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COMMENTARY

PHOSPHOLIPASE A_2: FUNCTION AND PHARMACOLOGICAL REGULATION

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Phospholipases are a class of enzymes that catalyze the hydrolysis of membrane phospholipids to release free fatty acids. The existence of these enzymes has been known for over 100 years (for historical review, see ref. 1), but it is only within the last 20 years that their importance in biological processes has been appreciated. An exhaustive review of the structure and biochemistry of all phospholipases is not possible in this commentary, but several excellent reviews of this general area have been published recently [2–4]. Instead, what follows is a commentary on a subset of phospholipases collectively defined as phospholipase A_2 (PLA_2) which cleave the sn-2-acyl bond of phospholipids producing equimolar amounts of lysophospholipids and free fatty acids. This specific class of phospholipases has attracted a lot of attention because of its ability to produce substrate for the generation of inflammatory lipid mediators. The fact that intracellular levels of arachidonic acid (AA) are extremely low [5, 6] further suggests that PLA_2 activation represents a rate-limiting step in the whole process of lipid mediator synthesis. Indeed, inhibition of PLA_2 activity offers an attractive therapeutic approach to the design of novel drugs for the treatment of inflammation and tissue injury. Additionally, the fact that anti-inflammatory steroids are thought to inhibit PLA_2 through the induction of a PLA_2 inhibitory protein called lipocortin [7–9] provides supportive evidence that direct PLA_2 inhibitors may lead to useful therapeutic agents.

On a cautionary note, PLA_2-mediated phospholipid hydrolysis is by no means the only source of free AA because other pathways may also lead to fatty acid release. For example, phosphatidylinositol is rich in AA, and the combined actions of phospholipase C and diglyceride lipase release AA [10–12]. For a detailed analysis on the regulation of intracellular AA levels, the reader is referred to an excellent review by Irvine [10].

Source and structure of PLA_2

Phospholipase A_2 enzymes are widespread, being found in nearly all cell types examined as well as in bacteria and protozoa. Over forty PLA_2 enzymes, principally from snake venoms and pancreas, have been sequenced and structurally defined [13–15]. In general, the molecular weights of the purified enzymes are low and within the range of 12,000–15,000. These enzymes contain a high degree of disulfide cross-linking and are extremely stable to heat and acid treatment; in fact, solubilization of membrane-bound enzymes can easily be achieved through mineral acid extraction [4]. Although studies have shown that there are clear structural differences among various PLA_2, the enzymes share some sequence homology, especially in their active site regions which bind calcium. Also, pH requirements are similar in that most of these enzymes are optimally active at neutral to alkaline pH. From an evolutionary viewpoint, these data suggest that the enzyme is a primitive molecule that is highly conserved within the animal kingdom and is derived from a common ancestral protein.

PLA_2 has a higher affinity for aggregated than non-aggregated phospholipid, and whether this is due to a conformational requirement by the enzyme or the substrate is as yet unclear. It should be stressed that kinetic studies with PLA_2 are problematic since classical Michaelis–Menten kinetics do not apply. It is frequently not recognized that enzyme activity increases exponentially when the substrate concentration reaches or exceeds the critical micelle concentration. In addition, PLA_2 can exist in either the inactivated or activated state. For example, pancreatic PLA_2 is secreted as the inactive zymogen and requires enzymatic cleavage by proteolytic enzymes for activation [16], whereas stimulated neutrophils release active PLA_2 [17–19]. The problem of determining enzyme activity is further compounded by the fact that the enzyme may exist in solution as monomers or dimers of varying stability. Unless experimental conditions are well-defined, these differences illustrate some of the potential pitfalls encountered with the study of this enzyme.

Activation of PLA_2

Various physiological stimuli have been suggested to activate PLA_2; angiotensin II, bradykinin, prolactin, and thrombin, for example, release AA when added to responsive cells [20–23]. However, in these studies release of free fatty acid is tacitly assumed to be indicative of PLA_2 action. This may or may not be the case since AA can also be derived from non-PLA_2-mediated phospholipid hydrolysis, as previously noted. Better proof that PLA_2 activation occurs after physiological stimulation was obtained recently when we showed that Interleukin 1 causes a marked increase in both cell-associated and extra-

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cellular PLA_2 activity in chondrocytes and synovial fibroblasts [24, 25]. In fact, the promotion of PLA_2 activity preceded the formation of prostaglandins by these cells. Since then, PLA_2 activation has also been demonstrated in sonicates of LTD_4-treated endothelial cells and smooth muscle cells [26]. This observation is especially intriguing because it suggests that end products of PLA_2 action can feedback to further activate PLA_2 and potentially amplify the production of lipid mediators.

Consequence of enzyme activation

Once activated, PLA_2 can mediate a variety of pathophysiological reactions either through a direct action or through subsequent transformation of its products (lysophospholipids and AA) to several potent biologically active substances such as prostaglandins, leukotrienes and platelet-activating factor (PAF). Lysophospholipids, the co-products of PLA_2 activation, are cytotoxic substances and membrane fusogens [27] and have been implicated in several human inflammatory conditions [28, 29]. Acetylation of lysophospholipid at the 2-hydroxy group gives rise to another lipid mediator, PAF, a potent platelet-aggregating substance and inducer of various inflammatory reactions such as erythema, chemotaxis, and increased vascular permeability [30, 31]. Finally, prostaglandins, thromboxane and leukotrienes, products formed from cyclooxygenase- and 5-lipoxygenase-catalyzed AA transformation, complete the family of inflammatory and allergic mediators derived from PLA_2 activation [32]. Suffice it to say, because of the potent effects of these lipid products on a variety of tissues, they have been implicated as major contributors to processes involving inflammation and tissue injury. Thus, inhibition of PLA_2 provides a "one step" approach to interfere with the production of these mediators and possibly lead to an alleviation of the disease process.

Apart from providing substrate for the generation of the mediators mentioned above, PLA_2 activation can modify cell membrane dynamics. The concept of phospholipid turnover has superceded our previous impressions that phospholipids are merely inert building blocks within membranes and lack functional importance. Instead, phospholipids are now known to undergo cycles of deacylation and reacylation and, depending on the phospholipid, the turnover rate may be rapid or gradual. Why membrane phospholipid turnover is constantly occurring under normal physiological conditions is unclear but may be related to the necessity of replacing auto-oxidized phospholipids as proposed by Dawson [33]. This turnover phenomenon may also reflect a fundamental signalling mechanism by which a cell responds to the changing extracellular environment. If so, total inhibition of PLA_2 activity could lead to undesirable effects such as phospholipid accumulation and lipid storage disorders.

Relevance of PLA_2 in disease

Involvement of PLA_2 in the pathophysiology of snake and bee venom toxicity is well documented [34–36]. Purified PLA_2 from snake venom has been shown to reproduce all the symptoms of snake bite such as acute pain, edema, hypotension, hemorrhage and neuromuscular junction blockage [36]. By contrast, the evidence is less clear in human disease, and a causal relationship between PLA_2 and the pathology of disease remains to be established. Available data suggest that PLA_2 is most likely involved in inflammation and tissue injury associated with various diseases.

Lysophospholipids induce gastric ulceration in rats and induce an inflammation similar to acute cholecystitis in the gall bladder mucosa [37]. Increases in net flux of Na^+ and acid secretion are observed after lysophosphatidylcholine administration [38–40], suggesting that PLA_2 may be an important factor in gastrointestinal disorders.

Increased levels of PLA_2 have also been found in rheumatoid arthritic joints [41], psoriatic skin [42], and serum from patients with pancreatitis [43, 44] and septic shock [45]. Moreover, exogenous administration of PLA_2, lysophospholipids, eicosanoids or PAF to animals has reproduced many of the symptoms associated with inflammatory disorders. In our own laboratory, we have demonstrated that an intradermal injection of PLA_2 elicits an intense acute inflammatory reaction [46].

Myocardial ischemia is postulated to be a disease involving PLA_2 activation, and an increase in lysophospholipids is found in damaged tissues [47, 48] with a parallel decrease in membrane phospholipids [49–51]. More recently, leukotrienes have also been implicated in myocardial infarction because of their potent vasoconstrictive properties [52]. These data strongly suggest that PLA_2 is activated during ischemia, although the source of PLA_2 is unclear and may either originate directly from cardiac tissues [53] or infiltrating neutrophils, a common feature of an infarcted area.

PLA_2 may also be critically involved in lung pathophysiology, especially asthma. A phospholipase A_2 is thought to hydrolyze disaturated phosphatidylcholine, a major component of lung surfactant, suggesting that it may be a causal factor in acute respiratory distress syndrome [54]. Lysophospholipids, leukotrienes and PAF have all been implicated in airway hyperreactivity [55] and PLA_2 activation may also modulate histamine release from basophils [56].

Endogenous regulation of enzyme activity

The regulation of PLA_2 activity is complex, and several factors modulate its activity. To what extent each factor contributes to the overall control of PLA_2 is undefined, although the existence of multiple mechanisms suggests that modulation of PLA_2 may vary from tissue to tissue. Nevertheless, there is little doubt that modulation of PLA_2 activity is highly desirable within the cellular environment because of the serious consequences that could result from random degradation of membrane phospholipids. Rather than listing all the possible factors that are believed to be involved in regulating PLA_2 activity [2], it is more useful to emphasize those factors that could potentially play important roles in controlling enzyme activity.

One obvious PLA_2 regulatory mechanism is the

formation of a proenzyme that requires proteolytic cleavage to generate an active enzyme. This has been demonstrated for pancreatic PLA_2 where conversion of the zymogen into active PLA_2 is accomplished by trypsin-catalyzed cleavage of an arginyl—alanine bond at the N-terminal [57]. It should be stressed that this type of regulation is unidirectional and has been implicated, but not unequivocally demonstrated, in the activation of PLA_2 derived from non-pancreatic tissues [58–60].

Calcium is a prime candidate for consideration as a PLA_2 regulator. A calcium binding site has been identified on PLA_2, and addition of calcium increases enzyme activity, whereas addition of Quin 2, a calcium chelator, inhibits enzyme activity [61, 62]. Furthermore, the calcium ionophore A23187 potentiates AA release from intact cells [63]. Although such studies demonstrate that phospholipase A_2 requires calcium for activity, its actual role is uncertain. Polarization of the substrate carbonyl by the divalent cation has been suggested on the basis of its close proximity to the scissile site in the crystal structure [64].

An area that is receiving increasing attention is the possible existence of endogenous peptides that can modulate PLA_2 activity. The discovery [65, 66] that steroids induce the synthesis and release of a phospholipase A_2 inhibitory protein, now called lipocortin, raises the possibility that endogenous proteins may play an important role. Depending on the cell type, one or more of these proteins may be produced to restrict PLA_2 activity [67, 68]. An extensive review of the discovery and pharmacology of this class of endogenous regulators has been published [69]. By analogy to other systems, the existence of an inhibitor usually suggests that a stimulatory counterpart should exist. Although stimulatory proteins have not been structurally defined, hints of their existence are available in the literature. For example, a peptide described as RCS-RF (rabbit aorta contracting substance releasing factor) induces the release of thromboxane A_2 from isolated perfused guinea pig lung [70]. Very recently, a peptide that activates PLA_2 (which is inhibitable with protein synthesis inhibitors) has been proposed to explain the LTD_4-mediated induction of prostaglandin synthesis by cultured smooth muscle cells [26]. PAF has also been suggested to stimulate PLA_2 in fibroblasts [71]. Thus, as more data become available on these proteins it will be possible to determine whether they contribute significantly to the overall control of PLA_2 activity.

Finally, several molecular entities involved in transmembrane signalling may also play a role in regulating PLA_2 (Fig. 1). Factors such as protein kinase C, guanine nucleotide regulatory proteins, inositol triphosphate and diacylglycerol derived from phospholipase C catalyzed reactions and cyclic AMP can all influence PLA_2 activity [72–74]. These intracellular second messengers can either operate independently of each other or form a network of signals that ultimately determine the level of enzyme action. Undoubtedly, more intracellular signals will be discovered, and advances in these seemingly unrelated areas of research will have a major impact on our understanding of the regulation of PLA_2 actions.

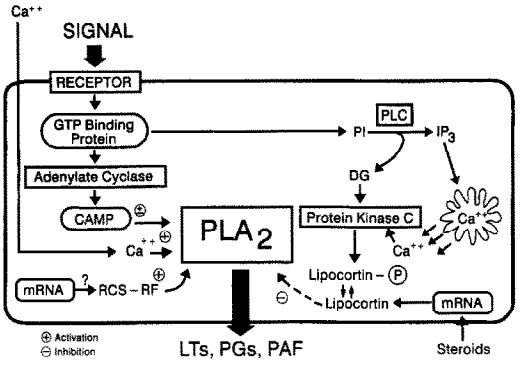

Fig. 1. Possible mechanisms leading to PLA_2 activation.

PLA_2 inhibitors

Numerous agents inhibit PLA_2 via different mechanisms and represent a diversity of structural types. To provide a cogent discussion of these putative PLA_2 inhibitors, it is necessary to classify these compounds by their chemical structures and/or modes of action. However, it should be stressed that overlap is inevitable, and this adopted format provides only a basis for speculating on the possible design of novel agents.

Agents that affect substrate–enzyme interface

The ability to interfere with the substrate–enzyme interface is shared by many PLA_2 inhibitors. PLA_2 activity is not only a function of substrate concentration and pH, but it is greatly influenced by the physicochemical state of the phospholipid substrate. As previously mentioned, PLA_2 is essentially inactive against monomeric substrate whereas enzyme activity is greatly increased at concentrations equal or above the critical micelle concentration. Even with substrate concentrations far exceeding the critical micelle concentration, any perturbation of the lipid–water interface diminishes enzyme activity [75]. Therefore, any agent that disturbs the architecture of the lipid–water interface could physically prevent PLA_2 from hydrolyzing phospholipid substrate. For example, compounds that make a membrane more fluid (e.g. halothane) or those that make the membrane more rigid (e.g. cholesterol) inhibit PLA_2 [76].

The critical importance of substrate architecture to expression of enzyme activity is further highlighted by studies where PLA_2 activity was examined at different temperatures [77]. It was demonstrated that the transition phase between a partially randomized state (with the consistency of a gel) and a highly ordered state (much like a liquid crystal) that is optimal for PLA_2 activity is temperature dependent.

Complex formation between phospholipid substrate and drug also prevents enzyme attack of the substrate. The following compounds [78–81] have all been reported to interfere with substrate–enzyme interface: local anesthetics (cincaine, butacaine and lidocaine), antimalarial agents (chloroquine, mepacrine), polyamines (spermine, spermidine and putrescine), antipsychotic agents (chlorpromazine, promethazine, and fluphenazine), *n*-alkanols

(methanol, *n*-tetradecanol and ethanol), antibiotics (streptomycin and gentamycin), and organic solvents (tetrahydrofuran and chloroform). Under the strictest terms, it is inaccurate to consider these agents as direct enzyme inhibitors, and conclusions drawn from their use in physiological experiments should be considered carefully. Clearly, the substrate used to detect PLA_2 inhibitors should not be influenced by the compound and should mimic closely the physicochemical state of membrane phospholipids. In our opinion, *Escherichia coli* membranes offer the optimal choice as the best alternative substrate to synthetic phospholipids for inhibitor studies [82].

Agents that modulate calcium levels

Since PLA_2 is dependent on calcium for activity, it is not surprising that compounds which influence cellular levels of calcium have been examined for inhibitory activity. Calcium antagonists such as bepridil and verapamil inhibit PLA_2, but the observed inhibition has been attributed to the formation of non-specific drug–phospholipid complexes rather than blockage of slow calcium channels [83]. Moreover, the concentration required to achieve PLA_2 inhibition far exceeds that required to inhibit slow calcium channels. Trifluoperazine and W-7, calmodulin antagonists, inhibit calcium ionophore induced-AA release by rabbit platelets [84]. However, calmodulin does not activate purified PLA_2 directly [85], suggesting that calmodulin antagonists probably work through an indirect mechanism. Collectively, these data suggest that the formation of drug–phospholipid complexes is more important than modulation of calcium levels by these drugs in terms of PLA_2 inhibition.

Non-steroidal anti-inflammatory agents

Anti-inflammatory agents are believed to owe their therapeutic efficacy to inhibition of cyclooxygenase. However, the following compounds also inhibit PLA_2 activity at higher concentrations: sulindac sulfide, tiaramide, indomethacin, sodium meclofenate and sodium flufenamate [86–88]. Franson and coworkers [89] have further shown that this inhibition is especially sensitive to calcium concentrations, the inhibitory activities of indomethacin and sodium meclofenamate being greatly diminished in the presence of high calcium concentrations (5 mM). More recently, lipoxygenase inhibitors, such as 4,8,11,14-eicosatetraynoic acid, nordihydroguaiaretic acid, and quercetin, have also been demonstrated to inhibit PLA_2 [90]. However, it should be emphasized that inhibition of PLA_2 by these agents can only be observed at concentrations that may not be easily attainable under *in vivo* conditions.

Agents derived from screening

Screening programs to uncover specific and potent PLA_2 inhibitors that will exert anti-inflammatory actions *in vivo* are currently ongoing. The first systematic description of such compounds was reported by Wallach and Brown [91] where a series of butyrophenone derivatives was reported to inhibit hog pancreas, cobra venom and bee venom PLA_2. The butyrophenone analog U-3,585 (Fig. 2), a non-competitive PLA_2 inhibitor, also inhibits collagen-induced platelet aggregation, prostanoid synthesis by isolated perfused guinea pig lung, and u.v.-induced erythema. Since then, other compounds such as KF-4,939 and CGP 35,949B have been reported to inhibit PLA_2 [92, *]. However, neither compound is specific because of their abilities to inhibit phospholipase C and antagonize leukotriene receptors respectively. Nonetheless, screening will continue as an important source of novel agents and, with the aid of computer substructure search, drug companies can use their libraries of compounds and select test compounds that stand a greater chance of being PLA_2 inhibitors.

Agents isolated from natural sources

Several natural products including glycyrrhizin, alpha-tocopherol and polymyxin B inhibit PLA_2 [93–95]. Recently, plastatin and plipstatin (Fig. 2) were reported to inhibit pancreatic but not snake venom PLA_2 [96, 97]. However, both inhibitors lack specificity since phospholipases C and D are equally inhibited.

As previously stated, anti-inflammatory steroids induce the synthesis of an endogenous PLA_2 inhibitory protein called lipocortin which has been sequenced recently [98]. Lipocortin is thought to interact directly with PLA_2, and there is a stoichiometric relationship between inhibition and enzyme. Since the anti-inflammatory action of steroids may be due to the release of lipocortin, it may be possible to design traditional organic molecules as lipocortin mimics provided a small active fragment (mol. wt < 2000) can be identified. The potential exists for the side-effects of steroids to be duplicated by such inhibitors.

Agents that covalently bind to PLA_2

One of the most frequently used PLA_2 inhibitors is para-bromophenacylbromide (pBP), and the proposed PLA_2 involvement in several physiologic processes has been based on the effect of this reagent. This reactive organic reagent forms a covalent bond with the histidine residue on PLA_2 and, because of the spatial proximity of this amino acid to the calcium binding site, alkylation of histidine will lead to diminished enzyme activity [99]. Interestingly, calcium can protect the inactivation by pBP of a number of PLA_2. It should be stressed that pBP will also covalently bind to any nucleophilic group and, *in vivo*, would be expected to alkylate thiol and amine groups of many proteins. Unless other supporting data are available, conclusions based solely on pBP can be misleading. Furthermore, it is unlikely that agents that irreversibly bind to PLA_2 will be useful as therapeutic drugs, because of hapten formation that leads to blood dyscrasia.

A recent natural product, manoalide, has been described as a PLA_2 inhibitor [100]. This sesterterpenoid isolated from the sponge, *Luffanella variabilis*, inhibits PLA_2 at low concentrations and has demonstrated *in vivo* anti-inflammatory activity. Manoalide reacts with several lysine residues on

* M. A. Bray, A. Beck, P. Wenk, F. Manki, U. Niederhauser, M. Kuhn and A. Sallmann, *Sixth International Conference on Prostaglandins and Related Compounds*, Florence, Italy, Abstr. 252 (1986).

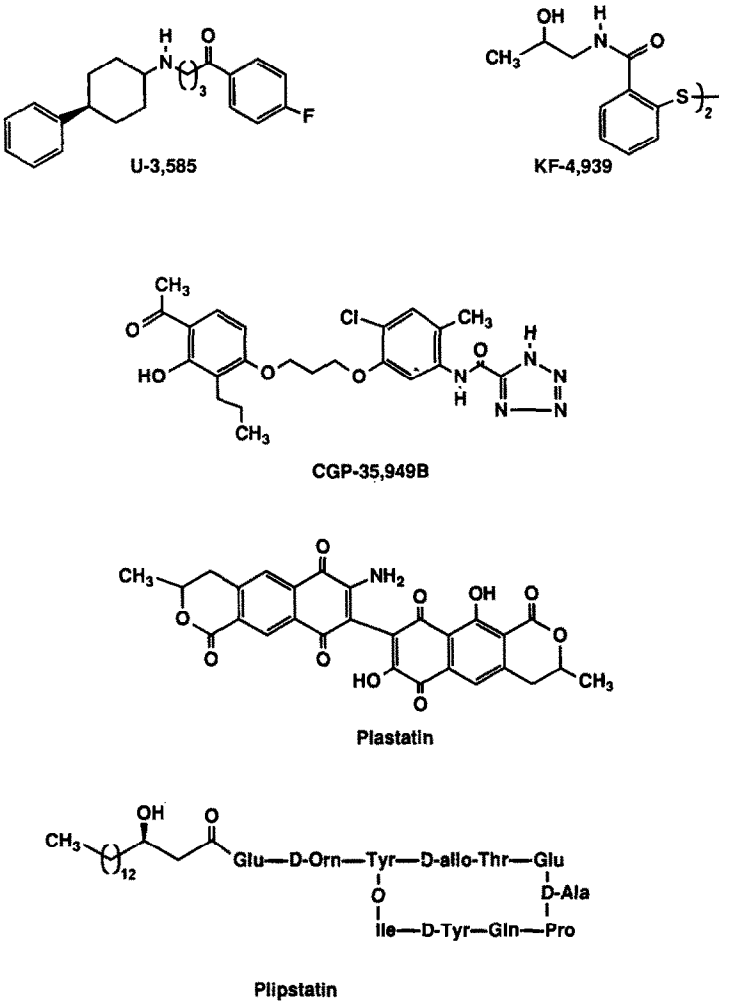

Fig. 2. Agents derived from screening.

PLA_2, which reduces the hydrolysis of phosphatidylcholine but not phosphatidylethanolamine by PLA_2 [101]; in fact, phosphatidylethanolamine hydrolysis is enhanced. This differential effect suggests that at least one of the lysine residues is at or near the substrate binding site of PLA_2. It has been shown that manoalide first forms a noncovalent complex with the enzyme which is then converted to the open form containing an α,β-unsaturated aldehyde and a cross-conjugated aldehyde/carboxylic acid (Fig. 3). The amine groups of the lysine residue then form a covalent bond with manoalide via Michael addition to the double bond adjacent to either aldehyde. Clearly, if PLA_2 catalyzes the conversion, manoalide can be considered a natural suicide enzyme inhibitor.

Substrate analogs

The design of active site-directed inhibitors has been explored extensively by Van Deenen and De Hass [102]. They observed that, in order to be a PLA_2 substrate, a fatty acid ester bonded vicinally to a phosphate ester is required (Fig. 4). By systemically modifying the structure of phosphatidylcholine, these workers have synthesized several analogs that are PLA_2 inhibitors. These include compounds with opposite stereochemical configuration, two position ester bond modifications, and an amide

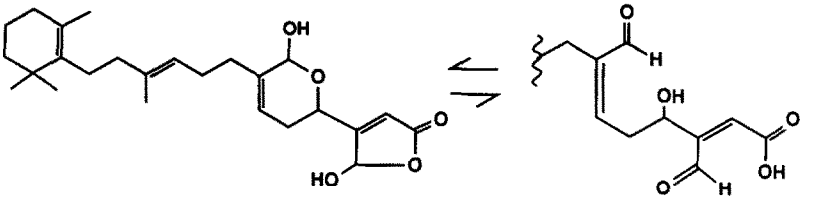

Fig. 3. Manoalide—natural and open forms.

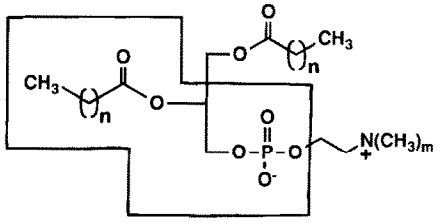

Fig. 4. Minimal requirements for a PLA_2 substrate.

linkage in place of an ester bond (example **1**, Fig. 5). Of particular interest is a series of *n*-alkylglycoletherphosphorylcholines (example **2**) which contain all the structural features that Van Deenen has postulated as minimal substrate requirements except that the alkyl ester is replaced with an alkylether [103]. Transition-state-like inhibitors have also been synthesized [104], and one such example is the difluoromethylene ketone phospholipid analog (example 3). The replacement of the susceptible ester with a polarized ketone provides evidence that isoteric replacement may be an effective strategy for designing PLA_2 inhibitors.

Conclusions

In this brief review, we have attempted to highlight the importance of PLA_2 in biological systems and the various pharmacological agents that purportedly inhibit PLA_2. There are gaps in our knowledge of this interesting enzyme, which behaves very differently from other esterases, and these differences, especially in terms of kinetics, perhaps explain why it has been difficult to obtain consistent results under all conditions. The availability of a potent and specific inhibitor will undoubtedly be a useful tool to unravel some of the uncertainties surrounding this enzyme but, unfortunately, none of the current crop of compounds is entirely satisfactory as a PLA_2 inhibitor. It is also not entirely clear whether PLA_2 derived from different sources have different drug sensitivities, and much work remains to be done on this important question. Nevertheless, the pharmacological data have provided clues to the direction that should be taken to develop better compounds and, with ingenuity, such compounds should be available in the near future. It is conceivable that understanding the role of PLA_2 in biological systems will lead to the use of PLA_2 inhibitors as a new class of drugs in a variety of diseases.

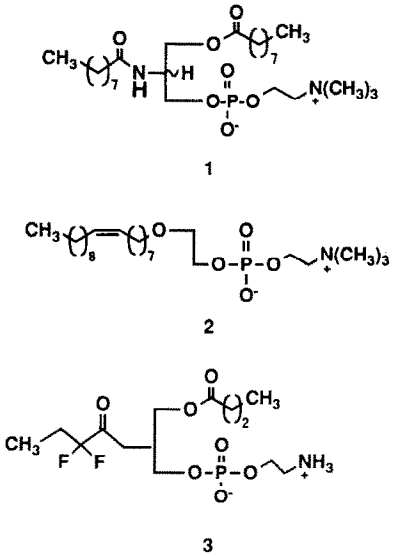

Fig. 5. Phospholipid analogs as PLA_2 inhibitors.

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