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Cellular Signalling 17 (2005) 1052–1062

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Review

Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A_2 in activated cells

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Received 28 February 2005; accepted 9 March 2005
Available online 7 April 2005

Abstract

Mammalian cells contain several calcium-independent phospholipase A_2 (PLA_2) enzymes. The best studied of them is the so-called Group VIA PLA_2 ($iPLA_2$-VIA), which is an 85-88 kDa enzyme with unique structural features among the PLA_2 superfamily of enzymes, and has been found to play a key role in homeostatic membrane phospholipid metabolism in various cell types. Growing evidence suggests that, in addition to its homeostatic function, $iPLA_2$-VIA may also play distinct roles in cellular signaling. This review focuses on the biochemical mechanisms that regulate the activity of $iPLA_2$-VIA in activated cells, and the biological functions proposed for this enzyme during stimulus-response coupling.
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Keywords: Phospholipase A_2; Arachidonic acid; Eicosanoids; Membrane phospholipid; Ca^{2+}-independent; Lysophospholipid; Signal transduction

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Abbreviations: PLA_2, phospholipase A_2; $iPLA_2$, calcium-independent phospholipase A_2 activity (undefined molecular identity); $iPLA_2$-VIA, Group VIA phospholipase A_2; $cPLA_2\alpha$, cytosolic Group IVA phospholipase A_2; AA, arachidonic acid; PAF, platelet-activating factor; MAPK, mitogen-activated protein kinase; BEL, bromoenol lactone; MAFP, methyl arachidonyl fluorophosphonate; PC, phosphatidylcholine; lysoPC, lysophosphatidyl-choline.

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doi:10.1016/j.cellsig.2005.03.002

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1. Introduction

Phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of the fatty acid at the *sn*-2 position of glycerophospholipids, leading to the production of a free fatty acid and a 2-lysophospholipid. This reaction is particularly important when the fatty acid liberated is arachidonic acid (AA), which can be converted into the biologically active compounds called eicosanoids, which include prostaglandins, leukotrienes, and lipoxins. The other reaction product, the 2-lysophospholipid, may also possess biological activity on its own or can be used as a precursor for the biosynthesis of other biologically active compounds such as platelet-activating factor (PAF).

Since PLA_2-derived products appear to control a large number of extracellular stimuli-mediated signaling pathways, a first step in understanding these processes is to establish the regulatory features, mechanisms of action and cell regulation of the different PLA_2 forms present in cells. Conversely, a frequently overlooked fact is that, in addition to signaling roles, PLA_2s often play important roles in maintaining cell membrane homeostasis by participating in the recycling of fatty acid moieties within membrane phospholipids or helping regulate phospholipid mass.

At present, no less than 20 different proteins possessing PLA_2 activity have been identified and cloned in mammals. According to the nucleotide and amino acid sequence criteria established by Six and Dennis [1], these PLA_2s are systematically classified into several group types. The reader is referred to Refs. [2] and [3] for the latest versions of this classification (Group IVD PLA_2, discovered last year [4], has yet to be included). However, the PLA_2s are also frequently grouped by biochemical commonalities, which results in a less systematic but sometimes more straightforward classification. According to this classification, there are 4 major PLA_2 families, namely the secreted PLA_2s, the cytosolic Ca^{2+}-dependent PLA_2s ($cPLA_2$), the platelet-activating factor acetylhydrolases, and the cytosolic Ca^{2+}-independent PLA_2s ($iPLA_2$). Although this functional classification has numerous caveats—e.g. Group IVC is known as $cPLA_2\gamma$ despite the enzyme being neither cytosolic nor Ca^{2+}-dependent—it remains very popular because of its usefulness to generalize biochemical properties, and when the identity of a particular PLA_2 activity is not clearly defined.

At present, the $iPLA_2$ family only consists of two members in mammals. The better studied of them, Group VIA PLA_2 ($iPLA_2$-VIA), appears to be the first PLA_2 for which dual homeostatic and activating roles have been demonstrated. The involvement of $iPLA_2$-VIA in regulating homeostatic phospholipid levels via fatty acid deacylation/reacylation reactions has been the subject of recent reviews [5–8]. In this review we will focus on the signaling properties of $iPLA_2$-VIA by summarizing the recent advances in the regulation of enzyme activity and cellular function during stimulation.

The one other mammalian $iPLA_2$ family member [9–11] is classified as Group VIB PLA_2 ($iPLA_2$-VIB) [1–3]. This enzyme is presumably membrane-bound, and shows very little homology with $iPLA_2$-VIA at the N-terminus. However, several highly conserved sequences between $iPLA_2$-VIA and $iPLA_2$-VIB are clustered in the C-terminal half. Very little is yet known about this enzyme and its cellular functioning [12,13].

Recently, sequence data base searches for proteins containing the nucleotide and lipase consensus sequence motifs present in $iPLA_2$-VIA, resulted in the identification of three novel proteins possessing PLA_2 activity but also robust triacylglycerol lipase and acylglycerol transacylase activities [14]. It has not been defined at this time whether these three proteins should be regarded as true PLA_2 enzymes or as general lipases of broad specificity.

2. Structural and enzymological features of $iPLA_2$-VIA

$iPLA_2$-VIA was purified and cloned from different mammalian sources in 1997 [15–18], and classified as Group VIA PLA_2 [1]. Although $iPLA_2$-VIA bears no sequence homology with $cPLA_2\alpha$, both enzymes share some common biochemical features, such as the cytosolic location in resting cells, a molecular mass in the 85 kDa range, and the existence of a catalytic serine. However, $cPLA_2\alpha$ shows a marked preference for AA-containing phospholipids, whereas $iPLA_2$-VIA does not [1]. In some papers, $iPLA_2$-VIA is also referred to as $iPLA_2\beta$ ($iPLA_2\alpha$ being used for patatin, a non-mammalian enzyme with significant structural similarity to $cPLA_2\alpha$ [19]) or, simply $iPLA_2$. In this review we have utilized the abbreviation $iPLA_2$-VIA in the belief of it being more precise than the other two.

$iPLA_2$-VIA occurs in at least 5 different splicing variants (Fig. 1). Two of them have been demonstrated to possess enzymatic activity and are termed VIA-1 and VIA-2. $iPLA_2$-VIA-1 is an 85 kDa enzyme that possesses eight ankyrin-like repeated domains in the N-terminal half of the

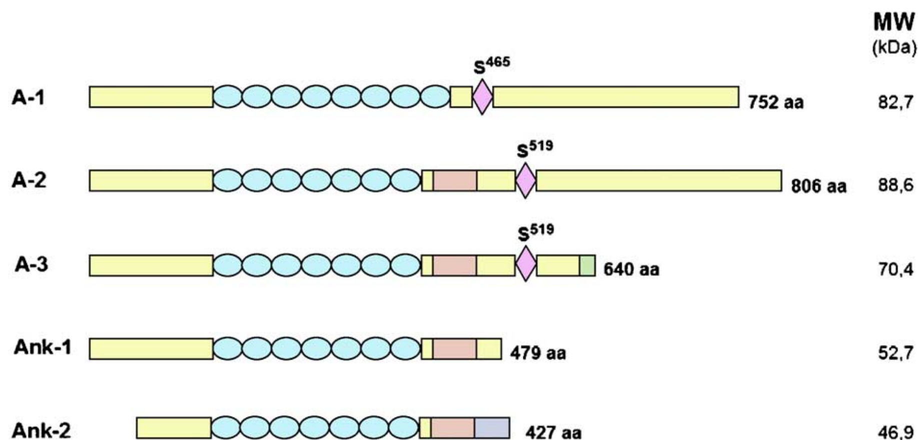

Fig. 1. Schematic representation of the multiple iPLA$_2$-VIA splicing variants. Identical sequences in all forms are shown in yellow. Sequence changes with respect to iPLA$_2$-VIA-1 are shown as orange, green, and purple inserts. The ankyrin-like sequences are represented as blue circles, and the catalytic Ser are shown as pink diamonds, Amino acid (aa) number and the calculated molecular mass of each variant are also shown.

protein, followed by a classical GXSXG lipase consensus motif that defines Ser^{465} as the catalytic nucleophile in the active site. iPLA$_2$-VIA-2 is almost identical to iPLA$_2$-VIA-1 except for the fact that the eighth ankyrin domain is interrupted by a proline-rich 54-amino acid stretch. Because of this insertion, the catalytic Ser shifts to position 519 (Fig. 1). Interestingly, this proline-rich sequence shares a consensus motif with the proline-rich middle linker domains of Smad. Both isoforms contain a glycine-rich nucleotide binding motif before the catalytic Ser, and a putative binding site for calmodulin near the C-terminus. These motifs may contribute to the regulation of the enzymatic activity of iPLA$_2$-VIA in cells (see Sections 4.1 and 4.3).

Two of the iPLA$_2$ variants possess only the ankyrin-repeated domains, and thus lack enzymatic activity. These proteins have been suggested to act as dominant-negative inhibitors for the active variants (see Section 4.4).

The fifth iPLA$_2$-IVA splicing variant, termed iPLA$_2$-VIA-3, is identical to iPLA$_2$-VIA-2 but exhibits a truncated C-terminus as a consequence of a premature stop codon due to the insertion of a 158-nucleotide stretch. Since iPLA$_2$-VIA-3 contains the lipase consensus motif, it probably has enzymatic activity; however this has not been verified experimentally.

In addition to its intrinsic PLA$_2$ activity, iPLA$_2$-VIA also exhibits lysophospholipase and phospholipid trans-acylase activities although, when measured under comparable conditions, the PLA$_2$ activity is significantly higher [20]. iPLA$_2$-VIA shows no substrate specificity for the fatty acid present in the *sn*-2 position or the headgroup present in the *sn*-3 position of phospholipids. Remarkably, the enzyme can efficiently hydrolyze platelet-activating factor and/or oxidized phospholipids, mimicking in this regard the actions of PAF acylhydrolases [15]. Thus, in addition to its well defined properties in homeostatic phospholipid metabolism [5–8] and in signaling in activated cells (this review), iPLA$_2$-VIA could function as a signal terminator as well.

3. Inhibition of iPLA$_2$

iPLA$_2$-VIA is readily inhibited by hydrophobic serine-reactive inhibitors such as bromoenol lactone (BEL) [15,21], methyl arachidonyl fluorophosphonate (MAFP) [16,22], and fatty acyl trifluoromethyl ketones and tricarbonyls [21,23]. Among these, only BEL selectively targets iPLA$_2$s over other PLA$_2$s [24,25] and thus, has found much use in defining possible roles for iPLA$_2$ in cell functioning. However, when applied to cells, BEL is also likely to interact with many other cellular enzymes [6,15], so caution must be exercised when interpreting results based on BEL inhibition alone. In addition to iPLA$_2$-VIA, other hydrolases that are known to be inhibited by BEL include chymotrypsin and related serine proteases [26], Mg^{2+}-dependent phosphatidate phosphohydrolase [27–30], anandamide hydrolase [31] and, notably, iPLA$_2$-VIB [10].

To distinguish the effects of BEL on iPLA$_2$-VIA from those exerted on Mg^{2+}-dependent phosphatidate phosphohydrolase, propranolol has been used with some success [28,32]. Propranolol at relatively high concentrations (150 μM and above) inhibits the phosphohydrolase but does not affect iPLA$_2$-VIA. Thus, if a given process is inhibited by BEL but not by high propranolol concentrations, the involvement of Mg^{2+}-dependent phosphatidate phosphohydrolase can be certainly ruled out. Note that the lack of involvement of the phosphohydrolase in a BEL-inhibited effect does not necessarily mean that such an effect is mediated by iPLA$_2$-VIA. Note as well that propranolol concentrations in the low micromolar range, as used in some studies, are unlikely to inhibit cellular phosphatidate phosphohydrolase.

Recently, Jenkins et al. [33] separated BEL into its two enantiomers and found the *S*-isomer to be considerably more potent on iPLA$_2$-VIA than on iPLA$_2$-VIB. Conversely, the *R*-isomer inhibited iPLA$_2$-VIB much better than iPLA$_2$-VIA [33]. Thus, the use of the BEL enantiomers might provide a straightforward means to differentiate the

contributions of iPLA$_2$-VIA and iPLA$_2$-VIB to a given cellular process [33].

The combined use of BEL and a hydrophobic serine-reactive inhibitor such as MAFP and/or a fatty acyl trifluoromethyl ketone may help strengthen the evidence favoring the involvement of iPLA$_2$-VIA in a given process. If a reaction is inhibited by BEL but not by MAFP and/or the fatty acyl trifluoromethyl ketone, the effector involved is most likely not iPLA$_2$-VIA, and another undefined iPLA$_2$ activity present in the cells may be responsible for the effects. An iPLA$_2$ activity sensitive to BEL but not to MAFP has been detected in renal proximal tubule cells [34]. It is unfortunately not uncommon that negative results with MAFP and/or the fatty acyl trifluoromethyl ketones are interpreted as a proof for the lack of involvement of cPLA$_2\alpha$ only, when under these conditions iPLA$_2$-VIA will almost certainly be inhibited as well.

Since the use of chemical inhibitors is unlikely to provide definitive answers to the involvement of iPLA$_2$-VIA in a given cellular process, the use of more selective approaches to inhibit iPLA$_2$-VIA has recently gained much attention. Use of antisense oligonucleotide [35–38] and small interfering RNA [39,40] technologies has proven to be valid techniques to specifically block iPLA$_2$-VIA under certain conditions. Use of cells over-expressing iPLA$_2$-VIA has also been used with success [41–43]. Recently, mice with targeted disruption of the gene encoding for iPLA$_2$-VIA have been generated [44]. It is anticipated that studies with these mice will highlight the importance of iPLA$_2$-VIA in a wide variety of physiological and pathophysiological conditions. It is remarkable that, so far, the only obvious phenotypic defect reported in these animals is that males produce spermatozoa with impaired motility, resulting in greatly reduced fertility [44].

4. Mechanisms of regulation of iPLA$_2$ activity

In some cells, basal iPLA$_2$-VIA activity appears to be quite significant, as judged by the measurable decrease of the steady-state level of cellular lysoPC after acute inhibition of the enzyme. For example, treatment of P388D$_1$ macrophages with BEL or an antisense oligonucleotide reduces cellular lysoPC levels by 50–60% [35,45], and in pancreatic islets, which naturally contain exceedingly high levels lysoPC, BEL lowers them down by approx. 20–25% [29]. However, little is known about how cells control and regulate iPLA$_2$-VIA activity during cellular signaling. This contrasts with the great deal of information that is currently available on the mechanisms of activation of cPLA$_2\alpha$ by Ca^{2+} transients and MAPK-mediated phosphorylation [46]. Since various iPLA$_2$-VIA splice variants co-exist in cells [6], it is plausible that the enzyme be regulated by multiple mechanisms that differ among cell types and stimulation conditions.

Diverse studies have measured increases in iPLA$_2$ activity in subcellular fractions and/or homogenates obtained from activated cells [37,47–51]. Although iPLA$_2$ activities different from that of iPLA$_2$-VIA enzyme might account for the activity changes, these studies raise the interesting possibility that under certain conditions, iPLA$_2$-VIA undergoes changes that stably modify its specific activity and/or subcellular localization. Several mechanisms have been proposed to account for agonist-induced iPLA$_2$ activation under different conditions, and the best described ones are discussed in this Section.

4.1. Ca^{2+}/calmodulin

iPLA$_2$-VIA is known to physically interact with calmodulin in vitro in a calcium-dependent manner [52]. In the presence of Ca^{2+}, calmodulin binds tightly to iPLA$_2$-VIA resulting in inhibition of the enzymatic activity. In the absence of Ca^{2+}, calmodulin would separate from and relieve its tonic inhibition of iPLA$_2$ [52]. Whether such a calmodulin-mediated mechanism operates in intact cells is currently under active investigation (see Section 5.8). A 15 kDa region near the C-terminus of the Group VIA (around residues 694–705) has been identified to participate in the Ca^{2+}-dependent binding of calmodulin to iPLA$_2$-VIA [52]. Although this C-terminal region of iPLA$_2$ appears to constitute the major calmodulin-binding site, the possibility that other binding sites exist within the iPLA$_2$-VIA sequence has not been ruled out.

4.2. Phosphorylation

Although no phosphorylation consensus sequences have been described in iPLA$_2$-VIA [15–18], different studies suggest the involvement of phosphorylation reactions in regulating the activity of the enzyme. It is possible that some of the effects measured are not due to direct phosphorylation of iPLA$_2$-VIA but of an associated regulatory protein. It is also conceivable that a kinase acts as a co-factor for proper iPLA$_2$-VIA activation independent of the intrinsic kinase activity, in a manner similar to that described for protein kinase C-regulated-phospholipase D [53]. In this regard, recent data utilizing the yeast two-hybrid system have indicated that Ca^{2+}/calmodulin-dependent protein kinase IIβ interacts with iPLA$_2$-VIA in pancreatic islet β-cells, and as a result of the association, the activities of both enzymes increase [54]. Since iPLA$_2$-VIA is also reported to directly interact with calmodulin (see Section 4.1, above), elucidation of the mechanisms regulating the interaction in vivo of these three proteins should prove to be a most exciting task.

Conversely, it has been reported that, in interferon γ-primed U937 monocytes stimulated through cross-linking of Fcγ-RI, iPLA$_2$-VIA can be immunoprecipitated by a specific anti-phosphoserine antibody in a protein kinase C-dependent manner [55]. Protein kinase C regulation of

$iPLA_2$-VIA has also been reported in zymosan-stimulated $P388D_1$ macrophages [38], where the α isoform of the kinase is suggested to regulate the translocation of $iPLA_2$-VIA to membrane fractions [56]. Similar to these findings, the ε isoform of protein kinase C has been reported to mediate the increased association of $iPLA_2$ activity to membrane fractions [47]. However, in this report the $iPLA_2$ activity measured was not identified to belong to the $iPLA_2$-VIA enzyme; thus it could arise from another enzyme.

Regarding other kinases, studies in thrombin-stimulated vascular smooth muscle cells have provided evidence for the implication of p38 MAPK in the cellular regulation of $iPLA_2$-VIA. In this system, $iPLA_2$-VIA appears to be involved in fatty acid release and DNA synthesis [48], and these actions are strongly blunted by inhibitors of p38 MAPK. Importantly, thrombin is found to increase the $iPLA_2$ specific activity of cell extracts, and such an increase is blocked if the extracts are prepared from cells pretreated with MAPK inhibitors [48].

4.3. ATP

In vitro assays have shown that under certain experimental conditions [18] the specific activity of $iPLA_2$-VIA is several fold higher if assayed in the presence of ATP. A glycine-rich, nucleotide-binding motif (GXGXXG) prior to the catalytic site has been identified in the two active variants of Group VIA PLA_2. However this ATP effect has not been observed under all conditions [15]. Other lines of evidence suggest that ATP does not directly activate the $iPLA_2$ but actually acts to protect the enzyme from losses of activity [20]. Direct evidence that in intact cells ATP binding to $iPLA_2$-VIA may function to modulate enzyme activity is lacking.

4.4. Ankyrin domain-mediated oligomerization

A prominent structural feature of $iPLA_2$-VIA is the presence of seven or eight ankyrin-like repeated domains in the N-terminal half of the protein (eight in the VIA-1 variant, and seven in the VIA-2 variant). These ankyrin repeats are present in vast number of proteins, and have been implicated in facilitating protein–protein interactions [57]. Interestingly, radiation-inactivation experiments have shown that the $iPLA_2$-VIA active species is a tetramer [58]. Furthermore, deletion of the ankyrin repeats by removing the first 150 amino acids yields an inactive $iPLA_2$-VIA [15]. Thus it appears likely that the ankyrin repeats enable oligomerization of the enzyme and hence expression of full enzymatic activity. Since many cells express truncated $iPLA_2$ protein fragments containing the ankyrin repeats but lacking activity, it has been suggested that these inactive fragments might function as regulators of $iPLA_2$-VIA by interfering with the ankyrin-mediated oligomerization of the active fragments [17,59]. In support of this proposal, it has been found that co-transfection of one of these inactive fragments with full-length $iPLA_2$-VIA leads to a significant reduction of the activity of the latter [17,59]. Further evidence for this model of $iPLA_2$-VIA activity regulation has come from the recent studies by Manguikian and Barbour [60], demonstrating by immunoprecipitation that the truncated $iPLA_2$-VIA inactive fragments physically associate with $iPLA_2$-VIA and down-regulate its activity during the G_1 phase of the cell cycle (see Section 5.1).

4.5. Proteolytic processing

The $iPLA_2$-VIA sequence contains several putative consensus sequences for caspase cleavage (DXXD), and at least three of them have been reported to be utilized in cells, leading to the generation of $iPLA_2$-VIA fragments with increased biological activity. Atsumi et al. [61,62] showed that in vivo cleavage of $iPLA_2$-VIA at Asp^{183} (Fig. 2), near the N-terminus, within the first ankyrin repeat, yields a 70-kDa protein that exhibits increased biological functioning.

Two other caspase cleavage sites yielding active proteins were identified by Lauber et al. [63]. The first site is located very proximal to the catalytic Ser, and the second one occurs very near the C-terminus of the molecule (Fig. 2). Caspase cleavage at the two sites yields a 24–26 kDa fragment, and cleavage only at the first site yields a 32 kDa fragment. These fragments correspond to amino acids 514–806 and 514–733 in the $iPLA_2$ VIA-2 sequence depicted in Fig. 2 (amino acids 459–752 and 459–679 in the $iPLA_2$-VIA-1 sequence). Over-expression of the two fragments increased the $iPLA_2$-VIA-mediated lysoPC production [63]. This is a striking finding since neither of the two fragments contains any ankyrin repeat, and earlier mutagenesis studies had suggested that the ankyrin repeats are required for enzymatic activity [15].

Recent evidence suggests that $iPLA_2$-VIA may also be cleaved by proteases other than caspases. Contrary to previous belief, the predominant $iPLA_2$ form in glucose-responsive 832/13 INS-1 cells, parental INS-1 cells, and pancreatic islets appears not to be the classical 85-kDa $iPLA_2$-VIA but a 70-kDa form [64]. Analysis of the tryptic map of the 70-kDa form reveals fragments that are identical to those obtained after tryptic digestion of the classical 85-kDa enzyme, indicating that both proteins are closely related. Since the 70-kDa protein does not appear to arise from an exon-skipping mechanism or alternate splicing, it is suggested to derive from an undetermined proteolytic processing mechanism that takes place at the C-terminal half of the molecule [64]. The 70 kDa variant is fully active and, as a matter of fact, it is now thought that the biological roles previously attributed to the classical 85 kDa $iPLA_2$-VIA in the native β cell (see Section 5.4), might be attributable to the 70 kDa form [64].

```
  1 MQFFGRLVNT FSGVTNLFSN PFRVKEVAVA DYTSSDRVRE EGQLILFQNT
 51 PNRTWDCVLV NPRDSQSGFR LFQLELEADA LVNFHQYSSQ LLPFYESSPQ
101 VLHTEVLQHL TDLIRNHPSW SVAHLAVELG IRECFHHSRI ISCANCAENE
151 EGCTPLHLAC RKGDGEILVE LVQYCHTQMD VTDYKGETVF HYAVQGDNSQ
201 VLQLLGRNAV AGLNQVNNQG LTPLHLACQL GKQEMVRVLL LCNARCNIMG
251 PNGYPIHSAM KFSQKGCAEM IISMDSSQIH SKDPRYGASP LHWAKNAEMA
301 RMLLKRGCNV NSTSSAGNTA LHVAVMRNRF DCAIVLLTHG ANADARGEHG
351 NTPLHLAMSK DNVEMIKALI VFGAEVDTPN DFGETPTFLA SKGRLVTRKA
401 ILTLLRTVGA EYCFPPIHGV PAEQGSAAPH HPFSLERAQP PPISLNNLEL
451 QDLMHISRAR KPAFILGSMR DEKRTHDHLL CLDGGGVKGL IIIQLLIAIE
501 KASGVATKDL FDWVAGTSTG GILALAILHS KSMAYMRGMY FRMKDEVFRG
551 SRPYESGPLE EFLKREFGEH TKMTDVRKPK VMLTGTLSDR QPAELHLFRN
601 YDAPETVREP RFNQNVNLRP PAQPSDQLVW RAARSSGAAP TYFRPNGRFL
651 DGGLLANNPT LDAMTEIHEY NQDLIRKGQA NKVKKLSIVV SLGTGRSPQV
701 PVTCVDVFRP SNPWELAKTV FGAKELGKMV VDCCTDPDGR AVDRARAWCE
751 MVGIQYFRLN PQLGTDIMLD EVSDTVLVNA LWETEVYIYE HREEFQKLIQ
801 LLLSP
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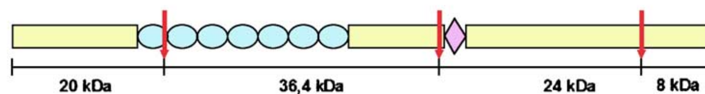

Fig. 2. Caspase cleavage sites in human iPLA$_2$-VIA-2. Amino acids within ankyrin repeats are shown in blue, and each repeat is underlined with a different color. Amino acids in red show the three caspase cleavage sites that have been positively identified to date. The first one is placed in between the first ankyrin repeat, the second is very proximal to the catalytic center, and the third is placed close to the C-terminus. The catalytic center is shown within a purple box. Below, the three caspase cleavage sites and the size of the products generated are further highlighted using the iPLA$_2$-VIA schematic representation described in Fig. 1.

4.6. Substrate availability

A fact that is not always immediately recognized is that, if cellular membranes are modified and become more susceptible to PLA$_2$ attack, increased phospholipid hydrolysis may occur in the absence of changes in the specific activity of the enzyme. Evidence has been provided that a mechanism such as this operates for iPLA$_2$-VIA in cells subjected to oxidative stress [65].

Oxidative damage induced by H_2O_2 is often associated with mobilization of various free fatty acids including AA, but the molecular mechanism responsible for these effects appears to vary from cell to cell. For example, in kidney epithelial cells [66] or Her14 fibroblasts [67] cPLA$_2\alpha$ has been found to mediate the process. However, in alveolar macrophages [68] and vascular smooth muscle cells [69] increased free fatty acid mobilization occurs through inhibition of its incorporation into phospholipids. Finally, in uterine stromal cells [49], murine macrophages [50], and U937 phagocytes [65], it is the BEL-sensitive iPLA$_2$ that appears to play a role. The mechanism for H_2O_2-induced, iPLA$_2$-mediated fatty acid mobilization was studied in detail in the latter system. Enzyme assays conducted under a variety of conditions revealed that the iPLA$_2$ specific activity did not change as a result of H_2O_2 exposure [65]. However, lipid hydrolysis by iPLA$_2$ occurred more readily in H_2O_2-treated cells because of changes in the physical state of membrane substrate, subsequent to lipid peroxide accumulation. Thus the oxidant acted to perturb membrane homeostasis in a way that the enzyme susceptibility/accessibility to its substrate increased, resulting in free fatty acid release [65].

5. Cellular signaling functions

Since our previous review on iPLA$_2$-VIA in which the significant role of this enzyme in regulated homeostatic phospholipid deacylation/reacylation reactions was delineated [6], compelling evidence has accumulated to indicate that Group VIA PLA$_2$ may also be involved in signaling. Importantly, the signaling roles of iPLA$_2$-VIA have now been substantiated not solely by using the unspecific inhibitor BEL, but also by more direct approaches such as inhibition of iPLA$_2$-VIA expression by antisense oligonucleotides or RNA silencing, and transient and stable over-expression of the enzyme. Unless otherwise indicated, we will discuss results where the involvement of iPLA$_2$-VIA was ascertained not just by using BEL but also by more selective approaches.

5.1. Cell growth

Phosphatidylcholine (PC) is the most abundant phospholipid in mammalian cell membranes, and as such, serves an important structural role. Thus a tight regulation of biosynthetic and catabolic pathways is necessary to maintain PC homeostasis. In some cell types, PC levels appear to be maintained by the opposing actions of CTP:phosphocholine cytidylyl transferase, the rate-limiting step in the de novo pathway for PC biosynthesis, and a BEL-sensitive iPLA$_2$,

most likely iPLA$_2$-VIA [11]. Overexpression of CTP:phosphocholine cytidylyl transferase in several cell types increases PC synthesis, yet PC mass does not augment due to increased catabolism via iPLA$_2$ deacylation [70,71].

Barbour et al. [70] made the key observation that iPLA$_2$-VIA activity and mass were up-regulated in response to increased PC synthesis by CTP:phosphocholine cytidylyl transferase over-expression. Subsequent work demonstrated that iPLA$_2$-VIA is a cell-cycle regulated enzyme [60,72]. The mechanism for cellular regulation of iPLA$_2$-VIA under these circumstances was also addressed [60]. In proliferating T cells, highest iPLA$_2$-VIA activity is observed at the G_2/M phase, and treatment with BEL or a specific antisense oligonucleotide results in marked suppression of cell division [72]. Similar results have been observed in CHO-K1 cells [60]. In these cells, maximal iPLA$_2$ activity is found during G_2/M, and late S phases, and lowest is at the G_1/S transition and early S phase [60]. As expected, accumulation of PC correlates with decreased iPLA$_2$ activity, and changes in iPLA$_2$-VIA enzyme activity during the cell cycle were found to be due to cell-cycle-regulated expression of inactive iPLA$_2$-VIA splice variants which interfere with the normal oligomerization of iPLA$_2$-VIA [60].

Collectively, these results indicate that alterations in the activity and/or expression of iPLA$_2$-VIA exert profound effects on phospholipid metabolism that affect cell growth. It should be noted however, that this iPLA$_2$-VIA role in regulating membrane phospholipid metabolism may not be a general one. There are cellular systems where iPLA$_2$-VIA appears not to act as a major controller of PC degradation by deacylation [29,73]. In these systems, other PLA$_2$ enzymes might compensate for iPLA$_2$-VIA.

5.2. Eicosanoid metabolism

The eicosanoids are a family of bioactive compounds that derive from the enzymatic oxygenation of AA. A key role of eicosanoids in pathophysiology is the triggering of the inflammatory reaction, and understanding the biochemical pathways involved in eicosanoid generation during inflammation has been greatly aided by the generation of cPLA$_2\alpha$ null mice [74,75]. The inflammatory response of these animals is compromised by the lack of cPLA$_2\alpha$, highlighting the central role of this enzyme in eicosanoid biosynthesis during innate immunity and inflammation [46,76,77].

In keeping with the above, studies in phagocytic cells, which are cells typically involved in inflammatory reactions, have generally shown that the selective AA mobilization response and ensuing eicosanoid metabolism triggered by both receptor-directed and soluble agonists is insensitive to BEL, which rules out a role for iPLA$_2$-VIA in the process. Examples include PAF- and zymosan-stimulated P388D$_1$ macrophages (MAB clone) [25,78], H_2O_2-stimulated mesangial cells [66], oxidized lipoprotein-stimulated peritoneal macrophages [79], formyl peptide-stimulated human neutrophils [80], and concanavalin-A- and calcium-ionophore-stimulated U937 monocytes [30,81].

Notwithstanding, other studies have provided data to indicate that under certain conditions, AA mobilization and attendant eicosanoid production may also be under the control of iPLA$_2$-VIA. BEL has been shown to strongly blunt AA mobilization in nitric-oxide-treated RAW261.7 macrophages [82] and calcium-ionophore-stimulated neutrophils [83], suggesting the involvement of a BEL-sensitive iPLA$_2$. However, owing to BEL unspecificity in cells [6,15], these data need to be substantiated by more direct approaches. More recently, two other studies using both BEL and specific antisense oligonucleotides have provided stronger evidence for a role of iPLA$_2$-VIA in AA mobilization and prostaglandin production in phagocytic cells. iPLA$_2$-VIA inhibition by either BEL or antisense reduces AA mobilization and prostaglandin production in zymosan-stimulated P388D$_1$ macrophages (uncloned ATCC cells) [37], and in interferon-γ-primed U937 cells stimulated via cross-linking of FcγRI [55]. Interestingly, in the latter study, the effects of PAF were studied as well and found to be mediated by cPLA$_2\alpha$, not iPLA$_2$-VIA [55], raising the possibility that multiple AA mobilization mechanisms regulated by distinct PLA$_2$ effectors co-exist within a single cell type. Activation of either effector would depend on cell type and stimulus. The possibility also exists, that under certain stimulation conditions, cross-talk exists between iPLA$_2$-VIA and cPLA$_2\alpha$, so that inhibition of the former might affect the activity of the latter. A possible cooperation between these two enzymes in AA mobilization has been suggested by the recent studies of Atsumi et al. [61,62].

Eicosanoids are involved in the regulation of many other biological functions in addition to innate immunity and the inflammatory reaction. Thus, the involvement of iPLA$_2$-VIA in prostaglandin production in cells not of leukocyte origin has also been profusely investigated. A striking feature of iPLA$_2$-VIA is its lack of specificity for the fatty acid present at the *sn*-2 position of phospholipids, the enzyme being able to hydrolyze practically any fatty acid, including acetic acid or oxidized fatty acids [15]. Therefore, it would be expected that true activation of iPLA$_2$-VIA results in a significant release of various fatty acids in addition to AA. This appears to be so in several instances [48,61,62,65].

In iPLA$_2$-VIA-transfected HEK293 cells, calcium-ionophore-induced AA release is greatly increased, and the resultant fatty acid is converted to prostaglandins preferentially via cyclooxygenase-1 [41,84]. Interestingly, the response to interleukin-1β is not similarly augmented, highlighting the existence of multiple mechanisms to effect the AA release within a single cell [41]. Increased fatty acid release and prostaglandin production in response to calcium ionophore in iPLA$_2$-VIA-transfected cells is a very striking result, and suggests that under some conditions, the enzyme might be regulated by Ca^{2+} or Ca^{2+} -dependent factors (see

Sections 4.1 and 4.8). Note, however, that forced overexpression of $iPLA_2$-VIA in cultured cells may not reflect the true in vivo regulation of this enzyme. As a matter of fact, the AA response to Ca^{2+} ionophores in cells expressing normal $iPLA_2$-VIA levels has been generally found to be insensitive to BEL [30,81,85].

In pancreatic islets, $iPLA_2$-VIA has been suggested to mediate AA release and prostaglandin E_2 production in response to D-glucose plus the muscarinic receptor agonist carbachol [86]. These responses are enhanced by $iPLA_2$-VIA overexpression and ablated by BEL [42,86,87], and are thought to be instrumental in insulin secretion (see Section 5.4).

5.3. Apoptosis

A role for $iPLA_2$-VIA in apoptosis was first suggested by Atsumi et al. [61,62] in U937 promonocytes treated with fas ligand or tumor necrosis-α plus cycloheximide. Under these conditions, cells undergo apoptosis and release various fatty acids in an $iPLA_2$-VIA-mediated manner [61,62]. U937 cell apoptosis was also found to be associated with cleavage of the enzyme by caspase-3, a protease that is central to execution of certain types of apoptosis. As a result of this cleavage, $iPLA_2$-VIA loses its N-terminal region and becomes more active, thus accelerating membrane phospholipid destruction [62]. Furthermore, inhibition of $iPLA_2$-VIA by MAFP decreased early apoptosis although it has no effect at longer time periods [62]. Similar data were obtained by Pérez et al. [43] in H_2O_2-induced U937 cell apoptosis. In this study, $iPLA_2$-VIA overexpression was found to increase membrane phospholipid hydrolysis and augment the rate of apoptosis [43]. Importantly however, inhibition of $iPLA_2$-VIA activity by either MAFP or an antisense oligonucleotide did not prevent apoptosis, suggesting that although $iPLA_2$-VIA does participate in membrane phospholipid destruction during apoptosis, it is not absolutely required for the apoptotic process to fully develop [43]. It was suggested that $iPLA_2$-VIA may be involved in providing accessory signals, namely clearance or attraction signals [88], that are triggered along with the destructive process itself.

That $iPLA_2$-VIA plays a central role in generating the attraction signals that bring professional phagocytes in contact with the dying cell has recently been demonstrated by Lauber et al. [63]. In this study, lysoPC secreted by apoptotic cells was identified as a potent chemoattractant for phagocytes, and this metabolite was produced by caspase-3-cleaved $iPLA_2$-VIA fragments of 26 and 32 kDa [63].

Evidence for $iPLA_2$ involvement in generating signals for the optimal clearance of apoptotic cells by phagocytes, the so-called “eat me” signals, has also been provided [89]. Exposure of $iPLA_2$-derived lysoPC on the surface of apoptotic T cells is thought to facilitate binding of IgM and complement factors, leading to their eventual recognition and clearance by phagocytes [89]. It should be indicated that in this study, $iPLA_2$ involvement was ascertained only by the use of BEL, and thus it is not clear at this time whether $iPLA_2$-VIA, $iPLA_2$-VIB, both, or even another unidentified phospholipase are involved in the process.

Involvement of $iPLA_2$-VIA in apoptosis of INS-1 insulinoma cells induced by thapsigargin has recently been documented [90]. Overexpression of $iPLA_2$-VIA augmented the rate of apoptosis, and BEL suppressed it [90]. Apoptosis under these conditions was associated with the caspase-3-catalyzed cleavage of $iPLA_2$-VIA to a 62-kDa fragment that associated with the nucleus [90]. RNA silencing experiments in PC12 cells have also demonstrated translocation of $iPLA_2$-VIA to the nucleus during caspase-independent hypoxic cell death leading to nuclear shrinkage [41].

5.4. Secretion

PLA_2 enzymes can affect membrane rearrangement events in two different ways. In the first place, they could function indirectly to stimulate or inhibit signal transduction pathways through the generation of signaling molecules. Secondly, PLA_2s could also directly affect membrane structure and function by promoting the accumulation of lysophospholipids and free fatty acids, both of which can stimulate the fusion of biological membranes by perhaps increasing local areas of membrane fluidity or promoting non-bilayer structures that would facilitate bilayer mixing. In recent years, a number of reports have linked $iPLA_2$-VIA activity to secretion of a number of proteins by either of these mechanisms.

Accumulating evidence suggests that glucose-stimulated insulin secretion in pancreatic β-cells and related cell lines is associated with increased hydrolysis of phospholipids [29,86]. Involvement of $iPLA_2$-VIA in the process was suggested by initial observations that BEL completely suppressed both phospholipid hydrolysis and insulin secretion [86]. These findings were later extended by studies utilizing INS-1 cells transfected with $iPLA_2$-VIA [42,87]. Mild improvement in glucose responsiveness but robust insulin secretion in response to cAMP-elevating agents was evident in the $iPLA_2$-VIA-overexpressing cells [42]. Intriguingly, the cAMP-elevating agents induced translocation of the enzyme to the nuclear region [42,87]. In this system, AA, acting as a signaling molecule, is postulated to mediate the $iPLA_2$-VIA actions on insulin secretion. Very recently, a report suggests that the $iPLA_2$-VIA involved in this process may not be the classical 85 kDa enzyme, but a novel 70 kDa closely related form, perhaps derived from proteolytic processing of the former [64].

Treatment of U937 cells with an $iPLA_2$-VIA antisense oligonucleotide produced cells with diminished levels of lysoPC and impaired lysozyme secretory responses to phorbol myristate acetate [91]. AA mobilization in the $iPLA_2$-VIA-deficient cells, a process that is mediated by $cPLA_2\alpha$, was unchanged [91]. Reconstitution experiments

revealed that lysoPC, but not other lysophospholipids or fatty acids, restored lysozyme secretion [91]. From these data it was concluded that $iPLA_2$-VIA-mediated phospholipid fatty acid recycling of membranes and concomitant generation of lysoPC is important for full secretion to take place. $cPLA_2\alpha$, on the other hand, appears not to play a role [91].

5.5. Chemotaxis

$iPLA_2$-VIA may be implicated in the chemotactic response of monocytes to monocyte chemoattractant protein-1 [36]. This response appears to involve $cPLA_2\alpha$ and $iPLA_2$-VIA, since specific inhibition of both enzymes by oligonucleotide antisense technology blocks it [36]. Lysophosphatidic acid completely restored MCP-1-stimulated migration in $iPLA_2$-VIA-deficient monocytes, although it was without effect in restoring migration in $cPLA_2\alpha$-deficient monocytes. Conversely, AA fully restored migration of $cPLA_2\alpha$-deficient monocytes, while having no effect on the $iPLA_2$-VIA-deficient monocytes. Additional studies revealed that neither enzyme appeared to be upstream of the other, suggesting that $iPLA_2$-VIA and $cPLA_2\alpha$ represent parallel regulatory pathways [37].

5.6. Regulation of gene expression

Several lines of evidence suggest that an $iPLA_2$-like activity may be involved in the transcriptional regulation of certain genes [51,92,93]. However, the available evidence linking $iPLA_2$ to gene expression has been obtained with BEL studies. Thus it is not possible at this time to define whether $iPLA_2$-VIA, $iPLA_2$-VIB, both or neither are involved in the above described events. An as yet undefined BEL-sensitive $iPLA_2$ might also be responsible for the actions described in this Section.

Double-stranded RNA, which accumulates at various stages of viral replication, appears to play a primary role in the activation of the antiviral response in virally infected cells. The antiviral response of macrophages includes the expression of several proinflammatory cytokines such as interleukin-1α and 1β as well as inducible nitric oxide synthase [51]. In murine macrophages and RAW264.7 macrophage-like cells, BEL is reported to prevent double-stranded RNA- and virus-induced expression of inducible nitric oxide synthase [51]. Possible effects of BEL on proteases or phosphatidate phosphohydrolase were ruled out and, interestingly, methyl lysoplasmenyl choline, a lysoPC analog, partially overcame the inhibitory effects of BEL on nitric oxide expression [51].

In another study, long-term incubation of cardiac myocytes with interleukin-1β was found to stimulate nitrite production, inducible nitric oxide synthase, AA release, and prostaglandin E_2 production, and all the responses were inhibited by BEL [92]. However, interleukin 1β decreased the expression of an 80–85 kDa $iPLA_2$ (most likely $iPLA_2$-VIA) by approx. 50%, as assessed by immunoblot, suggesting that the BEL-sensitive step in this system may not be $iPLA_2$-VIA.

In human monocytes, an $iPLA_2$ has been suggested to play a central role in controlling the processing of pro-interleukin-1β [93]. In these cells, nigericin-induced potassium leakage leads to acceleration of interleukin 1β maturation and glycerophosphocholine formation, and both of these actions are blocked by BEL. Possible contribution of phosphatidate phosphohydrolase to BEL effects was excluded, but BEL inhibition was not overcome by exogenous supplementation with lysoPC [93].

5.7. Cardiac ischemia

Accelerated phospholipid catabolism is known to occur early after the onset of myocardial ischemia. McHowat et al. [94], reported that hypoxia increases a membrane-associated BEL-sensitive $iPLA_2$ activity in isolated ventricular myocytes, resulting in increased arachidonic acid release and lysophospholipid accumulation, both of which could contribute to ischemic heart disease. The molecular identity of the $iPLA_2$ enzyme responsible for these effects was not established [94].

Using heart-specific $iPLA_2$-VIA transgenic mice, Mancuso et al. [95] have provided strong evidence to suggest that acute cardiac ischemia activates $iPLA_2$-VIA in intact myocardium and that the ensuing phospholipid hydrolysis can result in lethal malignant ventricular tachyarrhythmias. Coronary artery occlusion in Langendorff perfused hearts from these mice resulted in the massive release of various fatty acids, and the accumulation of lysophosphatidylcholine in ischemic zones. These effects could be prevented by adding BEL just minutes prior to induction of ischemia [95].

5.8. Calcium entry

Agonist-induced depletion of intracellular Ca^{2+} stores is known to activate store-operated channels and capacitative Ca^{2+} entry [96]. How the filling state of Ca^{2+} stores is communicated to the plasma membrane remains largely unknown. One possibility is that a diffusible mediator is generated as a consequence of Ca^{2+} loss from intracellular compartments and traverses cytosol to interact with plasma membrane targets. Identification of the molecular nature of such a diffusible mediator, usually referred to as calcium-influx factor, remains elusive [96].

The first indications of a possible link between $iPLA_2$-VIA and calcium signaling were provided by work from the Gross [97] and Turk [98] laboratories, which reported that depletion of intracellular Ca^{2+} stores results in an increased $iPLA_2$-mediated phospholipid hydrolysis and accumulation of free fatty acids including AA in rat smooth muscle cells and pancreatic islets. However, $iPLA_2$ involvement in these studies was inferred only from the use of BEL, and the biological significance of these results has remained unclear.

By using a variety of techniques to block cellular iPLA$_2$-VIA in cells, Bolotina and co-workers recently described the requirement of this enzyme for activation of store-operated calcium channels and capacitative Ca^{2+} influx in a variety of cell types, including vascular smooth muscle cells, Jurkat T-lymphocytes, platelets and rat basophilic leukemia cells [38]. In subsequent work, a novel mechanism was proposed for store-operated Ca^{2+} signaling in which iPLA$_2$-VIA plays a central role [99]. According to these authors, production of calcium-influx factor subsequent to intracellular Ca^{2+} store depletion would act to displace calmodulin from iPLA$_2$-VIA, leading to its activation and the production of lysophospholipids, which in turn would activate the store-operated channels and capacitative Ca^{2+} influx. Upon refilling of the stores and cessation of calcium-influx factor production, calmodulin would reassociate with iPLA$_2$-VIA, thereby inhibiting it. This would terminate the activity of store-operated channels and capacitative calcium influx [99].

6. Concluding remarks

In this article we have reviewed the ample evidence currently available on the cellular regulation and signaling functions of iPLA$_2$-VIA. Homeostatic functions of this enzyme had previously been recognized. Furthermore, owing to its capacity to inactivate PAF, iPLA$_2$-VIA may also serve to terminate signaling. Thus iPLA$_2$-VIA appears to function as a multi-faceted enzyme, with multiple functions in cells. The homeostatic, signaling, and catabolic roles of iPLA$_2$-VIA may not be mutually exclusive, and it is conceivable that the enzyme plays the three kinds of roles in certain cell types depending on maturation and stimulation conditions.

Acknowledgment

Work in our laboratories was supported by the Spanish Ministries of Science and Technology (Grant BMC2001-2244) and Education and Science (Grants BFU2004-01886/BMC and SAF2004-04676), the Education Department of the Autonomous Government of Castile and León (Grant CSI-4/02), and Fundació La Marató de TV3 (Grant 011232).

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