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Function and Inhibition of Intracellular Calcium-independent Phospholipase A_2*

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Our previous Minireview (1) considered the three main kinds of phospholipase A_2 (PLA_2)[1]: the well characterized Groups I, II, and III small Ca^{2+}-dependent secretory phospholipase A_2s ($sPLA_2$), the 85-kDa Group IV Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$), and the 80-kDa Ca^{2+}-independent cytosolic phospholipase A_2 ($iPLA_2$). In the ensuing years, it has become clear that PLA_2 represents a growing superfamily of enzymes with many additional $sPLA_2$s (Groups IIC, V, and IX), further definition of the 80-kDa $iPLA_2$ (Group VI), and two Ca^{2+}-independent PLA_2s specific for platelet-activating factor (PAF) (Groups VII and VIII) (2).

All of the well studied $sPLA_2$s appear to use a His-Asp catalytic mechanism and require Ca^{2+} to be bound tightly in the active site of the enzyme. The well characterized $iPLA_2$ appears to require a central Ser for catalysis and of course, no Ca^{2+}. Interestingly, the Group IV $cPLA_2$ does not use Ca^{2+} for catalysis, but rather the Ca^{2+} dependence seems to relate to a calcium lipid-binding domain (CaLB or C-2 domain) at the N-terminal end responsible for association of the enzyme with the membrane. Thus, the catalytic mechanism and active site Ser do not involve Ca^{2+} (3–5); therefore a mechanistic distinction between the Group IV $cPLA_2$ and the $iPLA_2$s may not be warranted at this time. This is relevant because most of the inhibitors that work on the Group IV $cPLA_2$ also act on the Group VI $iPLA_2$ (6, 7). Inhibitor specificity will be discussed in the next section.

We (8) recently surveyed all of the reported Ca^{2+}-independent PLA_2 activities. While there exists a group of lysosomal $iPLA_2$s and a group of characterized ectoenzymes with broad specificity, which may actually be general lipases (8), sequenced and well characterized intracellular $iPLA_2$s are limited to the 80-kDa Group VI $iPLA_2$ and the 29-kDa Group VIII enzyme, which is a PAF acetyl hydrolase (9). The latter hydrolyzes the acetyl chain present at the *sn-2* position of PAF and perhaps acts on oxidized phospholipids as well but not on normal phospholipids carrying unoxidized long chain fatty acids at the *sn-2* position (9). This enzyme and a secreted Group VII PAF acetyl hydrolase, both of which are really $iPLA_2$s with a particular substrate specificity, have been considered elsewhere (2).

The Group VI 80-kDa $iPLA_2$ was first identified in $P388D_1$ macrophages (10), purified (11), further characterized (12), and then cloned and sequenced by Jones and co-workers (13) from CHO cells. The CHO $iPLA_2$ has been shown to represent a species variant of that present in $P388D_1$ macrophage-like cells, where the $iPLA_2$ has also been cloned and sequenced (14). The sequence of the Group VI $iPLA_2$ reveals the presence of eight ankyrin-like domains and the G-*X*-S-*X*-G motif commonly found in other lipases. Interestingly, no known consensus sequences for posttranslational modification, such as phosphorylation sites, are apparent in the Group VI $iPLA_2$ (13, 14). This is compatible with the possibility that the Group VI $iPLA_2$ acts to remodel membrane phospholipids as a sort of *housekeeping* enzyme as will be discussed later.

Inhibition of $iPLA_2$

The functional significance of intracellular $iPLA_2$ can most easily be investigated using selective inhibitors. Unfortunately, no specific $iPLA_2$ inhibitors currently exist. As indicated above, the apparent presence of an active site Ser residue in Group VI $iPLA_2$ is similar to that of the Group IV $cPLA_2$. Thus, the $cPLA_2$ inhibitors currently available, which were designed as site-directed inhibitors, all inhibit the Group VI $iPLA_2$ as well. These include arachidonyl trifluoromethyl ketone (6), arachidonyl tricarbonyl (6), and methyl arachidonyl fluorophosphonate (7). These three compounds contain an arachidonyl tail and function as transition-state analogues in a reversible or irreversible manner. The arachidonyl tail was intended to confer selectivity to the inhibitors and to facilitate their access to the $cPLA_2$ active site (15, 16), as this enzyme selectively hydrolyzes arachidonate-containing phospholipids (17, 18). Remarkably, even though the $iPLA_2$ is not AA-specific (11), these inhibitors work even better on the $iPLA_2$ than on the $cPLA_2$ (6, 7). Furthermore, palmityl trifluoromethyl ketone and palmityl tricarbonyl are as good inhibitors of both the Group IV $cPLA_2$ and Group VI $iPLA_2$ as their arachidonyl analogs (6, 12).

Due to the lack of selectivity of the aforementioned compounds, it is unlikely that they will find much use in defining the role of the $iPLA_2$ in cell function, unless the process under study is truly Ca^{2+}-independent. Inhibition studies employing the fatty acyl trifluoromethyl ketones, tricarbonyls, or fluorophosphonates in the absence of Ca^{2+} might selectively target the $iPLA_2$, as this is the only one of the well studied cellular PLA_2s that remains active under Ca^{2+}-depleted conditions.

One common feature of the two best characterized intracellular $iPLA_2$s, namely the Group VI enzyme present in $P388D_1$ macrophages (12) and CHO cells (14) and a 40-kDa $iPLA_2$ present in myocardial tissue and pancreatic islets (19), is their complete and irreversible inhibition by the mechanism-based inhibitor BEL. This inhibitor was first introduced as a serine protease inhibitor (20) but has been shown to be specific for $iPLA_2$ over Ca^{2+}-dependent $sPLA_2$s (19, 21) and Group IV Ca^{2+}-dependent $cPLA_2$ (21). In addition, BEL does not affect a number of enzyme activities directly involved in cellular AA metabolism (22). Thus BEL has received great attention because of its possible use as a selective $iPLA_2$ inhibitor in whole cell studies. As a matter of fact, much of what is currently believed to be mediated by $iPLA_2$ enzymes has been derived

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $sPLA_2$, secretory Ca^{2+}-dependent phospholipase A_2; $cPLA_2$, 85-kDa Ca^{2+}-dependent cytosolic phospholipase A_2; $iPLA_2$, Ca^{2+}-independent cytosolic phospholipase A_2; BEL, bromoenol lactone; PAP-1, Mg^{2+}-dependent phosphatidic acid phosphohydrolase; PAF, platelet-activating factor; AA, arachidonic acid; DAG, 1,2-diacylglycerol; CHO, Chinese hamster ovary.

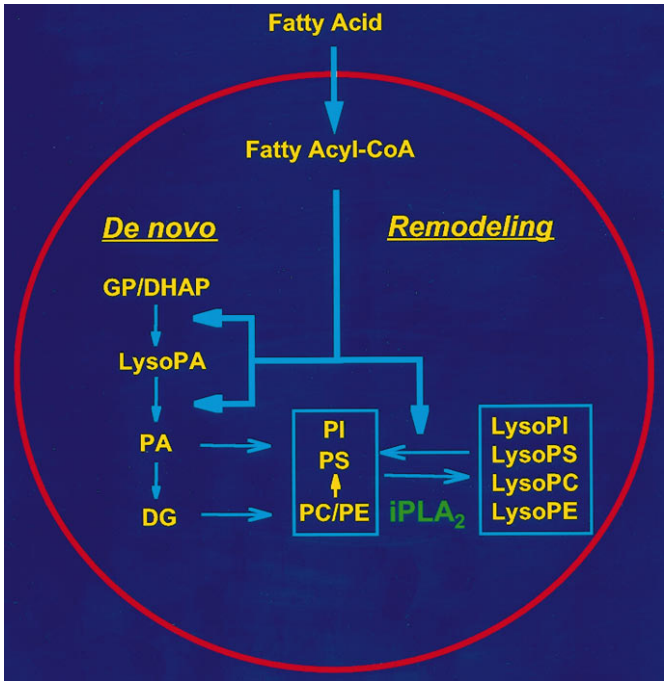

FIG. 1. ***De novo* and remodeling pathways for incorporation of free fatty acid into phospholipids.** In the *de novo* pathway free fatty acid, provided to the cell or liberated by endogenous phospholipases, is incorporated via fatty acyl-CoA into glycerol phosphate (*GP*) or dihydroxyacetone phosphate (*DHAP*) and into the resulting lysophosphatidic acid (*LysoPA*) by fatty acyl-CoA acyltransferases to form phosphatidic acid (*PA*). In mammalian cells, the phosphatidic acid can be converted to phosphatidylinositol (*PI*) or can be converted to diacylglycerol (*DG*), which is the precursor for phosphatidylcholine (*PC*) and phosphatidylethanolamine (*PE*), which in turn forms phosphatidylserine (*PS*). In contrast, in the remodeling pathway, preformed phosphatidylinositol, phosphatidylserine, phosphatidylcholine, or phosphatidylethanolamine is acted on by $iPLA_2$ to produce lysophosphatidylinositol, lysophosphatidylserine, lysophosphatidylcholine, or lysophosphatidylethanolamine; these can be reacylated by acyltransferases using fatty acyl-CoA. Adapted with permission from Ref. 22.

from studies using BEL. Unfortunately, BEL has recently been found to inhibit another key enzyme in cellular phospholipid metabolism, the Mg^{2+}-dependent phosphatidic acid phosphohydrolase (PAP-1) (23). The latter enzyme catalyzes the dephosphorylation of phosphatidic acid to yield 1,2-diacylglycerol (DAG), a central intermediate in glycerolipid synthesis as well as an important intracellular messenger molecule. Therefore, BEL cannot be used as a selective $iPLA_2$ inhibitor in whole cell studies unless it is demonstrated that the process under study is independent of variations in cellular DAG levels as well as Ca^{2+}; even then BEL is known to inhibit proteases and may affect other enzymes as well. The implications will be discussed in the next section.

Function of Group VI $iPLA_2$ in Membrane Phospholipid Remodeling

There exists in cells an ongoing deacylation/reacylation cycle of membrane phospholipids, the so-called Lands cycle, whereby a pre-existing phospholipid is cleaved by an intracellular PLA_2 to generate a 2-lysophospholipid, which in turn may be reacylated with a different fatty acid to generate a new phospholipid (22, 24). This remodeling cycle constitutes the major route for incorporation of free AA into the phospholipids of cells at nanomolar levels of the free fatty acid (Fig. 1) (22, 24). The *de novo* route or Kennedy pathway constitutes, in addition, a second route for incorporation of AA into cellular phospholipids (Fig. 1). However, the Kennedy pathway appears to be relevant in terms of AA incorporation only when high, micromolar levels of free AA are available (25).

Thus, AA incorporation into phospholipids under normal conditions is strikingly dependent on a PLA_2 that generates the 2-lysophospholipid used in the acylation reaction. Macrophages and macrophage cell lines possess a high capacity to incorporate AA into their membrane phospholipids (26, 27). This process occurs in a Ca^{2+}-independent manner (22, 26), suggesting the involvement of an $iPLA_2$. Consistent with this observation, BEL inhibits AA esterification in a dose-dependent and saturatable manner, and the decrease in AA incorporation directly correlates with inhibition of both cellular $iPLA_2$ activity and steady-state lysophospholipid levels (22). Although the lack of BEL specificity now raises some doubt about the firmness of this conclusion, it is important to stress here that AA esterification via phospholipid remodeling is independent of variations in DAG levels. Moreover, BEL does not reduce the cellular steady-state levels of DAG.[2] Thus, the possible parallel inhibition of PAP-1 by BEL should not affect the basal rate of AA incorporation into phospholipids.

The nucleotide sequence for the Group VI murine $iPLA_2$ is now available (14). This has enabled us to utilize more convincing molecular biological approaches, such as antisense inhibition studies, to ascertain the role of the Group VI $iPLA_2$ in cellular phospholipid metabolism. Antisense inhibition of the Group VI $iPLA_2$ has confirmed that this enzyme does play a role in phospholipid remodeling as $iPLA_2$-depleted cells show a significant reduction of their capacity to incorporate AA into membrane phospholipids.[2] Moreover, the decreased incorporation of AA into phospholipids that $iPLA_2$-depleted cells manifest is not further decreased by BEL,[2] demonstrating that this compound is indeed targeting the $iPLA_2$ in the previous experiments (22).

Collectively, these findings suggest that the Group VI $iPLA_2$ is responsible for phospholipid fatty acid remodeling under resting conditions. Hence this enzyme appears to regulate the main pathway through which the cells incorporate AA and other unsaturated fatty acids into their membrane phospholipids. In addition to its obvious importance in cellular metabolism, the rate of AA incorporation into phospholipids also determines the amount of free fatty acid available under resting conditions. This is relevant because free AA availability is a limiting factor for eicosanoid biosynthesis. By regulating basal AA esterification reactions, the Group VI $iPLA_2$ may also play a key role in regulating the amount of prostaglandins synthesized by resting cells.

On the other hand, there is now strong evidence for the existence of different AA pools within the cells (21, 28) that can be utilized by distinct Ca^{2+}-dependent PLA_2s during cell activation (21). The role of CoA-independent transacylase in regulating the asymmetric distribution of AA among different phospholipid subclasses has recently become evident (24). However, as the $iPLA_2$-mediated reaction precedes the action of the CoA-independent transacylase, it is possible that the $iPLA_2$ determines both the subcellular distribution of this fatty acid among the different cellular compartments and the relative amount of fatty acid present in each compartment before further remodeling reactions catalyzed by the CoA-independent transacylase take place (Fig. 2).

Other Proposed Functions for Intracellular $iPLA_2$s

Based solely on BEL effects, the $iPLA_2$ has been suggested to mediate AA release in different cells stimulated with various agonists (29–31). It is known, however, that during cell stimulation DAG levels rise appreciably, and this may influence

[2] J. Balsinde, M. A. Balboa, and E. A. Dennis, manuscript in preparation.

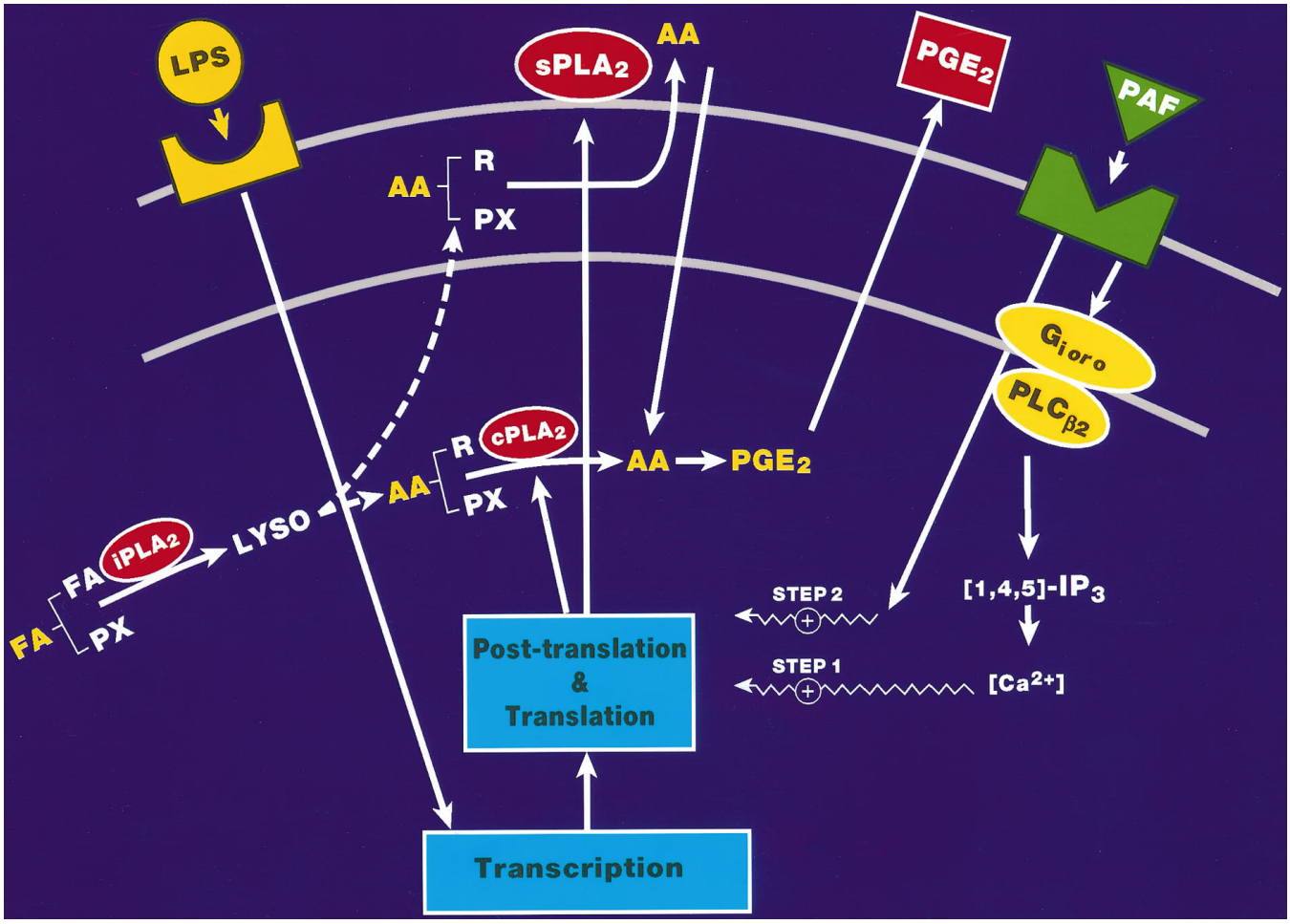

FIG. 2. **A model for PLA_2-mediated pathways for AA metabolism in $P388D_1$ macrophages.** PAF receptor-mediated AA mobilization in lipopolysaccharide (*LPS*)-primed $P388D_1$ macrophages involves the action of two distinct Ca^{2+}-dependent PLA_2s, *i.e.* $cPLA_2$ and $sPLA_2$, acting on different AA pools located at distinct cellular membranes. The $iPLA_2$ mediates incorporation of AA at these two intracellular locations before further remodeling reactions take place. *PGE_2*, prostaglandin E_2; *PLC*, phospholipase C; *FA*, fatty acid; *[1,4,5]-IP_3*, inositol 1,4,5-trisphosphate; *R*, fatty acid or alcohol; *PX*, phosphobase. Adapted with permission from Ref. 21.

agonist-induced AA release, either directly (by providing DAG substrate for the release) (32) or indirectly (by activating protein kinase C) (33). As discussed above, BEL has recently been found to inhibit cellular PAP-1 activity (23). It is likely that PAP-1 plays a role in raising intracellular DAG levels during signal transduction, particularly in those settings where the *de novo* phospholipid biosynthetic pathway is involved (34). Thus, the reported effects of BEL on agonist-induced AA release might also be due, at least in part, to inhibition of PAP-1 in addition to the $iPLA_2$.

The following example may better illustrate the uncertainty of BEL effects on agonist-induced AA mobilization. In studies with glucose- and carbachol-stimulated pancreatic islets, Ramanadham *et al.* (29) proposed the $iPLA_2$ as the major mediator of AA release on the basis of its inhibition by BEL. However, Konrad *et al.* (35) have suggested that the DAG lipase pathway constitutes the major route for AA release in the same system. The results by these two groups could be reconciled if BEL was inhibiting DAG generation in the work by Ramanadham *et al.* (29). On the other hand, it is interesting to note that in systems where agonist-stimulated AA release appears not to depend on DAG-induced protein kinase C activation, such as PAF-stimulated $P388D_1$ macrophages (36), BEL is ineffective in inhibiting this release (21).

Involvement of an $iPLA_2$ in stimulus-induced AA release has also been suggested by Lennartz and colleagues (37, 38) in studies with human monocytes. During immunoglobulin G-mediated phagocytosis, human monocytes release AA in a Ca^{2+}-independent manner (37). Consistent with the possible involvement of an $iPLA_2$, AA release in this system is blocked by BEL (37). The process was later found to be dependent on protein kinase C activation (38). Thus, the BEL effects could be partly due to protein kinase C dependence on DAG, although the finding that the process takes place in the absence of Ca^{2+} lends credence to the possible involvement of an $iPLA_2$.

It is generally difficult to rationalize the involvement of a Ca^{2+}-independent enzyme in processes such as AA release, which in most cases is strongly Ca^{2+}-dependent. Wolf and Gross (39) have recently reported that a 40-kDa myocardial $iPLA_2$ associates with calmodulin in a Ca^{2+}-dependent manner, providing a mechanism through which Ca^{2+} may regulate a Ca^{2+}-independent enzyme. According to these authors, myocardial $iPLA_2$ is inactive when associated with calmodulin. Dissociation of the complex due to decreases in the Ca^{2+} concentration or addition of calmodulin antagonists renders the $iPLA_2$ ready to attack phospholipids and release AA (39). This mechanism has been proposed to mediate the cardiac cycle-dependent alterations in PLA_2-catalyzed release of AA (39).

Before the reported association with calmodulin, the 40-kDa $iPLA_2$ activity from myocardial tissue and pancreatic islets was reported to associate to phosphofructokinase or an antigenically related protein (40, 41). Due to their very distinct biochemical properties and molecular sizes, it appears clear that the 40-kDa $iPLA_2$ activity identified in myocardium and pan-

creatic islets is different from the Group VI enzyme present in $P388D_1$ macrophages and CHO cells (41). However, as a common feature, the two proteins exist as catalytic complexes of about 340 kDa (11). The Group VI $iPLA_2$ has been shown to possess eight ankyrin repeats, which may mediate self-aggregation or interaction with other proteins (13). It is possible that the 40-kDa myocardial $iPLA_2$ activity possesses similar motifs that mediate its interaction with other proteins such as phosphofructokinase and calmodulin; however, the sequence of the 40-kDa protein has not yet been elucidated. Thus, its relationship with the Group VI $iPLA_2$, if any, cannot be ascertained at this time. However, analogous to the Group VI $iPLA_2$ (11), the 40-kDa activity is stimulated by ATP *in vitro* (40). Recent data by our laboratory have shown that, rather than stimulating enzyme activity, ATP stabilizes and protects the $iPLA_2$ from inactivation during the assay; hence, higher activity is found in the presence than in the absence of ATP and other nucleotides (14). Thus there is no evidence that ATP plays a regulatory role for the Group VI $iPLA_2$ *in vivo*.

Concluding Remarks

The importance of the intracellular $iPLA_2$ in control of cell function has not been clearly established at present, despite the fact that $iPLA_2$s have been found to exist in all cells and tissues examined. Currently, many new $iPLA_2$s are being purified and characterized (8, 42–44). The widespread occurrence of $iPLA_2$s suggests that this class of enzymes may play important roles in cell physiology. Fatty acid remodeling of membrane phospholipids in macrophages appears to be an event most likely mediated by intracellular $iPLA_2$s. Currently, much of the data available on cellular $iPLA_2$ function relies on the use of inhibitors that have been shown not to be selective for this class of enzymes. However, these inhibitors may offer leads for the development of more selective agents that may help to uncover new roles for intracellular $iPLA_2$s in cellular functioning.

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