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REGULATION AND INHIBITION OF PHOSPHOLIPASE A_2

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ABSTRACT

In recent years, there has been great interest in the study of phospholipid metabolism in intact cell systems. Such an interest arises mainly from the discovery that cellular membrane phospholipids serve not only in structural roles, but are also reservoirs of preformed second messenger molecules with key roles in cellular signaling. These second messenger molecules are generated by agonist-induced activation and secretion of intracellular and extracellular phospholipases, respectively, i.e. enzymes that cleave ester bonds within phospholipids. Prominent members of the large collection of signal-activated phospholipases are the phospholipase A_2s. These enzymes hydrolyze the *sn-2* ester bond of phospholipids, releasing a free fatty acid and a lysophospholipid, both of which may alter cell function. In addition to its role in cellular signaling, phospholipase A_2 has recently been recognized to be involved in a wide number of pathophysiological situations, ranging from systemic and acute inflammatory conditions to cancer. A growing number of pharmacologic inhibitors will help define the role of particular phospholipase A_2s in signaling cascades.

INTRODUCTION

Arachidonic acid (AA; 5,8,11,14-eicosatetraenoic acid) metabolism has been under active investigation in pharmacology, physiology, and biochemistry for over 25 years. In spite of the widespread recognition that AA metabolites have a large number of physiological roles, details regarding AA formation continue to be uncovered. As a free fatty acid, AA levels are very low in cells and thus

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its formation generally limits synthesis of AA metabolites. AA is found in the *sn-2* position of membrane phospholipids, where it can potentially be liberated by the deacylating action of a variety of different lipases. Direct cleavage of AA from the *sn-2* position via phospholipase A_2 (PLA_2) is a key step in deacylation in cells (and is the focus of this review), but it is important to note that theoretically AA could be generated by a number of other pathways as well. These include phospholipase C, which forms diacylglycerol that could be cleaved to generate AA via a mono- or diglycerol lipase, as well as phospholipase D–generating phosphatidic acid that could be further metabolized by phosphatidic acid phosphohydrolase to diacylglycerol. Less widely recognized is the fact that levels of AA can increase in cells when its utilization in the reacylation of lysophospholipids is inhibited.

While none of these pathways have been unequivocally demonstrated to be as relevant as the one controlled by PLA_2 under physiological conditions, all these reactions are important to consider when one examines the large number of articles in the literature in which cells or tissues are labeled with AA and then AA release or AA metabolites are measured. Investigators commonly assume that such release or metabolite formation is a direct measure of PLA_2, but this assumption must be directly demonstrated in each case, as other enzymatic reactions could contribute to AA formation. Confirmation of involvement of PLA_2 in AA release can be obtained by several different approaches. These include (*a*) parallel assessment of the generation of lysophospholipid, the other product formed by PLA_2 in addition to AA, even though lysophospholipids are readily metabolized and may be difficult to assess; (*b*) in vitro assay of PLA_2 activity in subcellular fractions prepared from treated cells; (*c*) the use of PLA_2 antibodies to immunoprecipitate and assess by immunoblotting specific isoforms of PLA_2; (*d*) Northern blot probing for the presence of PLA_2 message; and (*e*) the use of chemical or antisense inhibitors of PLA_2. In this review, we focus our presentation on the identity and role of different forms of PLA_2 and the use of PLA_2 inhibitors to define the structure and function of these forms.

Role of PLA_2 in Inflammation

A large number of different types of plasma membrane receptors, including many that act via heterotrimeric GTP-binding proteins or tyrosine kinases, have been demonstrated to induce activation of PLA_2. This enzyme cleaves the *sn-2* fatty acyl bond of phospholipids (Figure 1), producing a free fatty acid and a lysophospholipid (1, 2). AA is the precursor of a large family of compounds known as the eicosanoids (based on their derivation from the precursor), which includes cyclooxygenase-derived prostaglandins and lipoxygenase-derived leukotrienes (3). The eicosanoids possess a wide spectrum of biological

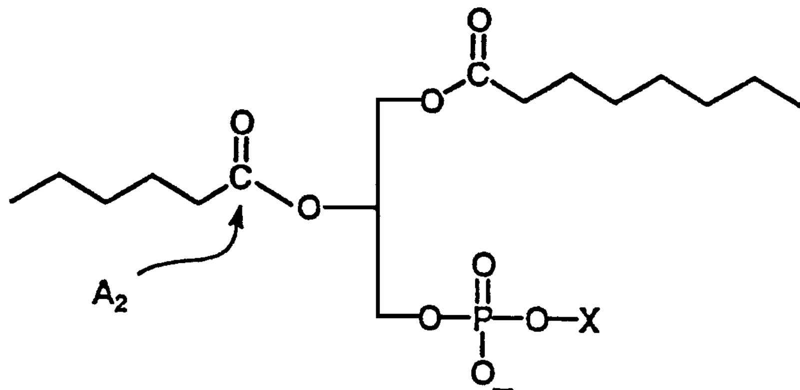

Figure 1 Phospholipid structure and phospholipase A_2 cleavage site

activities, among which is their ability to mediate a number of the signs and symptoms associated with inflammatory reactions (3). Aspirin and most of the widely known nonsteroidal, antiinflammatory drugs currently in use inhibit cyclooxygenases, thereby suppressing the synthesis of prostaglandins (4, 5). Other approved drugs or drugs in development block the lipoxygenase pathway or serve as leukotriene antagonists (5a).

In addition to being a required step for eicosanoid biosynthesis, PLA_2 plays another important role in inflammation (6). The other compound released by its action on membrane phospholipids, the 2-lysophospholipid, is in some settings utilized to form platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), another potent inflammatory mediator (7). Thus, PLA_2 is an attractive target for drug discovery because if one could inhibit PLA_2, the synthesis of all three inflammatory mediators (the prostaglandins, leukotrienes and PAF) could potentially be blocked.

ENZYMOLOGY

Phospholipase A_2 Groups

A major drawback in the development of a universal PLA_2 inhibitor is that mammalian cells generally contain more than one PLA_2, making it difficult to understand the regulation of AA mobilization and PAF synthesis at the molecular level. Nonselective inhibition of PLA_2 would also block other PLA_2-mediated reactions that, although not directly involved in inflammation, are needed for normal cell function (i.e. membrane remodeling and phospholipid catabolism). Thus, a key question is: Which PLA_2 is involved in the generation

of inflammatory lipid mediators? Answering this question requires knowledge of the characteristics of the currently recognized forms of PLA_2 (1). In 1997, an updated classification of the PLA_2s was presented (2), based on the comparison of nucleotide gene sequences. Since then, a new PLA_2 group, Group X, has been identified (8) and another form of PAF acetyl hydrolase, Group VIIB, has been established (9, 10). The characteristics of these PLA_2 group types are listed in Table 1. Recently, a paper appeared (11) that describes two new forms of Group IV $cPLA_2$, termed β and γ. These two forms have been classified as Groups IVB and IVC, and are included as such in Table 1 but are not discussed further in this review.

If instead of utilizing sequence data one focuses on biological properties, the classification of the PLA_2s is simplified to three main types: the secretory PLA_2 ($sPLA_2$), the cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), and the intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$). There is also a class of PLA_2s called PAF acetyl hydrolases, which appear to act on PAF and oxidized lipids (9, 10, 12, 13) and will not be discussed further here.

The $sPLA_2$s are all low molecular mass enzymes ($\sim$14 kDa) with a very rigid tertiary structure arising from the presence of 5–8 disulfide bonds. This confers on these enzymes both stability against proteolysis and resistance to denaturation, which allows them to retain activity in the extracellular fluids where they are found. The $sPLA_2$s do not manifest significant fatty acid selectivity in vitro, but they exhibit a requirement for millimolar Ca^{2+} (Table 1). In mammalian cells as many as five different $sPLA_2$ enzymes exist, i.e. those belonging to Groups I, IIA, IIC, V, and X (2, 14).

The $cPLA_2$, or Group IV PLA_2, is a high molecular mass (85-kDa) enzyme, found in the cytosolic fraction of practically all cell types that have been studied. This enzyme possesses characteristics that suggest it is involved in receptor-activated signaling cascades. The enzyme is phosphorylated by kinases of the mitogen-activated protein kinase cascade, which results in a mild increase in its specific activity. Moreover, the enzyme is able to translocate to membranes in response to increases in intracellular Ca^{2+} via a calcium-lipid binding (CaLB or C-2) domain within the protein. Finally, $cPLA_2$ possesses a preference for phospholipids containing AA (1, 15, 16). The $cPLA_2$ has recently been shown to possess a pleckstrin homology domain through which the enzyme is thought to strongly interact with phosphatidylinositol 4,5-bisphosphate. This interaction may help facilitate enzyme activation (17).

The $iPLA_2$s are the most recently identified members of the PLA_2 superfamily. Although numerous Ca^{2+}-independent PLA_2 activities have been reported from many tissues and cell homogenates (18), only one of them has been sequenced and characterized in detail. This $iPLA_2$ is the Group VI enzyme (19). It shares some characteristics with the $sPLA_2$s and others with the $cPLA_2$. Like the

Table 1 Phospholipase A_2 groups[a]

Group		Sources	Location	Size (kDa)	Ca^{2+} requirement	Disulfides	Molecular characteristics
I	A	Cobras, kraits	Secreted	13–15	mM	7	His-Asp pair
	B	Porcine/human pancreas	Secreted	13–15	mM	7	His-Asp pair, elapid loop
II	A	Rattlesnakes, vipers, human synovial fluid/platelets	Secreted	13–15	mM	7	His-Asp pair, carboxyl extension
	B	Gaboon viper	Secreted	13–15	MM	6	His-Asp pair, carboxyl extension
	C	Rat/mouse testes	Secreted	15	MM	8	His-Asp pair, carboxyl extension
III		Bees, lizards	Secreted	16–18	MM	5	His-Asp pair
IV	A	Raw 264.7/rat kidney, human U937/platelets	Cytosolic	85	$<\mu M$		Ser-228 in GLSGS consensus sequence, Arg-200, Asp-549 required; Ser-505 phosphorylation site; CaL B domain; PH domain
	B	Human brain	Cytosolic	100	$<\mu M$		N-terminal extension, Ser-228
	C	Human heart/skeletal muscle	Cytosolic	65	None		Prenylated; Ser-228; lacks CaLB domain and Ser-505/Ser-727 phosphorylation sites
V		Human/rat/mouse heart/lung, $P388D_1$ macrophages	Secreted	14	MM	6	His-Asp pair, no elapid loop, no carboxyl extension
VI		$P338D_1$ macrophages, CHO cells	Cytosolic	80–85	None		GXSXG consensus sequence, ankyrin repeats, 340-kDa complex
VII	A	Human plasma	Secreted	45	None		GXSXG consensus sequence, Ser-273, Asp-296, His-351
	B	Bovine brain	Cytosolic	42	None		Myristoylated at N terminus
VIII		Bovine brain	Cytosolic	29	None		Ser-47
IX		Marine snail	Secreted	14	MM	6	His-Asp pair
X		Human leukocytes	Secreted	14	mM	7	His-Asp pair

[a]Modified from Dennis (2)

$sPLA_2$s, the $iPLA_2$ exhibits no apparent substrate specificity for AA-containing phospholipids and appears not to be subjected to posttranslational covalent modifications. The $iPLA_2$ shares the size, intracellular localization, and perhaps elements of the catalytic mechanism with $cPLA_2$ (19). A unique feature of the $iPLA_2$, in addition to the absence of a Ca^{2+} requirement, is that it contains eight ankyrin motifs at the N-terminal half of the molecule (20–22).

Catalytic Mechanisms

Mechanistic studies by several laboratories have demonstrated that catalysis by the $sPLA_2$s does not take place via the formation of the classical acyl-enzyme intermediate of serine esterases (1). Instead, the $sPLA_2$s use a His residue, assisted by an Asp to polarize a bound H_2O, which then attacks the carbonyl group. The Ca^{2+} ion, bound to the conserved Ca^{2+} loop, is required to stabilize the tetrahedral transition state intermediate (1). Thus, for this class of PLA_2s, the Ca^{2+} plays an active role in catalysis.

Conversely, the catalytic mechanism of the Group IV $cPLA_2$ is completely independent of Ca^{2+}. As indicated above, $cPLA_2$ requires Ca^{2+} to interact with the membrane where its substrate is localized (1, 15, 16). The $cPLA_2$ appears to function as a serine hydrolase, acting via an acyl-enzyme intermediate (23, 24). Although its catalytic mechanism has not been fully clarified, $cPLA_2$ may define a new class of serine hydrolases, because a role for His, as in the classical Ser/His/Asp triad, has not (at least so far) been demonstrated (24).

By analogy with the $cPLA_2$, the $iPLA_2$ also appears to function as a serine hydrolase, with the active Ser residue located in the middle of the consensus sequence GXSXG, which is common to many other lipases (19–21). Recent evidence has suggested that this enzyme operates via an acyl-enzyme intermediate (25). Other $iPLA_2$ residues important for catalysis have yet to be described.

Cellular Regulation

Recent advances in the understanding of PLA_2 have revealed that, in general, not one but several PLA_2s are involved in cellular regulation and lipid messenger formation. A general mechanism for the role of multiple forms of PLA_2 has been suggested from studies carried out by several laboratories. This mechanism includes participation of at least two different PLA_2s, namely $cPLA_2$ and $sPLA_2$, in the generation of AA in cells. Activation of the $cPLA_2$ is the foremost event (26–28) and may be mediated by several signals, such as phosphorylation cascades (29–37), intracellular Ca^{2+} elevations (37, 38), and perhaps phosphatidylinositol 4,5-bisphosphate levels (17).

The synchronous coupling between these signals may converge to produce a prolonged activation of the $cPLA_2$ (26, 37). In cells not expressing $sPLA_2$, the

$cPLA_2$ probably accounts for the vast majority of AA mobilized during cellular activation. However, in those cells that contain $sPLA_2$, the bulk of AA release appears to be mediated by the $sPLA_2$, not the $cPLA_2$ (26–28, 39–41). Coincidentally, many types of eicosanoid-producing cells (e.g. phagocytes, mast cells, platelets) synthesize and secrete $sPLA_2$ (42). This enzyme, once secreted, associates with the outer surface of the cells and then releases AA, which can be captured by surrounding cells to produce eicosanoids (27, 39, 43–45). Recent studies have demonstrated that, despite its lack of AA specificity, the $sPLA_2$ releases AA in preference to other fatty acids (42). Why this is so is unknown. Interestingly, $sPLA_2$ action appears to be somehow dependent on an active $cPLA_2$ (26, 27, 40, 41, 45). Thus, $cPLA_2$ is key for AA signaling even in those settings where the $sPLA_2$ is the major effector of the response. This essential role of $cPLA_2$ in AA metabolism has been highlighted by recent experiments utilizing $cPLA_2$ knock-out mice, in which the cells generated significantly less AA-derived metabolites and PAF (46, 47).

What about the $iPLA_2$? Although this enzyme does not appear to be directly involved in effecting stimulated AA release, it is important for AA metabolism, in particular for phospholipid fatty acid remodeling (19, 28, 48, 49). Thus, the $iPLA_2$ participates in the main pathway through which the cells incorporate AA and other fatty acids into membrane phospholipids (19). This is an interesting concept because there is strong evidence that the AA-releasing PLA_2s use different AA pools for the release (26, 50). Thus, by regulating fatty acid remodeling reactions, the $iPLA_2$ may influence the subcellular distribution of AA among different compartments and the relative amount of fatty acid present in each compartment.

PHARMACOLOGICAL INHIBITION

Chemical Inhibitors

The most straightforward approach to assessing the implication of a specific PLA_2 in a given process is to inhibit its activity by using chemical inhibitors. As indicated earlier, inhibition of specific PLA_2s constitutes a potentially useful approach to treating both acute and chronic inflammatory disorders. Unfortunately, no potent and absolutely type-specific PLA_2 inhibitors are widely available to investigators. However, a number of compounds do behave as potent, reasonably type-selective inhibitors, and these may be prototypes for the development of more selective drugs.

The $sPLA_2$s were the first forms of PLA_2 to be identified, isolated, and characterized, and a large number of reports have appeared describing the properties of several reputed $sPLA_2$ inhibitors (51, 52). Typical compounds reported as "classical" PLA_2 inhibitors include antimalarial drugs (e.g. mepacrine),

aminoglycosides, alcohols, and polyamines (51, 52). These molecules generally do not inhibit PLA_2 per se, but act by blunting PLA_2 interaction with its substrates or even Ca^{2+} (51, 52). Therefore, the lack of specificity of such compounds indicates that they should not be used as "PLA_2 inhibitors". Articles reporting use of many of the above compounds continue to appear in the literature.

Similarly, nonspecific covalent-modifying PLA_2 agents such as manoalide (53–55) or p-bromophenacyl bromide (56, 57) have received great attention as PLA_2 inhibitors. Whereas these compounds do generally (and often potently) inhibit $sPLA_2$ in vitro, and not $cPLA_2$ or $iPLA_2$, this inhibition is due to the covalent blockage by these compounds of exposed Lys or His residues. Thus, in whole cell systems, these compounds will likely interact with many different proteins, making it impossible to draw definitive conclusions about their effects. In spite of these limitations, p-bromenacyl bromide is still commonly, and we believe incorrectly, referred to as a "selective PLA_2 inhibitor" in cellular studies, even in recent publications. Other compounds that are phospholipid substrate analogues do serve as reversible inhibitors of PLA_2 and may have a preference for $sPLA_2$; these include thioether amide phospholipids (58–60) and phosphonate transition state analogues (61–64). Unfortunately, none of these appears to be particularly potent either in vitro or in vivo. Furthermore, these compounds are amphipathic and usually aggregate or partition into micelles or membranes. Such reversible, competitive, active-site inhibitors require special kinetic evaluation (65) whereby their concentrations are expressed in mole fraction units (66). When IC_{50} values determined in this manner are converted into volume units, these compounds usually inhibit in the low millimolar range. Although such inhibition is not potent enough for pharmacological intervention, these compounds can be useful in mechanistic studies.

More specific $sPLA_2$ inhibitors have recently been described. Among them, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy) propane sulfonic acid (LY311727) (67) (Figure 2*A*) is probably the best characterized and has proven to be useful as a selective $sPLA_2$ inhibitor in studies aimed at clarifying the $sPLA_2$ role in AA mobilization in whole cell systems (25). LY311727 is an indole derivative, whose chemical structure was refined by X-ray crystallography, using the active site of the human Group IIA $sPLA_2$ as a template (67). This compound binds in the low nanomolar range and is selective for Group IIA $sPLA_2$ over Group IB $sPLA_2$; however, it does not necessarily distinguish among other $sPLA_2$ groups. Although it was designed as a Group IIA PLA_2 specific inhibitor, it has been found that LY311727 also inhibits Group V $sPLA_2$ (68).

Owing to the central role of the $cPLA_2$ in AA signaling, design of $cPLA_2$ inhibitors has recently been an area of great interest. Two mechanism-based

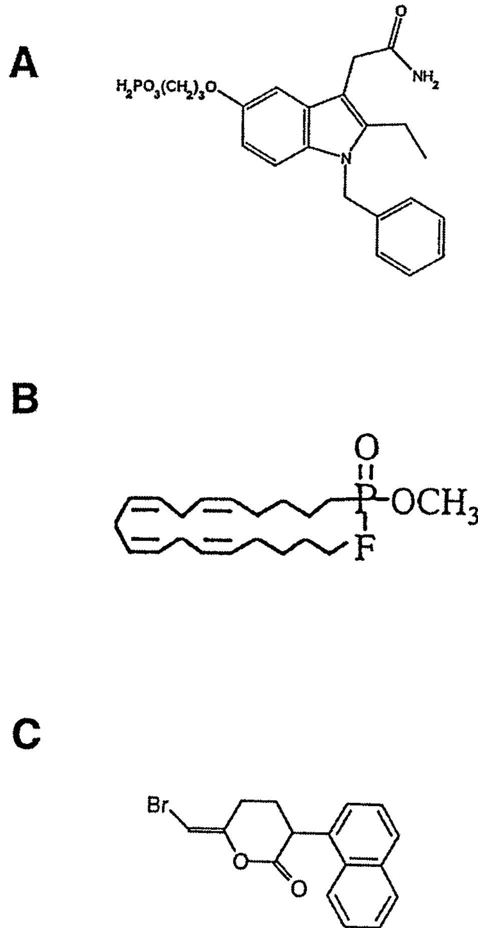

Figure 2 Phospholipase A_2 inhibitors. (*A*) indole derivative, LY311727 [3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy) propane sulfonic]; (*B*) methyl arachidonyl fluorophosphonate (MAFP); (*C*) bromoenol lactone (BEL, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one

$cPLA_2$ inhibitors have been widely used: arachidonyl trifluoromethyl ketone (AATFMK, also referred to as $AACOCF_3$) (69–72) and methyl arachidonyl fluorophosphonate (MAFP) (26, 73) (see Figure 2). The two compounds share a common chemical structure: an arachidonyl tail coupled to a Ser-reactive group. Whereas both AATFMK and MAFP are potent $cPLA_2$ inhibitors in vitro, the latter appears to be preferred over the former in whole cell systems. The reason is that AATFMK is a reversible, slow-binding inhibitor, i.e. it takes

a long time for its full inhibitory activity to develop (70). Whereas this may not be a problem in the in vitro situation and in certain cells such as platelets (71, 72), it might pose a significant problem in other cells, where it may act to inhibit other enzymes or may be reduced to an unreactive alcohol. MAFP does not have that problem. Moreover, AATFMK, but not MAFP, also inhibits cyclooxygenases (45, 72).

Unfortunately, both AATFMK and MAFP also potently inhibit the Group VI iPLA$_2$ (70, 74, 75). This is not a surprising finding because both the cPLA$_2$ and the iPLA$_2$ appear to use a central Ser for catalysis and probably similar catalytic mechanisms. There is one instance in which it appears to be possible to ascertain whether the inhibitory effects of AATFMK and MAFP are due to the cPLA$_2$ or the iPLA$_2$. This involves the parallel use of bromoenol lactone [BEL, also referred to as haloenol lactone suicide substrate (HELSS); Figure 2*C*], an inhibitor selective for the iPLA$_2$ among PLA$_2$ types (26, 76). Thus, if a given response is inhibited by AATFMK and MAFP but not by BEL, that would indicate the involvement of cPLA$_2$ (26). Though a fairly selective iPLA$_2$ inhibitor relative to the other PLA$_2$s, BEL is known to inhibit other important effectors in signal transduction, e.g. phosphatidate phosphohydrolase (77, 78) and cPLA$_2$ at high concentrations (20). Therefore, caution should be exercised in interpreting positive results with BEL in intact cells, and this caution applies to AATFMK and MAFP as well. Unfortunately, numerous papers in the literature have relied only on the inhibition of a process by BEL to implicate iPLA$_2$ and we believe this may be misleading.

Antisense Inhibition

The inherent lack of specificity problems associated with the use of currently available chemical inhibitors could, in principle, be circumvented by inhibiting PLA$_2$ expression with antisense oligonucleotides. This strategy is probably one of the most promising for obtaining pharmacologically active inhibitors with low toxicity that are specific for a particular PLA$_2$ form. However, even these oligonucleotides may not be devoid of undesired side effects; they have delivery problems, and their adequacy must be established on a case-by-case basis (79).

Antisense oligonucleotide inhibition of PLA$_2$ was first described for sPLA$_2$ (80) and has since been described for all major PLA$_2$ forms present in cells, Group V sPLA$_2$ (39, 81), cPLA$_2$ (82–84) and iPLA$_2$ (49). These studies have generally confirmed evidence obtained with chemical inhibitors and have thus provided some of the strongest evidence that each of the PLA$_2$ types plays a different role in AA signaling and cell functioning. However, antisense oligonucleotides do not easily translate into pharmacologically useful inhibitors in humans.

CONCLUDING REMARKS

The importance of PLA_2 enzymes in the control of key aspects of cellular physiology is well established but it is anticipated that new PLA_2 forms and roles will be uncovered. The challenge is to identify the role(s) that each of the multiple PLA_2 forms plays in cells. Much of the data available on PLA_2 regulation at the cellular level rests on the use of chemical inhibitors that are not totally type-specific. However, these inhibitors may offer leads for the development of more selective agents that can specifically target a single PLA_2 enzyme. On the other hand, antisense RNA inhibition of specific PLA_2s has proven to be an alternative and sometimes very successful approach to uncover new roles for PLA_2 in cellular function.

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