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Review

Calcium-independent phospholipase A_2 and apoptosis

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Abstract

Apoptosis or programmed cell death is associated with changes in glycerophospholipid metabolism. Cells undergoing apoptosis generally release free fatty acids including arachidonic acid, which parallels the reduction in cell viability. The involvement of cytosolic group IVA phospholipase $A_2\alpha$ ($cPLA_2\alpha$) in apoptosis has been the subject of numerous studies but a clear picture of the role(s) played by this enzyme is yet to emerge. More recently, the importance of lipid products generated by the action of a second phospholipase A_2, the group VIA calcium-independent phospholipase A_2 ($iPLA_2$-VIA) in apoptosis has begun to be unveiled. Current evidence suggests that $iPLA_2$-VIA-derived lysophosphatidylcholine may play a prominent role in mediating the chemoattractant and recognition/engulfment signals that accompany the process of apoptotic cell death, and gives possibility to the efficient clearance of dying cells by circulating phagocytes. Other lines of evidence suggest that perturbations in the control of free arachidonic acid levels within the cells, a process that may implicate $iPLA_2$-VIA as well, may provide important cellular signals for the onset of apoptosis.

Keywords: Phospholipase A_2; Apoptosis; Arachidonic Acid; Calcium-independent; Free fatty acid; Eicosanoid

1. Introduction

The phospholipase A_2 (PLA_2) superfamily constitutes a heterogeneous group of enzymes whose common feature is to hydrolyze the fatty acid at the *sn*-2 position of glycerophospholipids [1,2]. To date 22 genes, encoding structurally diverse PLA_2 proteins, have been identified in mammals. These enzymes have traditionally been classified into groups according to their nucleotide sequence. At the time of writing this review, 14 groups have been described, and many of them contain several members [1,2]. Alternatively, there is a much utilized classification that arranges the PLA_2 enzymes on the basis of biochemical commonalities. According to this criterion the PLA_2s have been grouped into four major families, namely the Ca^{2+}-dependent secreted enzymes ($sPLA_2$), the Ca^{2+}-dependent cytosolic enzymes ($cPLA_2$), the Ca^{2+}-independent cytosolic enzymes ($iPLA_2$), and the platelet-activating factor acetyl hydrolases (PAF-AH) (Table 1). Note that, despite its obvious usefulness, this classification is not devoid of significant caveats (e.g. despite being referred to as secreted enzymes, some $sPLA_2$s may indeed work intracellularly; Group IVC is referred to as $cPLA_2\gamma$, in spite of the fact that the enzyme is Ca^{2+}-independent, etc). The presence of PLA_2 enzymes in all cells and tissues underscores their key role in a number of physiological and pathophysiological processes, including the mobilization of free arachidonic acid (AA), and the regulation of membrane phospholipid homeostasis via deacylation/reacylation reactions.

Apoptosis is a type of cell death that does not involve an inflammatory response, and occurs in a tightly controlled manner. There are two main pathways through which apoptosis may occur. In the first pathway, apoptosis can be induced by a death receptor such as the tumor necrosis factor receptor; in the second one, apoptosis can be induced by cytotoxic agents that cause a mitochondria-dependent cascade of events [3,4]. In the death receptor-mediated apoptosis, a

Abbreviations: PLA_2, phospholipase A_2; AA, arachidonic acid; $iPLA_2$, calcium-independent phospholipase A_2 activity (undefined molecular identity); $iPLA_2$-VIA, Group VIA phospholipase $A_2\alpha$; $cPLA_2\alpha$, cytosolic Group IVA phospholipase $A_2\alpha$; BEL, bromoenol lactone; MAFP, methyl arachidonyl fluorophosphonate; lysoPC, lysophosphatidylcholine; CoA-IT, CoA-independent transacylase

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death-inducing signaling complex is formed by the death receptors, adapter proteins, and caspases (cysteinyl aspartate-specific proteases) such as caspase-8 and caspase-10 [3–6]. In the mitochondria-dependent apoptosis, major alterations in mitochondrial membrane function occur [4,7]. It has been found that mitochondrial membrane permeabilization is an early event of apoptosis [8], resulting in the release of proteins from the soluble intermembrane space in a nonspecific manner. Cytochrome c is one of these proteins and, once released to the cytosol, interacts with Apaf-1, and pro-caspase-9 to form a molecular caspase activation complex, the apoptosome, where caspase-9 is in its active form [9]. Caspase-8, caspase-10 and caspase-9 are believed to be the initiator caspases at the top of the caspase signaling cascade that culminates with the cleavage and activation of caspase-3, the principal effector caspase [6].

Apoptosis is strikingly associated with changes in glycerophospholipid metabolism, resulting in the accumulation of lysophospholipids and various fatty acids, including AA [10]. Thus, the proposal was made that a PLA_2 is critically involved in cellular signaling leading to apoptotic cell death [10]. Given that $cPLA_2\alpha$ (Group IVA PLA_2), an AA-specific enzyme, is the major effector of AA release in mammalian cells, much attention has been focused on this particular enzyme. Currently, there is no clear consensus as to the precise role of $cPLA_2\alpha$ in apoptosis. Since strong evidence both in favor [11–14] and against [15–17] the involvement of $cPLA_2\alpha$ in apoptosis has been put forward, the role of this enzyme in apoptosis may be restricted to certain instances where particular stimuli and/or cell types are involved. In cells exposed to stimuli that promote non-apoptotic forms of cell death, $cPLA_2\alpha$ may act to protect the cells from injury [18–20].

Recent data have implicated another PLA_2 form, the Ca^{2+}-independent Group VIA enzyme, or $iPLA_2$-VIA, in cells induced to undergo apoptosis under various conditions [16,21–27]. Very interestingly, the bioactive product generated by $iPLA_2$-VIA may not only be free AA or other fatty acid, but also lysophosphatidylcholine (lysoPC). Moreover, the involvement of $iPLA_2$-VIA in apoptosis may not be restricted to a mere hydrolytic role, but the enzyme may also be exquisitely involved in the generation of the accessory signals (attraction –*find me*–, and recognition/engulfment –*eat me*–) that accompany the apoptotic process itself (Fig. 1). In this review we focus on several aspects of the involvement of $iPLA_2$-VIA in apoptotic events, from specific roles in providing accessory signals via lysoPC production to more general ones, as a participant in the homeostatic mechanisms of fatty acid deacylation/reacylation reactions in resting cells.

Table 1
PLA_2 classification according to biochemical properties

Family	Group types comprised	Calcium requirement	Cellular localization
$sPLA_2$	I–III, V, IX–XIV	Yes	Extracellular
$cPLA_2$	IV	Yes	Intracellular
$iPLA_2$	VI	No	Intracellular
PAF-AH	VII, VIII	No	Extra and intracellular

PAF-AH, platelet-activating factor acetylhydrolase.

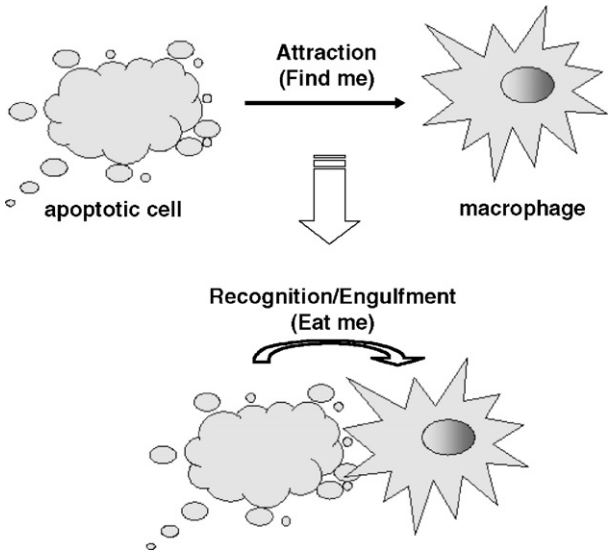

Fig. 1. Accessory signals for apoptotic cell clearance. The apoptotic cell generates signals that attract phagocytic cells (*find me* signals). Once in contact, the phagocyte recognizes the apoptotic cell as such and engulfs it (*eat me* signals).

2. $iPLA_2$-VIA role in apoptosis

$iPLA_2$-VIA is a cytosolic 85–88 kDa enzyme that shows no fatty acid selectivity. Distinguishing features of this enzyme include the presence of 7–8 ankyrin-like domains in the N-terminal half of the protein, the existence of multiple splicing variants, and the presence of multiple consensus sites for caspase cleavage (Fig. 2). A complete information on the structure and general biological functioning of $iPLA_2$-VIA and related enzymes falls out of the scope of this article, but thorough reviews on the topic are available, and the reader is kindly referred to these [28–32].

$iPLA_2$-VIA appears to play a significant role in maintaining the homeostatic levels of lysophosphatidylcholine (lysoPC) in resting cells [29,30]. Acute inhibition of the enzyme by the selective inhibitor bromoenol lactone (BEL) or antisense oligonucleotides results in marked decreases of the steady-state level of cellular lysoPC. The magnitude of this decrease appears to be dependent on cell type, ranging from 50–60% in phagocytic cells to 20–25% in rat pancreatic islet cells and uterine stromal cells [33–36]. Since lysoPC is the main acceptor of free AA for its incorporation into phospholipid pools [10], decreases in lysoPC levels as due to acute $iPLA_2$-VIA inhibition frequently result in decreases of the initial rate of AA incorporation into phospholipids [29,33,34]. Thus, $iPLA_2$-VIA has been implicated in phospholipid fatty acyl chain deacylation/reacylation reactions (i.e. the Lands cycle) in certain cells, notably phagocytes (see Section 4, below). It should be stressed however that, in addition to the contribution of $iPLA_2$-VIA to cellular lysoPC, the cells may possess other mechanisms to generate and maintain the appropriate levels of

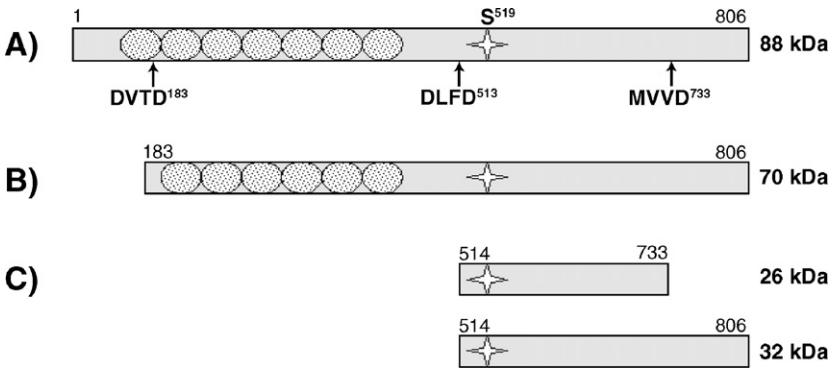

Fig. 2. Schematic representation of the iPLA$_2$-VIA truncated forms generated after caspase cleavage of native iPLA$_2$-VIA. (A) Full length iPLA$_2$-VIA (splice variant 2). Ankyrin repeats at the N-half of the molecule are represented as circles, and the catalytic Ser^{519} is shown as a star. (B) The 70 kDa fragment was identified by Atsumi et al. [16,21]. (C) The fragments of 26 and 32 kDa were identified by Lauber et al. [23].

lysophospholipid acceptors. Recent data in cells lacking iPLA$_2$-VIA by genetic ablation [37] found no evident alterations in the Lands cycle, suggesting that these cells may have adapted to the loss of iPLA$_2$-VIA by utilizing alternative routes for lysoPC generation. These results stress the importance of iPLA$_2$-VIA-regulated processes during normal physiological conditions, and the necessity to identify all the enzymes that contribute to the whole *sn-2* phospholipolytic activity of cells.

The first indications for a role of iPLA$_2$-VIA in apoptosis were provided by Atsumi et al. [16, 21] in their studies with U937 promonocytes treated with fas ligand or tumor necrosis factor α plus cycloheximide. Apoptosis induced by these agents was accompanied by the iPLA$_2$-VIA-mediated cleavage of membrane phospholipids and the subsequent release of various fatty acids, including AA. Caspase-3-mediated cleavage of the enzyme was detected, yielding a truncated enzyme of ~ 70 kDa lacking most of the first ankyrin repeat (Fig. 2). This cleavage resulted in increased iPLA$_2$ functioning, although changes in the specific activity of the enzyme were not detected. Interestingly caspase-3 cleavage of cPLA$_2$α was also detected under these conditions, although in this case cleavage resulted in inactivation of the enzyme [16,21].

In studies with insulinoma cells exposed to thapsigargin to induce apoptosis, Turk and co-workers [24] observed that overexpression of iPLA$_2$-VIA accelerates membrane hydrolysis and the extent of apoptosis. In contrast, inhibition iPLA$_2$-VIA by treating the cells with low micromolar BEL concentrations prevented thapsigargin from inducing apoptosis in these cells. In analogy with the results by Atsumi et al. [16,21], thapsigargin-induced apoptosis of insulinoma cells was found to result in the cleavage of iPLA$_2$-VIA by caspase-3 to a presumably more active 62-kDa fragment that associated with the nucleus [24].

Collectively, the above studies provide strong support for a general role for iPLA$_2$-VIA in apoptosis induced by different agents. Accordingly, iPLA$_2$-VIA might participate just by merely increasing membrane damage and, alternative or additionally, by providing bioactive lipid metabolites involved in apoptotic signaling cascades or apoptosis-related events. In studies with U937 monocytes exposed to hydrogen peroxide, an oxidative stress that causes apoptosis, Pérez et al. [26] found that, in agreement with the aforementioned data in insulinoma cells [24], overexpression of iPLA$_2$-VIA significantly increased the rate of apoptosis. Unexpectedly however, abrogation of U937 cell iPLA$_2$ activity by the inhibitor methyl arachidonyl fluorophosphonate (MAFP) or a specific antisense oligonucleotide did not decrease the extent of apoptosis at long incubation times (20–24 h), a finding that is in agreement with the results of Atsumi et al. [16,21]. Collectively, these data suggest that although iPLA$_2$-VIA-mediated phospholipid hydrolysis does occur during apoptosis, iPLA$_2$-VIA may actually be dispensable for the apoptotic process to occur. Thus, beyond a mere destructive role, iPLA$_2$-VIA may likely play other roles during apoptosis. The generation of accessory signals such as those of attraction (*find me*) and/or clearance (*eat me*) [38,39] has recently been identified as a preeminent role for iPLA$_2$-VIA during apoptosis.

Efficient clearance of apoptotic cells is a critical process for tissue homeostasis, and is carried out by phagocytic cells. The process is initiated by the display of *eat me* signals on the surface of the dying cells. Thus, *eat me* signals represent markers for phagocytes to identify and engulf dying cells. Different types of *eat me* signals have been identified, including new molecules that appear on the surface, surface molecules that undergo modifications, and indirect signals generated by interaction of serum components with the apoptotic cell surface [38–44]. With regard to the latter kind of *eat me* signals, Kim et al. [22] demonstrated that apoptotic cells generate a surface epitope recognized by natural IgM antibodies which results in complement activation and phagocytosis. The epitope recognized was the phosphoryl choline moiety of lysoPC [22]. Interestingly, enhanced IgM binding could be suppressed by the unspecific iPLA$_2$ inhibitor BEL, making it likely that lysoPC was being generated by an iPLA$_2$, although the precise molecular identity of the iPLA$_2$ form involved was not determined [22].

In our recent studies on hydrogen peroxide-induced apoptosis of U937 cells, we have found that a functionally active iPLA$_2$-VIA enzyme within the apoptotic cells is required for them to be efficiently recognized and engulfed by macrophages [26]. By either blunting or increasing the

levels of iPLA$_2$-VIA in the apoptotic cells, we have found a correlation between iPLA$_2$-VIA activity and the extent of phagocytosis of the dying cells by the macrophages, suggesting that a metabolic product of iPLA$_2$-VIA is involved in the process. We have identified this metabolite as lysoPC. Importantly, it is the lysoPC that is generated specifically by iPLA$_2$-VIA that functions as an *eat me* signal, since abrogation of the one other phospholipase A$_2$ present in the cells, cPLA$_2\alpha$, has no discernible effect [26]. These studies were conducted in the absence of serum in the incubation medium, making it likely that lysoPC was being directly recognized by the macrophages. In support of this view, phagocytosis of dying cells by macrophages could be inhibited by pre-incubating the macrophages with exogenous lysoPC. That lysoPC on the surface of the dying cell is directly [26] or indirectly [22] recognized by the phagocyte may probably depend on cell type and apoptotic stimulus used, and it is also possible that both types of recognition co-exist during phagocytosis.

The role of iPLA$_2$-VIA-derived lysoPC in the generation of *eat me* signals for phagocytes is striking in view of work by Lauber et al. demonstrating that lysoPC may also function as an attraction signal [23]. Conditioned media removed from apoptotic cells was found to induce migration of monocytic cell lines and primary human macrophages, and the chemotactic factor responsible for this effect was unequivocally identified as lysoPC. Importantly, lysoPC was found to be produced by caspase-3-cleaved iPLA$_2$-VIA fragments of 26 and 32 kDa [23] (Fig. 2).

Collectively, these studies highlight a fundamental role for iPLA$_2$-VIA and its by-product lysoPC in mediating the attraction of macrophages by apoptotic cells, and the subsequent recognition and engulfment of the latter by the former. An important molecular aspect that remains to be clarified is the mechanism of activation of the iPLA$_2$-VIA truncated form(s) that are produced during apoptosis. Atsumi et al. [21] failed to detect an increase of the iPLA$_2$ specific activity after caspase-3 cleavage, as determined by in vitro assay, and mutagenesis studies established that the ankyrin repeats are required to support iPLA$_2$-VIA enzymatic activity [45]. Yet, the iPLA$_2$-VIA truncated forms discovered by Lauber et al. [23] lack all of the ankyrin repeats and were clearly demonstrated to be biologically active. These observations raise very interesting hypotheses like e.g. certain apoptotic signals may render the membrane more susceptible to iPLA$_2$-VIA, or that a putative iPLA$_2$-VIA-regulatory protein may be modified during cell stimulation, leading to increased phospholipid hydrolysis and the accumulation of lysoPC. On the other hand, multiple mechanisms for iPLA$_2$-VIA activation have been described under general stimulation conditions [32]. Thus it is possible that mechanisms other than caspase-cleavage may also account for iPLA$_2$-VIA activation under apoptotic conditions. Likewise, whether lysoPC triggers specific signaling pathways in apoptotic cells that are different from those taking place under non-apoptotic activation conditions [46–49], is yet to be defined. It is anticipated that these molecular details will shortly be unveiled.

3. Induction of apoptosis by BEL

BEL is a member of a family of compounds known as haloenol lactones that were first described as suicide substrates of chymotrypsin and related serine proteases [50]. Later, this compound was found to potently inhibit myocardial iPLA$_2$ activity [51], and a purified iPLA$_2$ preparation from P388D$_1$ macrophages [52]. BEL is exquisitely selective for inhibition of iPLA$_2$-VIA versus other Ca^{2+}-dependent PLA$_2$ forms (including both sPLA$_2$ and cPLA$_2$ forms) when used in *in vitro* assays [51,53,54]. Due to this selectivity in vitro, BEL has been widely used in cellular studies as a specific tool to investigate iPLA$_2$ roles in cells. Unfortunately however, the selectivity of BEL when applied to cells is questionable, as it can inhibit a number of other key enzymes of lipid metabolism, including Mg^{2+}-dependent phosphatidate phosphohydrolase [35,55,56], cPLA$_2$ [45], iPLA$_2$-VIB [57], diacylglycerol lipase [58], and neuropathy target esterase (a lysophospholipase A) [59].

Relevant to the topic of this review, BEL has recently been found to induce apoptosis in some cell lines when applied at concentrations higher than 10 μM [60]. It is important to stress that BEL-induced apoptosis appears to be cell-type specific; some cell lines are affected and some others are not. The reasons for this differential sensitivity are not known. In the sensitive cells, long-term treatment with BEL (24 h) results in increased annexin-V binding to the cell surface and nuclear DNA damage, as detected by staining with both DAPI and propidium iodide. At earlier times (2 h), BEL induces the proteolysis of procaspase-9 and procaspase-3, and increases cleavage of poly(ADP-ribose) isomerase. These changes are preceded by variations in the mitochondrial membrane potential. All these effects are not mimicked by the general PLA$_2$ inhibitor MAFP or by treating the cells with a specific iPLA$_2$ antisense oligonucleotide, suggesting that this is not an iPLA$_2$-VIA-mediated effect. However, propranolol, a PAP-1 inhibitor, does reproduce the effects, suggesting that it is the inhibition of PAP-1 and not of iPLA$_2$ that is involved in BEL-induced cell death. Thus caution is advised when BEL is to be used in studies involving long incubation times, due to the capacity of the compound to induce apoptosis in a variety of cells.

4. Control of free arachidonic acid levels and the induction of apoptosis

One of the most widely studied functions of PLA$_2$ enzymes in cells is their ability to catalyze the hydrolysis of phospholipids in response to agonist stimulation to generate free arachidonic acid (AA) for eicosanoid biosynthesis [2]. Current evidence suggests that cPLA$_2\alpha$ is, by far and large, the major enzyme mediating AA mobilization in stimulated cells, with sPLA$_2$ family members playing accessory, often augmentating roles [2,61–63]. The participation of iPLA$_2$-VIA in stimulus-induced AA mobilization and attendant eicosanoid synthesis may be limited to a very specific set of experimental conditions. However, as discussed elsewhere [29, 30], iPLA$_2$-VIA may play important roles in modulating the small amounts

of free AA and other fatty acids that are continuously generated during resting conditions.

Since availability of free AA is in itself a rate-limiting factor for the formation of eicosanoids, the levels of this fatty acid must be tightly controlled by the cells. AA is an intermediate of a reacylation/deacylation cycle of membrane phospholipids, the so-called Lands pathway, in which the fatty acid is cleaved from phospholipid by PLA_2s and reincorporated by acyltransferases [5]. Whereas in resting cells reacylation dominates, in stimulated cells the dominant reaction is the deacylation. Nevertheless, increased AA reacylation during cellular activation is still very significant, as manifested by the fact that only a minor portion of the free AA released by PLA_2 is converted into eicosanoids, the remainder being effectively incorporated back into phospholipids [64].

AA incorporation into phospholipids is critically dependent on the availability of lysophospholipid acceptors, particularly lysoPC. In some cells, most notably phagocytes, the levels of lysoPC appear to be maintained by the continuing action of $iPLA_2$-VIA on cellular phospholipids [29,30,33,34]. Thus, a decrease in the activity of $iPLA_2$-VIA frequently results in the diminished production of lysoPC and hence in the inhibition of AA incorporation into phospholipids [29,30,33,34]. To be acylated into the sn-2 position of lysoPC, the AA needs to be in an active form, which is achieved by the enzyme-catalyzed linkage of the fatty acid to coenzyme A via a thioester bond. This critical step is catalyzed by the enzyme fatty acyl CoA ligase, or simply, arachidonoyl-CoA synthetase. Once in an active form, AA is incorporated into lysoPC by the action of CoA-dependent acyltransferases. Subsequently, AA is transferred from PC to certain lysophospholipids, particularly the ethanolamine lysophospholipids. Such a transfer is catalyzed by the enzyme CoA-independent transacylase (CoA-IT) [64]. Thus, for the AA to be efficiently incorporated into phospholipids, two kinds of lysophospholipid acceptors should be readily available (Fig. 3). The provider of the ethanolamine lysophospholipid acceptors for the CoA-IT reaction is assumed to be a PLA_2 [64], but its identity has not been ascertained [65,66].

Recent data have suggested that accumulation of free AA within the cells may constitute a signal for the induction of apoptosis. This includes the stimulated release of AA in response to external factors but, importantly, also to perturbations of the homeostatic mechanisms that control free AA availability in otherwise unstimulated cells [67].

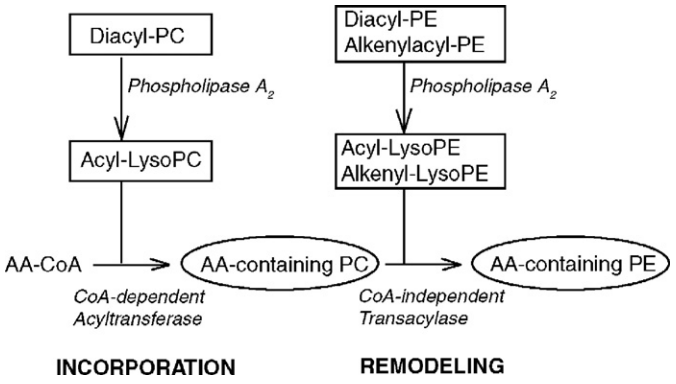

Fig. 3. AA incorporation into and remodeling among phospholipids. Adapted with permission from ref. [59] (© The Biochemical Society). PE, ethanolamine-containing glycerophospholipid; LysoPE, ethanolamine lysophospholipid.

The processes of phospholipid AA incorporation and remodeling have been described in detail in inflammatory cells, and to a much lesser degree in other cell types [64]. Inflammatory cells are endowed with highly efficient systems to maintain the levels of free AA at very low levels. Supplementation of these cells with exogenous AA generally results in the rapid incorporation of this fatty acid into membrane phospholipids. This process can be halted however by inhibitors of the AA reacylation pathway, resulting in the cells being exposed to high levels of free AA. In our recent study with human U937 promonocytes, manipulation of free AA levels was achieved by interfering with two major controlling steps of the phospholipid AA incorporation and remodelling pathway namely arachidonoyl-CoA synthetase activity, and CoA-IT. Incubation of the cells with exogenous AA plus inhibitors of either of these enzymes resulted in the accumulation of unesterified AA which correlated with induction of apoptosis [68]. The concentrations of AA that were able to induce apoptosis when combined with drugs inhibiting fatty acid incorporation and remodeling are in the low micromolar range. These concentrations are likely to be reached locally *in vivo* during an inflammatory process, and therefore may be of pathophysiological relevance.

Similar findings with regard to inhibition of CoA-IT have also been reported by Surette et al. [69] in HL60 promyelocytic cells and, regarding arachidonoyl-CoA synthetase, by Cao et al. in colorectal cancer cell lines [70]. Interestingly, in the latter study the mere addition of exogenous free AA was sufficient to induce apoptosis, and the effect could be blunted by overexpressing arachidonoyl-CoA synthetase, which acted to reduce free AA levels. These data suggest that the colorectal cancer cell lines may lack the highly efficient systems for AA incorporation into phospholipids that are present in inflammatory cells for the rapid attenuation of free AA levels [64]. Altogether, these findings have strongly suggested that it is the accumulation of unesterified intracellular AA that is inducing the apoptotic response. Clearly, much work will be needed to determine how increasing cellular free AA levels puts into motion the machinery leading to programmed cell death. In this regard, it is worth noting that AA at low micromolar concentrations is known to impact on a number of intracellular effectors and signaling pathways, including, e.g., the generation of apoptosis-inducing ceramides [12].

5. Concluding remarks

Currently available data indicate that $iPLA_2$-VIA is centrally involved in apoptosis induced by a variety of stimuli. $iPLA_2$-VIA not only functions to augment membrane damage by virtue of its hydrolytic activity, but also acts to generate lysoPC, a bioactive lipid signal that enables phagocytes to find, identify and engulf dying cells. On the other hand, AA another bioactive lipid that may be produced by $iPLA_2$-VIA is also known to induce apoptosis when its concentration inside the cells rises

above certain levels. Thus control of intracellular free AA levels, a process involving multiple enzymes including iPLA$_2$-VIA, may be crucial for cell survival.

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