Title
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Permalink
https://escholarship.org/uc/item/77r57844

Journal
The Journal of biological chemistry, 272(26)

ISSN
0021-9258

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Publication Date
1997-06-01

DOI
10.1074/jbc.272.26.16069

Peer reviewed
Minireview

Function and Inhibition of Intracellular Calcium-independent Phospholipase A2*

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Our previous Minireview (1) considered the three main kinds of phospholipase A2 (PLA2): the well characterized Groups I, II, and III small Ca2+-dependent secretory phospholipase A2s (sPLA2s), the 85-kDa Group IV Ca2+-dependent cytosolic phospholipase A2 (cPLA2), and the 80-kDa Ca2+-independent cytosolic phospholipase A2 (iPLA2). In the ensuing years, it has become clear that PLA2 represents a growing superfamily of enzymes with many additional sPLA2s (Groups IIC, V, and IX), further definition of the 80-kDa iPLA2 (Group VI), and two Ca2+-independent PLA2s specific for platelet-activating factor (PAF) (Groups VII and VIII) (2).

All of the well studied sPLA2s appear to use a His-Asp catalytic mechanism and require Ca2+ to be bound tightly in the active site of the enzyme. The well characterized iPLA2 appears to require a central Ser for catalysis and, of course, no Ca2+. Interestingly, the Group IV cPLA2 does not use Ca2+ for catalysis, but rather the Ca2+ 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 Ca2+ (3–5); therefore a mechanistic distinction between the Group IV cPLA2 and the iPLA2s may not be warranted at this time. This is relevant because most of the inhibitors that work on the Group IV cPLA2 also act on the Group VI iPLA2 (6, 7). Inhibitor specificity will be discussed in the next section.

We (8) recently surveyed all of the reported Ca2+-independent PLA2 activities. While there exists a group of lysosomal iPLA2s and a group of characterized ectoenzymes with broad specificity, which may actually be general lipases (8), sequenced and well characterized intracellular iPLA2s are limited to the 80-kDa Group VI iPLA2 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 iPLA2s with a particular substrate specificity, have been considered elsewhere (2).

The Group VI 80-kDa iPLA2 was first identified in P388D1 macrophages (10), purified (11), further characterized (12), and then cloned and sequenced by Jones and co-workers (13) from CHO cells. The CHO iPLA2 has been shown to represent a species variant of that present in P388D1 macrophage-like cells, where the iPLA2 has also been cloned and sequenced (14). The sequence of the Group VI iPLA2 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, are apparent in the Group VI iPLA2 (13, 14). This is compatible with the possibility that the Group VI iPLA2 acts to remodel membrane phospholipids as a sort of housekeeping enzyme as will be discussed later.

Inhibition of iPLA2

The functional significance of intracellular iPLA2 can most easily be investigated using selective inhibitors. Unfortunately, no specific iPLA2 inhibitors currently exist. As indicated above, the apparent presence of an active site Ser residue in Group VI iPLA2 is similar to that of the Group IV cPLA2. Thus, the cPLA2 inhibitors currently available, which were designed as site-directed inhibitors, will not inhibit the Group VI iPLA2 as well. These include arachidonyl trifluoromethyl ketone (6), arachidonyl tricarbonyl (6), and methyl arachidonyl fluorophosphate (7). These three compounds contain an arachidonyl tail 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 cPLA2 active site (15, 16), as this enzyme selectively hydrolyzes arachidonate-containing phospholipids (17, 18). Remarkably, even though the iPLA2 is not AA-specific (11), these inhibitors work even better on the iPLA2 than on the cPLA2 (6, 7). Furthermore, palmitoyl trifluoromethyl ketone and palmitoyl tricarbonyl are as good inhibitors of both the Group IV cPLA2 and Group VI iPLA2 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 iPLA2 in cell function, unless the process under study is truly Ca2+-independent. Inhibition studies employing the fatty acyl trifluoromethyl ketones, tricarbonyls, or fluorophosphonates in the absence of Ca2+ might selectively target the iPLA2, as this is the only one of the well studied cellular PLA2s that remains active under Ca2+-depleted conditions.

One common feature of the two best characterized intracellular iPLA2s, namely the Group VI enzyme present in P388D1 macrophages (12) and CHO cells (14) and a 40-kDa iPLA2 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 iPLA2 over Ca2+-dependent sPLA2s (19, 21) and Group IV Ca2+-dependent cPLA2 (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 iPLA2 inhibitor in whole cell studies. As a matter of fact, much of what is currently believed to be mediated by iPLA2 enzymes has been derived

* This minireview will be reprinted in the 1997 Minireview Compendium, which will be available in December, 1997. This is the third article of six in “A Thematic Series on Phospholipases.” Work in the authors’ laboratory was supported by National Institutes of Health Grants HD 26171, GM 26051, and GM 51606.

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1 The abbreviations used are: PLA2, phospholipase A2; sPLA2, secretory Ca2+-dependent phospholipase A2; cPLA2, 85-kDa Ca2+-dependent cytosolic phospholipase A2; iPLA2, Ca2+-independent cytosolic phospholipase A2; BEL, bromoeno lactone; PAF-1, Mg2+-dependent phosphatidic acid phosphohydrolase; PAF, platelet-activating factor; AA, arachidonic acid; DAG, 1,2-diacylglycerol; CHO, Chinese hamster ovary.
Minireview: Intracellular Calcium-independent Phospholipase A_2

**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 (DG, PC). In mammalian cells, the phosphatidic acid can be converted to lysophosphatidic acid (LysoPA) or diacylglycerol (DG), which is the precursor for phosphatidylcholine (PC) or phosphatidylethanolamine (PE). In contrast, in the remodeling pathway, preformed phosphatidylethanolamine (PS) and phosphatidylcholine, or pre-existing phospholipid is cleaved by an intracellular PLA2 (1PLA2) to produce lysophosphatidylethanolamine (LysoPE). These can be reacylated by acyltransferases using fatty acyl-CoA. Adapted with permission from Ref. 22.

Function of Group VI iPLA2 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 PLA2 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 PLA2 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 iPLA2. Consistent with this observation, BEL inhibits AA esterification in a dose-dependent and saturable manner, and the decrease in AA incorporation directly correlates with inhibition of both cellular iPLA2 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. 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 iPLA2 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 iPLA2 in cellular phospholipid metabolism. Antisense inhibition of the Group VI iPLA2 has confirmed that this enzyme does play a role in phospholipid remodeling as iPLA2-depleted cells show a significant reduction of their capacity to incorporate AA into membrane phospholipids. Moreover, the decreased incorporation of AA into phospholipids that iPLA2-depleted cells manifest is not further decreased by BEL, demonstrating that this compound is indeed targeting the iPLA2 in the previous experiments (22).

Collectively, these findings suggest that the Group VI iPLA2 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 iPLA2 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 PLA2s 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 iPLA2-mediated reaction precedes the action of the CoA-independent transacylase, it is possible that the iPLA2 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 iPLA2s

Based solely on BEL effects, the iPLA2 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. Baasinde, M. A. Balboa, and E. A. Dennis, manuscript in preparation.
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 iPLA2.

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 iPLA2 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 P388D1 macrophages (36), BEL is ineffective in inhibiting this release (21).

Involvement of an iPLA2 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 Ca²⁺-independent manner (37). Consistent with the possible involvement of an iPLA2, 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 Ca²⁺ lends credence to the possible involvement of an iPLA2.

It is generally difficult to rationalize the involvement of a Ca²⁺-independent enzyme in processes such as AA release, which in most cases is strongly Ca²⁺-dependent. Wolf and Gross (39) have recently reported that a 40-kDa myocardial iPLA2 associates with calmodulin in a Ca²⁺-dependent manner, providing a mechanism through which Ca²⁺ may regulate a Ca²⁺-independent enzyme. According to these authors, myocardial iPLA2 is inactive when associated with calmodulin. Dissociation of the complex due to decreases in the Ca²⁺ concentration or addition of calmodulin antagonists renders the iPLA2 ready to attack phospholipids and release AA (39). This mechanism has been proposed to mediate the cardiac cycle-dependent alterations in PLA2-catalyzed release of AA (39).

Before the reported association with calmodulin, the 40-kDa iPLA2 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 iPLA2 activity identified in myocardium and pan-

FIG. 2. A model for PLA2-mediated pathways for AA metabolism in P388D1 macrophages. PAF receptor-mediated AA mobilization in lipopolysaccharide (LPS)-primed P388D1 macrophages involves the action of two distinct Ca²⁺-dependent PLA2s, i.e. cPLA2 and sPLA2, acting on different AA pools located at distinct cellular membranes. The iPLA2 mediates incorporation of AA at these two intracellular locations before further remodeling reactions take place. PGE₃, prostaglandin E₃; PLC, phospholipase C; FA, fatty acid; [1,4,5]-IP₃, inositol 1,4,5-trisphosphate; R, fatty acid or alcohol; PX, phosphobase. Adapted with permission from Ref. 21.
Minireview: Intracellular Calcium-independent Phospholipase A<sub>2</sub>

The importance of the intracellular iPLA<sub>2</sub> in control of cell function has not been clearly established at present, despite the fact that iPLA<sub>2</sub>s have been found to exist in all cells and tissues examined. Currently, many new iPLA<sub>2</sub> s are being purified and characterized (8, 42–44). The widespread occurrence of iPLA<sub>2</sub> 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<sub>2</sub> s. Currently, much of the data available on cellular iPLA<sub>2</sub> 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<sub>2</sub> s in cellular functioning.

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Function and Inhibition of Intracellular Calcium-independent Phospholipase A$_2$

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J. Biol. Chem. 1997, 272:16069-16072.
doi: 10.1074/jbc.272.26.16069

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