# Control of Free Arachidonic Acid Levels by Phospholipases $A_2$ and Lysophospholipid Acyltransferases

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## Abstract

Arachidonic acid (AA) and its oxygenated derivatives, collectively known as the eicosanoids, are key mediators of a wide variety of physiological and pathophysiological states. AA, obtained from the diet or synthesized from linoleic acid, is rapidly incorporated into cellular phospholipids by the concerted action of arachidonoyl-CoA synthetase and lysophospholipid acyl transferases. Under the appropriate conditions, AA is liberated from its phospholipid storage sites by the action of one or various phospholipase $A_2$ enzymes. Thus, cellular availability of AA, and hence the amount of eicosanoids produced, depends on an exquisite balance between phospholipid reacylation and hydrolysis reactions. This review focus on the enzyme families that are involved in these reactions in resting and stimulated cells.

### Abbreviations

AA, arachidonic acid; ACS, acyl-CoA synthetase; PA, phosphatidic acid; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids, PI, phosphatidylinositol; $PIP_2$; phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PG, phosphatidylglycerol; $PLA_2$, phospholipase $A_2$; $cPLA_2\alpha$, group IVA cytosolic phospholipase $A_2\alpha$; $iPLA_2$, calcium-independent phospholipase $A_2$; $iPLA_2$-VIA, group VIA calcium-independent phospholipase $A_2$; $sPLA_2$, secreted phospholipase $A_2$; LPAAT, lysoPA:acyl-CoA acyltransferase; LPCAT, lysoPC:acyl-CoA acyltransferase; LPEAT, lysoPE:acyl-CoA acyltransferase; LPIAT, lysoPI:acyl-CoA acyltransferase; MBOAT, membrane bound O-acyltransferase; AGPAT, acyl glycerol phosphate acyltransferase.

## 1. Introduction

Arachidonic acid (5,8,11,14-eicosatetraenoic acid, ω-6) (AA) is an essential fatty acid that is obtained directly from dietary sources or indirectly, from conversion of linoleic acid. AA is the precursor of a large family of bioactive compounds called the eicosanoids, produced by oxygenation through cyclooxygenase and lipoxygenase pathways [1, 2]. Because of the potent biological actions of the eicosanoids cells keep this fatty acid at very low levels, by promoting its esterification into cellular lipids. As a matter of fact the availability of free AA is well described to constitute a rate-limiting step in the generation of eicosanoids by mammalian cells [3, 4]. In addition, free AA may also exert signaling functions by itself, e.g. as an inducer of apoptosis [5].

Under physiological conditions, AA is generally found esterified into the sn-2 position of glycerophospholipids, particularly choline glycerophospholipids (PC) ethanolamine glycerophospholipids (PE), and phosphatidylinositol (PI). The production of free AA is a highly regulated process that represents a balance between two competing reactions, namely, phospholipid deacylation by phospholipase $A_2$ ($PLA_2$) enzymes, and reacylation and transfer into various phospholipid pools by acyltransferases and transacylases [6]. Depending on the state of the cell (i.e. resting or activated) one kind of reaction will dominate over the other. Thus in resting cells reacylation dominates, and hence, the bulk of cellular AA is found in esterified form. In stimulated cells, the dominant reaction is the $PLA_2$-mediated deacylation, which results in dramatic releases of free AA that is now available for eicosanoid synthesis. However, under activation conditions AA reacylation is still very significant, as manifested by the fact that only a minor fraction of the AA released by $PLA_2$ is available for eicosanoid synthesis, and the

remainder is effectively incorporated back into phospholipids by acyltransferases [5, 6]. In this regard, various studies have shown that the rate of AA incorporation into cellular phospholipids is slightly increased following cellular stimulation [7-11]. Such an increase is generally thought to be important for the replenishment of the intracellular pools of AA being exhausted as a result of cellular stimulation [6]. However, increased influx of exogenous AA into phospholipids can also occur under conditions where no endogenous AA release occurs [12, 13], implying that this may actually be an independent process.

## 2. Regulation of AA Incorporation into Phospholipids

The pathways for AA incorporation into various classes of glycerophospholipids have been described in detail in various cells, particularly those involved in inflammatory reactions such as neutrophils and macrophages [6, 14]. Two distinct pathways appear to exist for the initial incorporation of AA. (Fig. 1) The first one is a high affinity pathway that incorporates low concentrations of AA into phospholipids via direct acylation reactions catalyzed by coenzyme A-dependent acyltransferases. This is thought to be the major pathway for AA incorporation into phospholipids under physiological conditions [6]; thus the $PLA_2$-dependent availability of lysophospholipid acceptors may constitute a critical regulatory factor [15, 16]. The second pathway operates under high levels of free AA, which may be pathophysiological, and leads to the incorporation of the fatty acid primarily via the *de novo* route for phospholipid biosynthesis, resulting ultimately in the accumulation of AA into triacylglycerols and diarachidonoyl phospholipids [6]. This "high-capacity, low affinity" pathway is thought to primarily operate after the high-

affinity deacylation/reacylation pathway has been saturated due to the high AA concentrations [6].

Once the AA has been incorporated into phospholipids, a remodeling process carried out by CoA-independent transacylase transfers AA from choline glycerophospholipids (PC) to ethanolamine glycerophospholipids (PE), in a process that generally takes several hours in primary cells but is strikingly fast in tumor cell lines, where it takes only minutes [17-20]. In inflammatory cells, a major consequence of the CoA-independent transacylase-driven remodeling reactions is that, despite PC being the preferred acceptor for exogenous AA, under equilibrium conditions AA is more abundant in PE than in PC [6, 14].

## 2.1. Lysophospholipid regulation of AA incorporation

For the efficient incorporation of AA into phospholipids, two kinds of lysophospholipid acceptors should be readily available in the cell. Lysophospholipids, particularly lysoPC, are needed for the initial incorporation of AA into phospholipids via the Lands pathway, and lysophospholipids are again required, particularly lysoPE, for AA remodeling between phospholipids via CoA-independent transacylation reactions (Figure 2) [15]. Given that AA preferentially incorporates into the sn-2 position of phospholipids, the lysophospholipid acceptors used for AA incorporation and remodeling are of the 2-lyso type, i.e. those produced by $PLA_2$s.

It is likely that several $PLA_2$ forms may contribute to the 2-lysophospholipid pool utilized for AA incorporation and remodeling and that their identity varies between cell types and tissues. In phagocytic cells, a significant part of the steady-state level of lysoPC

appears to be maintained by the continuing action of $Ca^{2+}$-independent Group VIA phospholipase $A_2$ (iPLA$_2$-VIA) on cellular phospholipids [21, 22]. Thus, a decrease in the activity of the iPLA$_2$–VIA frequently results in the diminished production of lysoPC and hence in the inhibition of AA incorporation into phospholipids [21, 22]

Earlier studies on the initial incorporation of AA into glycerophospholipids in mouse macrophages indicated that the process was essentially $Ca^{2+}$-independent [12], suggesting that the $PLA_2$ putatively responsible for generating lysophospholipid acceptors for AA incorporation would correspond to that of an iPLA$_2$-like enzyme [12]. Such an activity was later identified to belong to iPLA$_2$–VIA in studies carried out with murine P388D$_1$ macrophage-like cells [23, 24]. However, evidence has also been provided to indicate that iPLA$_2$-VIA does not serve this function in other cell types [25], suggesting that, like other iPLA$_2$-regulated processes, the involvement of iPLA$_2$-VIA in phospholipid AA incorporation may depend on cell type and, in particular, on the expression level of iPLA$_2$-VIA (i.e. how much the enzyme contributes to the steady-state lysophospholipid pool of a given cell). Based on studies of iPLA$_2$ inhibition by the inhibitor bromoenol lactone (BEL), the iPLA$_2$–VIA contribution ranges from ~90% in rat submandibular ductal cells [26], to 50-60% in phagocytic cells [16, 23, 24, 27-30], and to only 20-25% in rat uterine stromal cells [31]. Studies in rat pancreatic islets, where iPLA$_2$ inhibition by BEL does not result in diminished AA incorporation into phospholipids [25], have estimated that iPLA$_2$-VIA contributes to at least 20% of the steady-state lysophospholipid levels of these cells. Given that rat pancreatic islets maintain cellular lysophospholipid levels at high levels, it seems possible that the amount of lysophospholipid present in these cells even after iPLA$_2$ inhibition by BEL is

still high enough to account for a normal rate of AA incorporation into phospholipids. In agreement with this view, studies on AA incorporation utilizing cells overexpressing iPLA$_2$–VIA have indicated that the excess amount of lysophospholipid produced under those conditions does not increase the rate of fatty acid incorporation [16, 32, 33].

On the other hand, we believe that it is important to note that a slowed rate of AA incorporation into phospholipids due to diminished availability of lyso acceptors subsequent to iPLA$_2$ inhibition, does not necessarily imply that the profile or amount of AA-containing phospholipids may have to change under equilibrium conditions, as has been assumed in a number of papers. The distribution of AA among phospholipid classes ultimately depends on transacylation reactions that are essentially iPLA$_2$-independent, and do not change whether iPLA$_2$ is inhibited or not (see below).

Very few studies have focused on the PLA$_2$ enzyme providing lysophospholipid acceptors for AA remodeling reactions via CoA-independent transacylases. The nature of such a PLA$_2$ has been investigated in peripheral T lymphocytes [13] and U937 macrophages [15] by measuring the transfer of AA from PC to PE in the presence of different PLA$_2$ inhibitors. Inhibitors of group IVA cytosolic phospholipase A$_2\alpha$ (cPLA$_2\alpha$), and iPLA$_2$-VIA failed to exert any detectable effect on the transfer of AA from PE to PC in either cell type, raising the possibility that the PLA$_2$ implicated in this pathway might be an as yet undefined PLA$_2$. The $Ca^{2+}$-independent nature of the response suggests the involvement of an iPLA$_2$-like activity different from the group VIA enzyme. An iPLA$_2$ activity that is not inhibted by BEL and therefore is not a group VI enzyme, was recently identified in U937 macrophage-like cells [15, 34]. This activity appears to prefer PE as substrate, consistent with a presumed role in providing lysoPE

acceptors for transacylation reactions [15].

## 2.2. Acyl-CoA synthetases utilizing AA

The first enzymatic step for the incorporation of AA into phospholipids is catalyzed by the enzyme acyl-CoA synthetase (ACS), which activates the carboxyl group of AA by coupling a CoA moiety to it via a thioester linkage.

All enzymatically active ACSs contain at least two conserved amino acid sequence domains: a covalent AMP-binding domain (motif I) consisting of 10 residues highly conserved from bacteria to humans [35] and a 36-37-residue domain (motif II) containing a sequence that is thought to be essential for binding of the substrate [36]. The latter has been used to assign ACSs to subfamilies [35, 36]. Up to now, 26 different ACS isoforms, each encoded by a separate gene, have been identified in the genome of human cells [36], although 4 of them are still considered as candidates to exhibit ACS activity based on the presence of the two distinctive motifs (medium-chain ACS-2A, short-chain ACS-3, medium-chain ACS-5, and ACS family-4). Twenty-two of these ACSs are classified into 5 subfamilies considering the chain length of the fatty acid of their preferred acyl groups (short-chain ACSs, medium-chain ACSs, long-chain ACSs, very long-chain ACSs and “bubblegum” ACSs); the other four proteins do not belong to any subfamily and are denominated ACSF (ACS family) (Table 1). The ACS enzymes displaying some preference for AA are typically those of the ACSL family. Thus, a summary of the properties of the other ACS families is given below and the long-chain acyl-coenzyme A synthetase (ACSL) family is reviewed in more detail.

The family of short-chain acyl-coenzyme A synthetases (ACSs) are composed by three enzymes (ACSS1, 2 and 3) capable of activating acetate, propionate or butyrate. It has been described in bovine, murine and human tissues that this family uses mainly acetate as a substrate, presenting a weak selectivity for propionate or butyrate [37, 38].

The medium-chain acyl-coenzyme A synthetase family (ACSMs) consists of six enzymes, all localized almost exclusively in the mitochondrial matrix. These ACSs activate C4-10 fatty acids, although the selectivity can drastically differ between each isoform [39]. The metabolism of medium-chain fatty acids is poorly understood, but it is thought to play an important role in energy generation, given that the medium-chain fatty acids are probably generated from long- and very-long fatty acids by peroxisomal β-oxidation, and further degradation via mitochondrial β-oxidation after transportation into the mitochondrial matrix. [40].

The very long-chain acyl-coenzyme A synthetase family (ACSVLs) is composed of six membrane integral proteins (ACSVL-1 to -6) that are capable of activating long-chain, branched-chain and very long-chain fatty acids containing more than 22 carbons. Members of this family are also designated as fatty acid transporter proteins (FATPs), and are thought to be involved in translocation of long- and very long-chain fatty acids across the plasma membrane [41]. Thus, these proteins could play a dual role in the transport and esterification of their substrates, with the exception of ACSVL3/FATP3, which has been demonstrated not to exhibit fatty acid transport activity [42].

The first member of the "bubblegum" acyl-coenzyme A synthetases (ACSBGs) were originally discovered in the Drosophila mutant "*bubblegum*", characterized by neurodegeneration and high tissue levels of saturated very long-chain fatty acids [43].

Overexpression of human ACSBG1 led to the finding that this enzyme activates both long- and very long-chain fatty acids [35]. More recently, a second member (ACSBG2) has been located in murine and human testis and brainstem, showing a high degree of homology to ACSBG1 [44].

The long-chain acyl-coenzyme A synthetases (ACSLs) are the best characterized of the ACS families and play a key role in remodeling of membranes and *de novo* lipid synthesis. To date, five ACSL isoforms have been described in mammalian cells, ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6 [45], with at least two spliced transcript variants per isoform. Based on sequence homologies, the ACSL enzymes have been subdivided into two major groups, ACSL1/ACSL5/ACSL6 and ACSL3/ACSL4 [46]. ACSL1 was the first cloned human ACSL family gene [47]. Originally it was considered to be different from ACSL2 [48], but later it was found to be the same gene, which went on to be denominated ACSL1. As a consequence, the rat ACS2 gene was renamed *Acsl6*, because of its high homology with human ACSL6. A remarkable characteristic of ACSL1 is that the rodent protein is one residue longer than the human protein (699 and 698 amino acids, respectively). This enzyme is predominantly located in heart, liver and adipose tissue and uses a wide range of fatty acids, although with a slight preference for palmitic, oleic, and linoleic acids [49].

ACSL3 is one of two ACSL isoforms highly expressed in brain [50, 51]. It is located in the endoplasmic reticulum and lipid droplets [52]. ACSL3 presents a marked selectivity for AA and eicosapentaenoic acid over other unsaturated fatty acids, although its preference also for myristic acid and lauric acid makes this isoform less specific than ACSL4 with regard to AA and eicosapentaenoic acid.

ACSL4 shows close homology to ACSL3, sharing 68% of their amino acids. It is expressed predominantly in steroidogenic tissue and located in peroxisomes and mitochondrial membrane. With regard to substrate preference, murine and human cell ACSL4 utilizes AA and eicosapentaenoic acid with marked preference over all other fatty acids, indicating a critical function in AA metabolism [53, 54].

ACSL5 is the only ACSL located in the outer mitochondrial membrane, suggesting a preferential role in activating acyl groups for mitochondrial β-oxidation. ACSL5 is highly expressed in small intestine, and to a lesser extent in liver, and uses a wide range of saturated and unsaturated fatty acids [55].

Together with ACSL3, ACSL6 is the major ACSL expressed in brain. Murine and human ACSL6 show a clear preference for docosahexaenoic acid (22:6) and AA [56, 57]. The fact that this isoform presents a preference for the most abundant polyunsaturated fatty acids in brain suggests an important role in the synthesis of lipids in neuronal membranes, which experience a rapid phospholipid turnover. In addition, ACSL6 is also present in the plasma membrane of mature erythrocytes, where it activates long-chain fatty acids for remodeling of lipids and acylation of proteins [58].

In addition to the differences in fatty acid preference, subcellular location and tissue distribution, ACSLs also show different responsiveness to pharmacological inhibitors. It has been shown that ACSL1, ACSL3 and ACSL4 are sensitive to triacsin C, while ACSL5 and ACSL6 activity are not [51, 56, 59]. Moreover, thiazolidinediones, a type of oral insulin-sensitizing agents formerly used to treat type 2 diabetes, can specifically inhibit the activity of ACSL4 [56, 60]. The initial experiments with these inhibitors suggest that the various ACSL isoforms can drive acyl-CoAs to different lipid

metabolism pathways with some selectivity. It was described in human fibroblasts that triacsin C inhibits the *de novo* synthesis of diacylglycerol, triacylglycerol, cholesterol esters and phospholipids from glycerol but not the reacylation of fatty acids into phospholipids [61]. These findings have led to the suggestion that triacsin C-sensitive ACSLs supply acyl-CoA for the *de novo* synthesis of glycerolipids, whereas isoforms resistant to triacsin C would be involved in reacylation of phospholipids and β-oxidation.

Studies in human monocyte-derived macrophages have indicated that rosiglitazone, a type of thiazolidinedione, inhibits the incorporation of oleic acid into diacylglycerol and triacylglycerol, but not into phospholipids, whereas triacsin C inhibits the partitioning of these fatty acids into all lipid classes [60]. These data confirm a role for ACSL4 in the partitioning of fatty acids into diacylglycerol and triacylglycerol, and suggest additional roles for ACSL1 and ACSL3. On the other hand, studies in rat demonstrate that ACSL5, a triacsin C-resistant form, is also implicated in activating acyl-CoA for the *de novo* synthesis of triacylglycerol [62].

## 2.3. Lysophospholipid Acyltransferases

Mammalian cells contain a number lysophospholipid acyltransferases that exhibit distinct acyl-CoA and lysophospholipid acceptor specificities. The recent availability of genomic information and sequence data has led to the identification of many lysophospholipid acyltransferases that may potentially be involved in AA recycling, either specifically or as part of a general function in homeostatic phospholipid metabolism.

Two families of lysophospholipid acyltransferase enzymes have been recognized, namely the membrane bound *O*-acyltransferase (MBOAT) family, and the 1-acyl-

glycerol-3-phosphate *O*-acyltransferase (AGPAT) family. [63-65]. While the MBOAT family comprises members specifically involved in the Lands cycle of phospholipid fatty acid remodeling, members of the AGPAT family are typically involved in the *de novo* pathway for phospholipid biosynthesis, but some members may also be involved in remodeling reactions.

The MBOAT family includes acyltransferases that can use not only lysophospholipids as acceptors, but also diacylglycerol, cholesterol or even a protein [63-65]. Only the MBOAT enzymes using lysophospholipids as acceptors will be considered in this review. Characteristic features of the MBOAT lysophospholipid acyltransferases include the existence of several membrane-spanning domains and a conserved His residue in a hydrophobic region that could constitute the catalytic site [66].

Members of the AGPAT family were first hypothesized to utilize lysoPA specifically as acceptor, and thus were classified as acyltransferases of the *de novo* phospholipid biosynthetic pathway. It was realized later that these enzymes possess broader substrate specificity being able to utilize other lysophospholipids such as lysoPC and lysoPE. Common structural features of AGPAT acyltransferases include the presence of four conserved domains (motifs I-IV) that are important for catalytic activity and substrate binding [67, 68].

### 2.3.1. Acyltransferases using lysoPA as acceptor

Three lysoPA acyltransferases have been cloned and characterized, namely LPAAT1 [69-71], LPAAT2 [69, 72] and LPAAT3 [73]. LPAAT1 and LPAAT2 may utilize several acyl-CoA as donors [74], and are expressed in a wide number of tissues. LPAAT3 shows

selectivity for AA and, interestingly, also possesses LPIAT activity [73].

### 2.3.2. Acyltransferases using lysoPC as acceptor

To date, three enzymes, called LPCAT1, LPCAT2, and LPCAT3 have found to utilize preferentially lysoPC as acceptor. LPCAT1 (also known as AGPAT9) [63, 64] was identified and characterized independently by two different groups in murine alveolar type II cells [75, 76]. LPCAT1 is highly expressed in lung, where it is suggested to play an important role in the synthesis of surfactant phospholipids, particularly dipalmitoyl glycerophosphocholine, the major component of pulmonary surfactant. Activity assays show high selectivity for medium-chain saturated acyl-CoAs (6:0-16:0) and lysoPC substrates, although the enzyme also displays significant activity towards lysoPA and lysoPG [76]. LPCAT1 appears to play an important role in the remodeling of PC in erythrocytes [77]. Recently it has been described that LPCAT1 is implicated in platelet-activating factor synthesis under $Ca^{2+}$ independent, non-inflammatory conditions [78]. Human LPCAT1 is also abundant in lung, and it seems to have the same properties as mouse LPCAT1. Other authors have described upregulation of human LPCAT1 in colorectal cancer adenocarcinomas. [79].

LPCAT2 has recently been cloned and characterized in mouse, and is believed to constitute the main enzyme involved in the formation of platelet-activating factor under inflammatory conditions [80]. The enzyme belongs to the AGPAT family, and is highly expressed in inflammatory cells, mainly in resident macrophages and casein-induced neutrophils. It shows marked preference for lysoPC. Using RAW264.7 cells overexpressing LPCAT2, it was found that under resting cell conditions the enzyme

shows activity for acetyl-CoA and, strikingly, for arachidonoyl-CoA. Under these conditions, the enzyme appears to have more affinity for arachidonoyl-CoA than for acetyl-CoA. However, upon receptor stimulation the acetyltransferase activity of LPCAT2 was found to be significantly increased, while the arachidonoyl-CoA acyltransferase was not [80].

LPCAT3, also known as MBOAT5, is expressed at high levels in all murine tissues, especially testis [81]. The enzyme shows selectivity for lysoPC, although it can also utilize lysoPE and lysoPS. As for fatty acyl donors, the enzyme utilizes AA and linoleic acid with preference over other fatty acids [81]. In humans, the enzyme is expressed at high levels in liver, pancreas and adipose tissue. In terms of specificity a preference for linoleic acid over AA was noted [82, 83].

### 2.3.3. Acyltransferases using lysoPE as acceptor

To date, three different LPEAT acyltransferases have been found, designated as LPEAT1 (also known as MBOAT1), LPEAT2 (also known as AGPAT7), and MBOAT2. LPEAT1 was extensively characterized in mouse and exhibits preference for oleoyl-CoA. The enzyme can also utilize lysoPS as an acceptor, although lysoPE is the preferred substrate [81]. The human enzyme displays similar properties, although in this case a higher preference for lysoPS over lysoPE was found [83].

LPEAT2 was identified in human tissues by Cao et al [84]. The enzyme is highly expressed in brain and inflammatory cells. It has selectivity for long-saturated acyl-CoAs as donors (16:0, 18:0, 18:1) and shows selectivity towards lysoPE, although the enzyme can also utilize lysoPC, lysoPG and lysoPS. Because of the brain is a tissue highly

enriched in PE, LPEAT2 has been suggested as a crucial enzyme in PE remodeling, and it could be implicated in neurological disorders, like Alzheimer or multiple sclerosis.

MBOAT2 has been extensively characterized in mice, and is highly expressed in epididymis, brain, testis and ovary and it shows preference for oleyl-CoA as donor and can use both lysoPE and lysoPC as acceptors. Human MBOAT2 has clearly been shown to exhibit preference for lysoPE over lysoPC, and also for oleoyl-CoA [82]. Mouse MBOAT2 has also been called LPCAT4, because it utilizes lysoPC and lysoPE equally well [81].

### 2.3.4. Acyltransferases using lysoPI as acceptor

MBOAT7 was identified in *C. elegans* as an acyltransferase specific for lysoPI [85]. In addition, MBOAT7 exhibits high selectivity for AA and eicosapentaenoic acid, making it an obvious candidate for mediating AA recycling into PI via the Lands pathway. The homolog in humans is also called BB1/LENG4, and displays the same substrate specificity as the *C. elegans* enzyme [82].

The one other LPIAT described to date is LPAAT3 which, as indicated above, may utilize either lysoPA or lysoPI as acceptors [73].

### 2.3.5. Other lysophospholipid acyltransferases

LCLAT1, cloned in mice, is involved in the remodeling of cardiolipin, a glycerophospholipid abundant in mitochondria [86]. LCLAT1 possesses both acyl-CoA:monolysocardiolipin acyltransferase and acyl-CoA:dilysocardiolipin acyltransferase activities, and uses oleic and linoleic acids with preference as donors.

LPGAT1 was identified in human as an acyltransferase using PG as acceptor [87] PG is a major component of lung surfactant; thus LPGAT1 is presumed to play an important key role in lung physiology. The enzyme appears to show some selectivity for palmitic, stearic, and oleic acids.

## 3. Role of $PLA_2$ in AA Mobilization

Stimulation of cells via receptor agonists frequently results in the activation of phospholipid hydrolysis by phospholipase $A_2$ enzymes. An immediate consequence of this is the net accumulation of free AA that can be used for various cellular functions, e.g. the biosynthesis of eicosanoids. Various routes for AA release have been described, including a phospholipase C/diacylglycerol lipase pathway or the inhibition of phospholipid AA reacylation; however, there is general agreement that the $PLA_2$-mediated hydrolysis of phospholipids is the major pathway controlling AA mobilization in stimulated cells, and that all major AA-containing phospholipid classes, namely PC, PE, and PI contribute to this release [88-91].

Mammalian cells contain multiple structurally diverse $PLA_2$ enzymes capable of hydrolyzing sn-2 fatty acids from phospholipids. $PLA_2$s have been systematically classified according to their nucleotide sequence. In the latest update to this classification, the $PLA_2$ enzymes were classified into fifteen group types, according to their primary sequence [92]. Additionally, a sixteenth $PLA_2$ group has been reported very recently [93]. However, a second classification of the $PLA_2$ enzymes, sometimes more useful, also exists which categorizes the enzymes into five major families attending to biochemical commonalities [89, 94]. These families are the $Ca^{2+}$-dependent secreted enzymes, the

$Ca^{2+}$-dependent cytosolic enzymes, the $Ca^{2+}$-independent cytosolic enzymes, the platelet-activating factor acetyl hydrolases, and the lysosomal $PLA_2$s. Of these families, the two first ones have been repeatedly implicated in AA mobilization in response to a variety of immunoinflammatory stimuli [88, 89, 95]. Today, it is firmly established that the calcium-dependent cytosolic group IVA $PLA_2\alpha$ ($cPLA_2\alpha$) is the critical enzyme in AA release [96] and that, depending on cell type and stimulation conditions, a secreted $PLA_2$ —in particular that belonging to groups IIA, V and X— may also participate by amplifying the $cPLA_2\alpha$-regulated response [17, 97-100]. In addition, recent data have also indicated that the $Ca^{2+}$-independent cytosolic $PLA_2$ (group VI enzymes) can also mediate AA release under certain conditions [101, 102].

A number of excellent reviews have recently been published covering various aspects of $cPLA_2\alpha$ biochemistry, including structure, catalysis, regulation by $Ca^{2+}$ availability, and physiological/pathophysiological functions [96, 102-106]. Thus, in the following sections we will focus on recent data on the cellular regulation of $cPLA_2\alpha$ by phosphorylation and anionic lipids.

### 3.1. Role of phosphorylation reactions in regulating $cPLA_2\alpha$ activity in cells

$cPLA_2\alpha$ can be phosphorylated on multiple residues under activation conditions, but only three of them, $Ser^{505}$, $Ser^{727}$, and $Ser^{707}$ appear to be relevant to the regulation of AA mobilization in agonist-stimulated cells [107-109]. Phosphorylation of $cPLA_2\alpha$ at these sites only modestly increases the activity of the enzyme *in vitro*, thus suggesting that such phosphorylation serves other regulatory functions in cells.

Phosphorylation of $cPLA_2\alpha$ at $Ser^{505}$ was the first to be described [107], and still is the most extensively studied, and perhaps the most controversial from a functional point of view. Phosphorylation of $cPLA_2\alpha$ at $Ser^{505}$ stably increases enzyme activity by 1.5-2-fold, and promotes a significant mobility shift of the protein in acrylamide gels [107]. Depending on cell type and stimulus, the phosphorylation reaction is catalyzed by extracellular-regulated kinases p42 and p48 [107], p38 [110] and SAPK/JNK [111, 112]. By studying *in vitro* membrane affinity of different phosphorylation-site mutants of $cPLA_2\alpha$, Cho and colleagues [113] have recently described that mutation at $Ser^{505}$ results in a lower affinity for PC membranes than the wild type enzyme, which is due to a faster desortion from the membrane. This difference is very much enhanced at low $Ca^{2+}$ concentrations compared with high $Ca^{2+}$ concentrations during the assays (2.5 and 50 μM respectively) [113]. In experiments with cells, this different behavior can also be observed at low intracellular $Ca^{2+}$ concentrations during cellular activation, a situation that is observed when the cells are stimulated with 2 μM $Ca^{2+}$ ionophore (0.4 mM intracellular $Ca^{2+}$). The interpretation of these results is that phosphorylation at $Ser^{505}$ enhances hydrophobic interaction of the enzyme with the membrane, by promoting membrane penetration of the hydrophobic residues $Ile^{399}$, $Leu^{400}$, and $Leu^{552}$. This effect probably occurs through a conformational change of the protein [113]. Other studies, however, have not found differences between the membrane translocation behavior of the wild type enzyme and the mutated enzyme in $Ser^{505}$ [114, 115]. Recently, it has been described that in lung fibroblasts activated with phorbol esters or serum, $Ser^{505}$ phosphorylation does not work to lower the $Ca^{2+}$ threshold levels necessary for $cPLA_2\alpha$ translocation, but only acts to increase the catalytic activity of the enzyme [116].

cPLA$_2\alpha$ phosphorylation at Ser$^{727}$ was first described in Sf9 cells overexpressing cPLA$_2\alpha$ [108], and later in agonist-stimulated human platelets [111]. In platelets, this phosphorylation is required for a full AA mobilization response, and the kinase involved appears to be MNK-1 or a closely related kinase [117]. Phosphorylation of cPLA$_2\alpha$ at Ser$^{727}$ increases the cellular activity of the enzyme by a mechanism that is not mediated by increased membrane affinity [117] but by a mechanism related with the interaction of cPLA$_2\alpha$ with a tetramer of p11 and annexin 2A in the cytosol [118]. *In vitro* activity assays and membrane binding measurements by surface plasmon resonance analyses showed that, in resting conditions cPLA$_2\alpha$ interacts with a tetramer of p11 and annexin 2A via the hydroxyl group of Ser$^{727}$, inhibiting the targeting of the enzyme to cellular membranes and the AA release. When cells are stimulated and phosphorylation in Ser$^{727}$ occurs, the tetramer is displaced from cPLA$_2\alpha$ and the enzyme can then interact with cellular membranes and effect phospholipid hydrolysis [118]. This mechanism has yet to be confirmed in agonist-stimulated cells.

Phosphorylation of cPLA$_2\alpha$ on Ser$^{515}$ was first described as the only residue phosphorylated by calcium/calmodulin-dependent protein kinase II *in vitro*, and leads to an increase in enzymatic activity of about two-fold [109]. The biological role of this phosphorylation has been defined very recently in norepinephrine-stimulated vascular smooth muscle cells, by using a specific antibody against the phosphorylated residue [119]. Phosphorylation of cPLA$_2\alpha$ at Ser$^{515}$ by calcium-/calmodulin-dependent protein kinase II appears to be a pre-requisite for the further phosphorylation of the enzyme at Ser$^{505}$ by ERK1/2, and both phosphorylated sites are required for a full AA mobilization response to response to norepinephrine. In this system, mutation of Ser$^{505}$, Ser$^{515}$ or both

(Ser$^{505}$/$^{515}$) to Ala does not change the ability of the mutated enzyme to translocate to the nuclear envelope [119].

### 3.2. Cellular Regulation of cPLA$_2\alpha$ by anionic phospholipids

The activating effect of anionic phospholipids on cPLA$_2\alpha$ was first described by Leslie and Channon [120] in studies utilizing a partially purified enzyme from RAW264.7 cells. It was shown that PS, PA, PI and phosphatidylinositol-4,5-bisphosphate ($PIP_2$) have the capability to increase cPLA$_2\alpha$ activity *in vitro* when incorporated into the vesicle substrate. $PIP_2$ was the best activator, reaching a 7-fold increase in activity at 1 mol% [120]. This effect could be further enhanced to 20-fold by coincubating with diacylglycerol or PE, decreasing at the same time the requirement of $Ca^{2+}$ for enzyme activity from mM to nM [120].

The activating effects of $PIP_2$ were later confirmed by Mosior and colleagues $PIP_2$ utilizing human recombinant cPLA$_2\alpha$ [121]. Binding of cPLA$_2\alpha$ to large unilamellar vesicles of PC was enhanced 20-fold in the presence of 1% $PIP_2$, with a concomitant increase in activity of the same magnitude. The stoichiometry of binding was 1:1, and just 1 molecule per 2000 lipid molecules in the membranes was enough to double the binding of the cPLA$_2\alpha$ [121]. The binding effect produced by $PIP_2$ was so important that it supported measurable association with vesicles and activity even in the absence of $Ca^{2+}$ in the reaction mixture (presence of EGTA). Other related phospholipids like PI(3,4,5)$P_3$ and PI(3,4)$P_2$ also enhanced PLA$_2\alpha$ activity in their assay but at 60 and 63% of the $PIP_2$. However, other anionic lipids such as PI, PS or PA had little or opposite effects on the binding of cPLA$_2\alpha$ to the lipid vesicles [121]. Following on these observations, Das and

Cho [122] identified a polybasic cluster in the catalytic domain of cPLA$_2\alpha$ that, at least partially, accounted for $PIP_2$ binding ($Lys^{541}$, $Lys^{543}$, $Lys^{544}$, and $Arg/Lys^{488}$). Mutations of this cluster eliminate the specific activation of the cPLA$_2\alpha$ promoted by $PIP_2$. However, no effect of $PIP_2$ on the affinity of the enzyme for vesicles was appreciated in experiments of surface plasmon resonance [122].

In a cellular context, the first evidence for the regulation of cPLA$_2\alpha$ by $PIP_2$ was obtained in a macrophage-like cell line activated by UV radiation [123]. Inhibition of phosphoinositide increases during cellular activation also inhibited the release of AA. The biological relevance of these observations was that UV activated cPLA$_2\alpha$ in the cells in the absence of any apparent change in the intracellular $Ca^{2+}$ concentration [123]. In this regard, Das and Cho also observed that sequestration of cellular $PIP_2$ by over-expressing a pleckstrin homology domain of the phospholipase C-$\delta_1$, considerably decreased the amount of AA released by cellular activation with ionophore [122]. Later it was also demonstrated that exogenous $PIP_2$ shuttled into the cells by coupling it to cationic carriers, promoted the translocation of cPLA$_2\alpha$ to those membranes were $PIP_2$ was localized, mainly perinuclear membranes [124]. The effect was observed at basal intracellular $Ca^{2+}$ concentrations (50 nM), but it did not occur in the presence of EGTA, suggesting that the process of cPLA$_2\alpha$ translocation by $PIP_2$ is not $Ca^{2+}$-independent. In support of this, a mutant in the $Ca^{2+}$ binding site of the enzyme (D43N) did not translocated in response to $PIP_2$ [124]. It was also observed that intracellular increases in $PIP_2$ lowered the $Ca^{2+}$ requirements for enzyme translocation to intracellular basal levels. Furthermore, mutations in the cationic cluster $Lys^{541}$, $Lys^{543}$, $Lys^{544}$, and $Lys^{488}$ did not

change the translocation pattern of the enzyme to intracellular membranes but inhibited the release of AA, indicating a non-productive membrane binding [124].

In contrast with these observations, by using yeast-based assay that tests the ability of proteins to bind to membrane lipids, Le Berre and colleagues [125] have found that only the $Ca^{2+}$-binding domain of cPLA$_2\alpha$ interacts with lipids, including $PIP_2$. Because inhibitory effects on cPLA$_2\alpha$ activity were observed by overexpressing the pleckstrin homology domain of PLC$\delta$1, and $PIP_2$-specific 5'-phosphatase in stimulated cells, the conclusion was made that cPLA$_2\alpha$ activity can be modulated by sequestration or depletion of cellular $PIP_2$, but not by direct binding [125].

Another anionic phospholipid that appears to have profound effects on the activity and physical state of cPLA$_2\alpha$ is ceramide 1-phosphate. Chalfant and colleagues demonstrated that ceramide kinase and its product ceramide 1-phosphate mediated the activation of cPLA$_2\alpha$ during cellular stimulation with IL-1 or calcium ionophore [126]. It was also observed that ceramide 1-phosphate induces the translocation of cPLA$_2\alpha$ to intracellular membranes, mainly Golgi and perinuclear membranes [127]. Moreover, the C2 domain of the enzyme itself also translocated in response to ceramide 1-phosphate. In vitro studies demonstrated that ceramide 1-phosphate binds cPLA$_2\alpha$ through the C2 domain (at the cationic $\beta$-groove $Arg^{57}$, $Lys^{58}$, $Arg^{59}$) and that such an interaction increases the enzymatic activity in a calcium-dependent manner [127, 128]. By using surface-dilution kinetics and surface plasmon resonance it has been described as well that ceramide 1-phosphate activates cPLA$_2\alpha$ not by affecting the Michaelis-Menten constant, but by increasing the residence time of the enzyme on membranes, decreasing the dissociation constant of the enzyme to membrane PC [129, 130].

### 3.3. $sPLA_2$ Role in AA Release

There is much data suggesting that certain $sPLA_2$ form are involved in mediating AA mobilization in a variety of cells, most notably those involved in immunoinflammatory reactions, like macrophages and mast cells [89, 94]. However, the mode how $sPLA_2$ participates in this process is still a very controversial issue. This is due in part to the fact that most of the evidence implicating $sPLA_2$ in AA mobilization derives from studies utilizing exogenous enzymes or cells overexpressing certain $sPLA_2$ forms, and limited information is available on the role of the "endogenous" relevant $sPLA_2$.

Exogenous $sPLA_2$, particularly that belonging to Groups IIA, V, and X, or $sPLA_2$ over-expressed in various cells can amplify the essential role of $cPLA_2\alpha$ in eicosanoid biosynthesis by augmenting the release of AA and other fatty acids under various experimental conditions [98, 131-135]. The $sPLA_2$ enzymes could potentially be involved in the $cPLA_2\alpha$-dependent AA mobilization through three pathways, one involving re-internalization via caveolin-rich domains [136, 137], the second involving direct interaction with PC-rich outer membrane domains [136, 138, 139], and the third one involving an undefined intracellular action prior secretion of the enzyme [140]. Nonetheless, $sPLA_2$s may also act to release AA in a $cPLA_2\alpha$-independent manner, as demonstrated by studies in cells from mice lacking $cPLA_2\alpha$ by genetic disruption [141].

Regarding the endogenous enzyme, studies using mice in which the gene encoding group V $sPLA_2$ was deleted have provided conclusive evidence for the role of this enzyme in eicosanoid production by macrophages and mast cells *in vivo* [142]. Interestingly, the effect of $sPLA_2$ is observed in cells on a C57BL/6 genetic background, while in cells on a BALB/c background no $sPLA_2$ effect could be ascertained [143].

These data provide evidence that two different phenotypes may exist in cells regarding the involvement of $sPLA_2$ in eicosanoid generation. Whether these two phenotypes may also manifest in cells depending on culture conditions is unknown at present.

On the other hand, studies on the role of $sPLA_2$ in AA release are complicated by the existence of cross-talk between the $sPLA_2$ and the main effector of the response, the $cPLA_2\alpha$. This cross-talk may work in both directions, i.e. $cPLA_2\alpha$ may regulate the action of $sPLA_2$ or *vice versa*.

In some cell types such as human and murine macrophage-like cell lines, $cPLA_2\alpha$ has been shown to regulate $sPLA_2$ activity by a mechanism involving the rapid generation of hydroperoxyeicosatetraenoic acid [144, 145]. At long incubation times, activation of $cPLA_2\alpha$ is also required for the increased expression of group V $sPLA_2$ that is characteristically induced by immunoinflammatory stimuli such as lipopolysaccharide and, as with the short-term response, may involve the production of hydroperoxyeicosatetraenoic acid [146-150] .

The regulation of $cPLA_2\alpha$ by $sPLA_2$ has been characterized in detail in some instances. In murine mesangial cells, an adenoviral infection technique was used to stably express group IIA and/or group V $sPLA_2$ into the cells [100]. $cPLA_2\alpha$ was found to effect the AA release and, when present, both $sPLA_2$ forms amplified the $cPLA_2\alpha$-mediated response, this resulting in increased AA mobilization [100]. Moreover, a correlation was found to exist between the expression level of $cPLA_2\alpha$ and the magnitude of AA release. Such a correlation did not occur between the expression level of $sPLA_2$ and the extent of AA release. Recent work in mouse mast cells from mice lacking group V $sPLA_2$ by genetic deletion has provided conclusive evidence that $sPLA_2$ modulates the activity of

cPLA$_2\alpha$ by regulating its phosphorylation via extracellular-regulated kinases [151]. Utilizing inhibitors, similar observations have also been made in recent work with murine macrophage-like cells [152, 153].

### 3.4. iPLA$_2$ Role in AA Release

The group VIA PLA$_2$ (iPLA$_2$-VIA) is ubiquitously expressed and has the potential to participate in AA release under some conditions [101, 102]. However, the role of this enzyme in AA release has traditionally been inferred from studies using the inhibitor BEL, a compound that manifests high selectivity for iPLA$_2$ *in vitro* but not *in vivo* [154-156]. In some studies, BEL was found to inhibit the AA release, but in others, notably in phagocytes, no significant effect was detected [89]. It appears likely that the involvement of group iPLA$_2$-VIA in AA release is markedly cell- and stimulus-dependent, as most of the roles attributed to this enzyme in cell physiology appear to be [22, 101, 157]. Since various iPLA$_2$–VIA splice variants co-exist in cells [22, 101, 157], it is possible that the enzyme is subject to multiple regulatory mechanisms that differ among cell types and stimulation conditions. This in turn could also explain the multiplicity of functions that this enzyme appears to serve depending on cell type.

Recently, mice with targeted disruption of the gene encoding for iPLA$_2$–VIA have been generated [158]. Use of cells from these animals has reinforced the idea that the involvement of iPLA$_2$ in AA mobilization notably differs depending on cell type and stimulation conditions. Thus, peritoneal macrophages from iPLA$_2$–VIA null mice appear to release AA in response to zymosan in a manner that is indistinguishable from that of cells from wild type animals [159]. In contrast however, iPLA$_2$–VIA appears to be

crucial for AA mobilization in macrophages upon free cholesterol loading [159] and for the eicosanoid response of macrophages stimulated via class A scavenger receptors [160].

## 4. Conclusions

In the last two decades much effort has been made to elucidate the mechanisms by which AA is liberated and incorporated into phospholipids. This review has dealt with the relatively high number of enzymes with acyl-CoA synthetase, CoA-dependent acyltransferase or phospholipase $A_2$ activities that have been described to participate in the regulation of cellular AA availability. Some of these enzymes show a marked selectivity for AA. Clearly, this is a very complex issue involving multiple enzymes and pathways, and there is still much to be learned about the interplay between some of the AA-utilizing enzymes and the regulatory mechanisms involved.

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## Figure Legends

Figure 1. Pathways for the incorporation of arachidonic acid into glycerolipids. LPLAT, lysophospholipid acyl-CoA acyltransferase.

Figure 2. Arachidonic acid incorporation into and remodeling among phospholipids. Adapted with permission from ref. 15 (© The Biochemical Society).

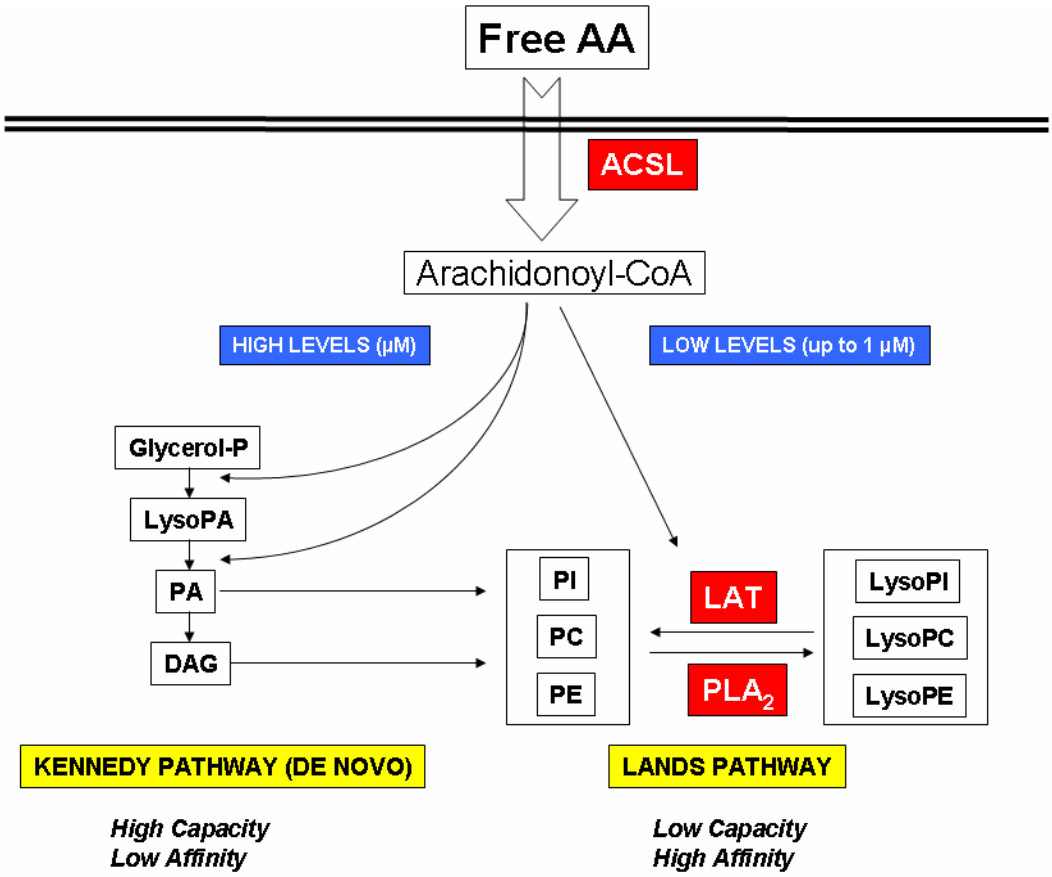
Free AA
ACSL
Arachidonoyl-CoA
HIGH LEVELS (µM)
LOW LEVELS (up to 1 µM)
Glycerol-P
LysoPA
PA
DAG
PI
PC
PE
LAT
PLA2
LysoPI
LysoPC
LysoPE
KENNEDY PATHWAY (DE NOVO)
LANDS PATHWAY
High Capacity
Low Affinity
Low Capacity
High Affinity

Figure 1.

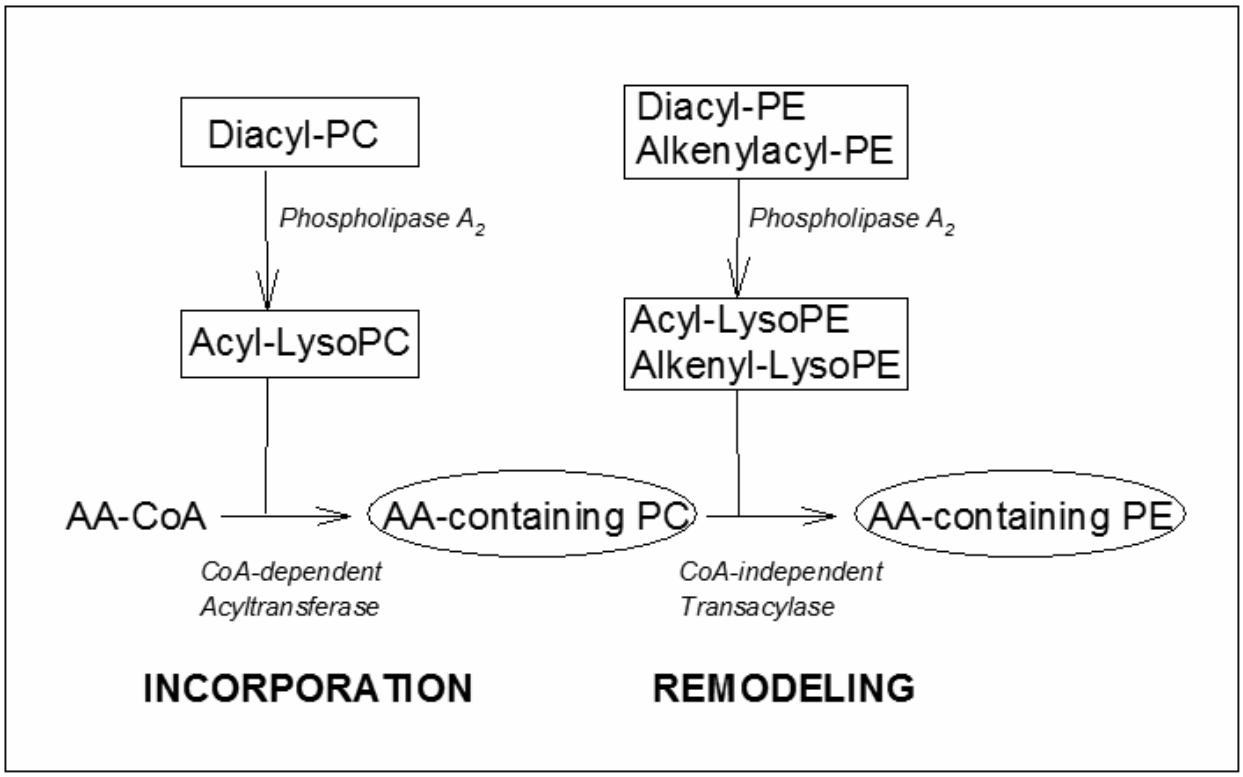
Diacyl-PC
Phospholipase $A_2$
Acyl-LysoPC
Diacyl-PE
Alkenylacyl-PE
Phospholipase $A_2$
Acyl-LysoPE
Alkenyl-LysoPE
AA-CoA
AA-containing PC
AA-containing PE
CoA-dependent
Acyltransferase
CoA-independent
Transacylase
INCORPORATION
REMODELING

Figure 2.