA2 by stimulus-activated kinases. Cellular mobilization of arachidonic acid and eicosanoid production are hallmarks of inflammation, and increased eicosanoids mediate both pathophysiological alterations and cellular processes which lead to inflammatory injury. There is substantial evidence to indicate that cPLA2 may be an important component in the cascade of events leading to the production of pro-inflammatory and injurious mediators in inflammatory and degenerative disease states. Accordingly, cPLA2 is an attractive target for the development of novel therapies.

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