review

Approaches to the study of mammalian cellular phospholipases

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Abstract The purpose of this article is to present some problems encountered in the study of cellular phospholipases and some approaches used in answering these problems. Examples of a number of phospholipases that differ in their origin and positional specificity are given. Consideration is given to enzyme purification, mode of action, and interaction with substrate and function in situ. — **Waite, M.** Approaches to the study of mammalian cellular phospholipases. *J. Lipid Res.* 1985. **26:** 1379–1388.

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The purpose of this review is to describe the general problems faced in studying cellular phospholipases and some of the approaches that have been used to meet these challenges. The enzymes chosen to be discussed represent a variety of types derived from different origins. Some are soluble or easily removed from membranes while others are integral membrane proteins that require extraction with detergents or organic solvents. Since only selected enzymes are covered, recent reviews on phospholipases are recommended for a more comprehensive coverage of the area (1–3).

Cellular phospholipases exist in almost every type of cell studied thus far and many cells contain a multitude of phospholipases. They are present in limited amounts so that a high-fold purification is required to achieve purity of the enzyme. Major problems in purification have resulted from their hydrophobic nature that results in aggregation or binding to lipid. The presence of detergents and/or glycerol provides a stabilizing environment and permits reasonable purification procedures.

Because of their low catalytic turnover numbers, the cellular phospholipases have most often been assayed using radiolabeled substrates. One of the earliest substrates for phospholipase assay was biosynthesized containing differently labeled fatty acids at positions one and two of the glycerol (4). Radiolabeled substrates are still widely used to differentiate between phospholipases A_1, A_2, C, and D, and to determine the relative activity of phospholipases A and the lysophospholipase that removes the second fatty acid. Recently, phospholipid analogs that contain thio- rather than oxyester have been employed permitting a more rapid spectrophotometric assay by coupling the product(s) that contain a free sulfhydryl group with Elman's reagent (4).

Major problems are encountered when cellular phospholipases are characterized with respect to their specificity and mode of action. Although often attempted, analysis using classical Michaelis-Menten kinetics is not valid since the substrates are used above their critical micellar concentration and, consequently, solution kinetics do not apply. Kinetic analysis of phospholipases is further complicated by the fact that the aggregate of the substrate phospholipid varies in size and nature of organization that is dependent upon acyl and polar head group of the lipid as well as the composition of the aqueous environment. For example, phosphatidylcholine and phosphatidylethanolamine under most conditions form entirely different types of aggregates, and phosphatidylethanolamine can form either bilayer or hexagonal array-like structures, depending on the pH and ionic composition of the medium (5).

Other approaches for the kinetic analysis of phospholipases have been used for the extracellular enzymes. These include monolayer systems (6), micelles composed of a mixture of detergent and phospholipid (7), micelles of pure phospholipid (8), and short-chain soluble phospholipids (9). The mixed micellar substrate system appears to hold considerable promise for the kinetic analysis of cellular phospholipases. In this case, the affinity for the mixed micelle (detergent plus phospholipid) as well as the affinity for phospholipid molecule in the micelle can be determined from experiments in which the total lipid and the ratio of detergent to phospholipid are independently varied. There are some notable problems with this approach, however, that have been dis-

cussed by Dennis (2). If the affinity of the enzyme for the Triton X-100 is sufficiently low and saturation levels of substrate can be achieved within the micelle, this is a good system to compare the relative activities of the enzyme on different substrates since the structures of the substrate micelles are comparable (10).

The functions of the cellular phospholipases are as varied as their cellular and tissue localization and properties. Over two decades ago it was postulated that a major function of phospholipases was to regulate the acyl composition of membrane phospholipid via a deacylation-reacylation cycle (11). Undoubtedly, the restructuring of phospholipids synthesized de novo by the insertion of long-chain polyunsaturated fatty acids into position two is essential for maintaining the required properties of the membrane.

Recently, considerable excitement about cellular phospholipases has been generated over their role in initiating the "arachidonate cascade." In this cascade, arachidonate that is released by the action of a phospholipase(s) is oxidatively metabolized by the lipooxygenase or prostaglandin H_2 synthase (12). Phospholipase C degrades phosphatidylinositol or its phosphate derivatives, phosphatidylinositol phosphate or phosphatidylinositol bisphosphate, to diacylglycerol that activates the protein kinase C (13). Likewise, it has been postulated that phospholipase A_2 is involved in the metabolism of platelet activating factor by forming the acyl group from position two of 1-O-alkyl lysophosphatidylcholine that is rapidly acetylated to form platelet activating factor (14).

The regulation of cellular phospholipases presents some very interesting challenges. Potentially, the cellular phospholipases can degrade and disrupt the membrane in which they are situated. Clearly, the regulatory processes for phospholipases are stringent. Up to this point very little is known about their regulation, although recent reports have suggested that a protein, macrocortin, is a regulator of the phospholipases involved in the "arachidonate cascade" (15). Also, since many cellular phospholipases are either stimulated by or require Ca^{2+}, changes in Ca^{2+} fluxes probably are regulatory for the action of phospholipases.

Phospholipases A_1 and lysophospholipases

Phospholipases A_1 have been noted in a wide variety of tissues and subcellular organelles. Sources used primarily for their study include brain, liver, pancreas, plasma, and tumors. A few have been purified and partially characterized and some general characteristics are known. First, some have lysophospholipase and lipase activity in addition to their phospholipase A_1 activity. Also, many do not have absolute requirement for Ca^{2+} and, as is the case with the lysosomal phospholipase A_1, can be inhibited by Ca^{2+}. Third, some phospholipases such as the plasmalemma phospholipase A_1 (hepatic lipase) have relatively high esterase activity on monomeric substrate.

In this section those enzymes having high phospholipase A_1 relative to lysophospholipase activity will be covered first, followed by a description of those enzymes having lysophospholipase activity.

Two phospholipases A_1 with high lipase activity were purified from guinea pig pancreas (16). These were extremely cationic with pI = 9.3 but differed in their molecular weights (39,000 vs. 43,000). Unlike the lysophospholipase purified from beef pancreas, these enzymes did not readily attack position two of diacyl phospholipid. The hydrolysis of diacyl but not monoacyl phospholipids by the guinea pig enzymes was stimulated by sodium deoxycholate, a characteristic of most lysophospholipases.

Quite a different phospholipase A_1 appears in both the homogenates and secreted juice of sheep pancreas (17). This enzyme preferentially hydrolyzed the acidic phosphatidylinositol and phosphatidic acid but attacked phosphatidylcholine poorly, even in the presence of deoxycholate. While there are notable differences in the preparation isolated from the pancreas of different species, it is difficult to compare their activities owing to the differences in the assays used. It must be assumed, however, that they serve different functions for the three species.

A number of phospholipases A_1 exist in rat liver. The cytosolic phospholipase A_1 has been purified some 10-fold and characterized with regard to its requirement for surface charge and possible preference for substrate in hexagonal arrays (18). However, until further purification of the enzyme(s) is carried out, these interesting findings could be the result of multiple activities.

Two phospholipases A_1 have been purified to near homogeneity from the lysosomes and plasmalemma of rat livers. The lysosomal enzyme is actually polymorphic, differing in its isoelectric points and reported molecular weights (19). Hostetler, Yazaki, and van den Bosch (19) found five isoenzymes that range in their pI values from 5.2 to less than 4.0. The variability in the pI could be the result of enzyme catabolism by other lysosomal enzymes. The isoenzyme representing the bulk of total activity recovered, 60%, had a molecular weight of 34,000, based on its behavior on a gel filtration column. This contrasts with the finding in our laboratory that the major fraction with pI = 4.8–5.0 had a molecular weight of 56,000. These molecular weight values must be further examined when greater quantities of enzyme are available since, as glycoproteins, some interaction with the column matrix is probable. The activity of the isoenzymes on different substrates did not vary significantly (19).

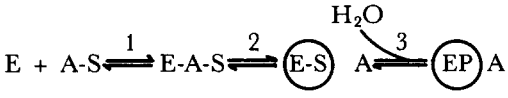
Hostetler et al. (19) report that acidic phospholipids were degraded most rapidly when used in mixed micelles prepared with Triton. Very low lysophospholipase activity was noted. On the other hand, we found that phosphatidylethanolamine was preferred and strongly acidic phospholipids were barely degraded when studied as pure lipid (20). This led to the appreciation that the physical

structure of the aggregated substrate is crucial in determining activity. It appears that the hexagonal array formed by phosphatidylethanolamine is preferred to the bilayers of other phospholipids but, when mixed with Triton to form mixed micelles, the specificity changes.

It was of interest to note that the acidic phospholipids do not exhibit the expected "surface dilution" kinetics seen with phosphatidylcholine (20) and predicted by the model of Deems, Eaton, and Dennis (7). Surface dilution of substrate occurs as the ratio of substrate to detergent decreases. The lack of surface dilution kinetics found with the acidic phospholipids probably is the result of charge dilution that occurs as the ratio of phospholipid to Triton decreases. The importance of decreasing the high negative surface charge is supported by the finding that Ca^{2+} stimulated the activity on the acidic phospholipids, yet had little effect on phosphatidylcholine hydrolysis. This effect of Ca^{2+} on the hydrolysis of the negative phospholipids would be predicted by the rather stringent surface charge (zeta potential) requirements of this enzyme when studied using phosphatidylethanolamine as substrate.

These and other studies suggest that the initial binding step to the bulk lipid is critical in the overall catalytic rate. We, therefore, can view the specificity in the three-step process as follows:

Scheme 1:



E = enzyme

A = aggregate

S = substrate

P = product

(E-S) = Michaelis-Menten complex

In this scheme the relative rates of steps 1 and 2 will be influenced by enzyme-aggregate formation which is dependent upon the state of the aggregate and the substrate-active site interaction. This scheme is similar to those adopted for the venom and pancreatic phospholipases A_2, although there is no evidence that the lysosomal phospholipase A_2 undergoes an activation upon lipid binding, as suggested for the pancreatic phospholipase A_2 (6). Likewise, no information is available to determine which is the rate-limiting step in this process.

The plasmalemma phospholipase A_1 was originally described in the early 1970s as a phospholipase A_1 that preferentially attacks phosphatidylethanolamine (21, 22). Subsequent studies showed that treatment of the plasmalemma with heparin released the phospholipase A_1 and the enzyme preparation also had activity on monoacylglycerol including transacylase activity converting two molecules of monoacylglycerol to diacylglycerol plus glycerol. This enzyme is distinct from the phospholipases A_1 (B) described by van den Bosch (1) in that its activity on lysophospholipids is low and does not attack position two of the glycerol backbone. However, controversy still surrounds its substrate specificity, primarily due to two factors: first, the assay conditions employed by different groups vary widely; and second, the enzyme is subject to proteolytic cleavage that reduces its action on triacylglycerol (23). A wide variety of substrates was recently studied and it was shown that both triacylglycerol and phospholipid in lipoproteins and emulsions or vesicles could be degraded (24). Because of its broad specificity, the name hepatic lipase was suggested to consolidate the different names used in the literature.

A recent study by Tsujita et al. (23) shed some interesting light on the effect of proteolysis on the relative esterase and lipase activities of the hepatic lipase. They found that hydrolysis of short-chain triacylglycerols below their critical micellar concentrations was not affected by trypsin treatment, whereas the activity on long-chain triacylglycerols in emulsions was drastically reduced or eliminated by proteolysis. Likewise, the activity on monoacylglycerol was unaffected by trypsin treatment. Although not reported in this study, it might be expected that the activity on phospholipids would not be affected by trypsin treatment.

The mixed micellar substrates appear to be ideal to study the kinetics of this enzyme using the kinetic model developed by Deems and coworkers (7). Mixed micelles of phosphatidylethanolamine and Triton X-100 are much more readily attacked than pure phosphatidylethanolamine (Kucera, G., Sisson, P., and Waite, M., unpublished data). When micelles composed of varying ratios of phosphatidylethanolamine and Triton X-100 were used, a family of curves was generated that could be used to determine the kinetic properties of the enzyme (K_S^A, the affinity for total lipid; K_m^B, the affinity for phospholipid in the micelle; and V, the velocity). The V of 100 μmol/min^{-1} per mg^{-1} is only 2.5% of that reported for the *N. naja naja* enzyme (7) whereas the K_m^B value of 6.7 $\times$ 10^{-11} mol/cm^{-2} is in the same range of that reported for the *N. naja naja* enzyme, 1–2 $\times$ 10^{-10} mol/cm^{-2}. It is important to note that the K_m^B is given in two dimensions rather than the conventional three dimensions since the substrate is present on the surface of the micelle. The K_S^A value, 0.11 mM, is lower than that for the *N. naja naja* enzyme, 0.5 mM. This probably is the result of the higher affinity of the hepatic lipase for Triton. It appears, therefore, that the major difference between the venom and hepatic phospholipases is not the affinity for substrate but the catalytic efficiency of the enzymes. It will be of interest to use this approach with other cellular phospholipases so

that substrate specificities can be compared between enzymes.

A role has been postulated for the hepatic lipase in the metabolism of lipoprotein phospholipid degradation (25). In this model the phospholipid of HDL_2 is degraded and removed from the surface coat, thereby reducing the surface area of HDL_2 and forcing the core cholesteryl ester into the liver cell. The product remaining in circulation is HDL_3. In that regard, Jansen et al. (26) have provided evidence that the hepatic lipase is associated with the endothelial cells primarily, even though the enzyme is thought to be synthesized in parenchymal cells. On the other hand, whole parenchymal cells rapidly degrade the monoacylglycerol of remnant lipoproteins (27) and much of the lipid from lipoproteins is taken up by the parenchymal cells (28, 29).

Lysophospholipase (phospholipase B)

As mentioned earlier, there is little distinction between phospholipase A_1 and some lysophospholipases. Some of the lysophospholipases have phospholipase B activity cleaving both acyl chains and some are capable of transacylation reactions in which the hydroxyl of lysophospholipid is used by the enzyme as the acyl acceptor rather than water (30).

Perhaps the best known of these cellular lysophospholipases are those isolated from beef pancreas and liver. In 1974, van den Bosch, Aarsman, and van Deenen (31) purified the phospholipase A_1 from beef pancreas 150-fold over crude homogenates. This enzyme had phospholipase B activity, attacking both positions one and two of the substrate, but lacked lipase activity under the conditions employed. They demonstrated that the attack at position two was not the result of migration of the acyl group from position two to position one by showing the hydrolysis of glycophosphatidylcholine that contains the acyl ester adjacent to the carbon containing the phosphoester. Also they observed that the phospholipase A_1 activity was stimulated, whereas the lysophospholipase activity was inhibited by sodium deoxycholate.

Two lysophospholipases, I and II, have been purified from beef liver. The activity on diacyl phospholipid, while low, does permit hydrolysis of both acyl chains. Both are sensitive to sulfhydryl agents, diisopropyl fluorophosphate, and to carboxyesterase inhibitors, and do not require Ca^{2+} for activity. They differ in the pI, molecular size, and pH optimum and are also immunologically distinct as shown by the lack of cross-reactivity of their antibodies (32). Both enzymes were active on thioacyl ethers of deoxylysophosphatidylcholine that allowed a spectrophotometric assay. They also had higher activity on the thioester than the oxyester, perhaps due to the more hydrophobic character of the former. This is reflected in a lower apparent "K_m" of II for the thio compound.

It was proposed by van den Bosch et al. (31) that an acyl intermediate was involved in catalysis (Scheme 2). Since the enzymes were sensitive to diisopropyl fluorophosphate it was thought that the acyl intermediate is esterification of the serine hydroxyl.

Scheme 2:

$$EOH + R_1COCOR_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EOH \cdot R_1COCOR_2 \xrightarrow[R_1COH]{k_2} \underset{k_{-2}}{}$$

$$EOCOR_2 \xrightarrow[H_2O]{k_3} EOH + HOCOR_2 \qquad k_{-3}$$

EOH = serine enzyme

R_1CO = alcohol moiety of substrate

R_2OC = acyl moiety

In this model the ester oxygen or sulfur remains with the alcohol rather than the acyl moiety, as shown by the production of 1-thiopropylphosphorylcholine when thiodeoxylysophosphatidylcholine was used as substrate. Although attempts were made to elucidate kinetic constants involved in the partial reactions, inconclusive results were obtained despite the kinetic advantages of the spectrophotometric assay. It was suggested, however, that a step prior to enzyme deacylation was rate limiting, that is, either k_1 or k_2 is lower than k_3.

Phospholipases A_2

Phospholipases A_2 from different sources have been highly purified. Tojo et al. (33) used a three-step procedure to purify a phospholipase A_2 from the supernatant of rat spleen homogenates 23,000-fold with a yield of 70%. Key to this procedure was a high pressure liquid chromatography step that gave a 5000-fold purification. The purification was achieved on a reverse-phase octadecasilyl silica gel column run with a gradient of 20–45% acetonitrile in 0.1% trifluoroacetic acid as the mobile phase. To show that this procedure might be applied to other phospholipases A_2, the authors demonstrated that they could separate the phospholipases from rat spleen, human pancreatic juice, and porcine pancreas. The spleen enzyme had a molecular weight of 14,700, required Ca^{2+}, and was not influenced by calmodulin (34). The specific activity reported for this enzyme, 335 μmol/min per mg protein, is one of the highest reported for a cellular phospholipase. It is possible that this method of preparation is sufficiently rapid to allow the enzyme to be isolated in a fully active form. It will be of interest to see whether comparable hydrolytic rates can be achieved with other

phospholipases employing the high pressure liquid chromatography procedure.

Elsbach and Rizack (35) recognized as early as 1963 that polymorphonuclear leukocytes contained an active phospholipase capable of degrading the phospholipids in *Escherichia coli,* primarily phosphatidylethanolamine. Since a number of bactericidal cationic proteins had been extracted from leukocytes with 0.15 M H_2SO_4, Weiss, Beckerdite-Quagliata, and Elsbach (36) used H_2SO_4 extraction as the first step in their isolation procedure. Overall, they achieved an 8000-fold purification with a 15% recovery. This protein is extremely cationic with a pI higher than 10.5. This enzyme has a number of properties in common with other phospholipases A_2 of comparable size, 14,000, including a requirement for Ca^{2+}, its amino acid composition, and its sensitivity to bromophenacyl bromide but not phenylmethylsulfonyl fluoride. The requirement for Ca^{2+} was rather stringent in that concentrations higher than 1 mM were inhibitory when phosphatidylethanolamine was the substrate, the result of a surface charge effect (37). Phospholipases A_2 of similar nature have been extracted from a number of cells using H_2SO_4, including macrophages, platelets, and sperm cells.

Most red cells appear not to contain phospholipases. The notable exception to this generality are red cells from sheep that contain a phospholipase A_2 that can be extracted by sodium dodecyl sulfate solutions (38). This study was of particular interest since the enzyme was most readily solubilized by sodium dodecyl sulfate, but cholate was required for purification and activation. This enzyme is somewhat larger than the leukocyte and venom phospholipases, having a molecular weight of 18,500. The substrate specificity was dependent upon the conditions employed; in liposomes of mixed phosphatidylcholine and phosphatidylethanolamine, both were hydrolyzed by red cell membranes in the presence of detergent. However, when detergent was omitted, only phosphatidylcholine was degraded indicating the importance of the aggregate state of the substrate. Sheep red cell membranes do not contain phosphatidylcholine and will degrade extracellular phosphatidylcholine since the phospholipase A_2 is on the exterior of the plasma membrane. When hydrolysis of extracellular phosphatidylcholine was blocked by EDTA, phosphatidylcholine accumulated in the membrane. This was accompanied by a shift in membrane microviscosity (39). Addition of Ca^{2+} to reactivate the phospholipase caused degradation of the phosphatidylcholine. This series of studies is probably one of the best examples demonstrating the role of a phospholipase in regulating membrane structure.

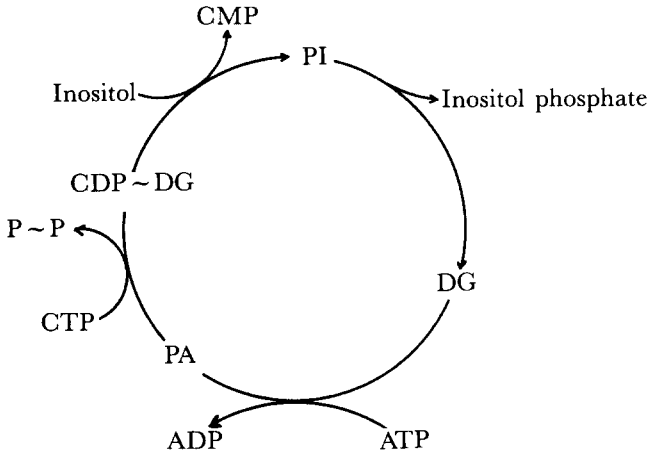
As early as 1963 (1) liver mitochondria were shown to have an active phospholipase A_2. This enzyme activity is located in both the inner and outer membrane compartments although it is not yet known whether the activity in the two fractions is the result of the same enzyme (40). Since the phospholipase A_2 is now known to exist in the mitochondria of many tissues and species, it could be an obligatory enzyme. It was shown to be an integral membrane protein requiring extraction of the mitochondria with acetone for solubilization (41). Stabilization of the enzyme with high salt and glycerol concentrations was essential to the successful purification procedure. Recently, Aarsman, Neys, and van den Bosch (42) simplified the purification procedure by introducing an affinity column step that employed 10-O-*p*-toluene sulfonyl decane-1-O-phosphocholine attached to Sepharose. It has a molecular weight of about 14,000 and is inhibited by *p*-bromphenacylbromide similar to many other phospholipases A_2. The enzyme purified by deWinter, Vianen, and van den Bosch (43) and Natori et al. (44) had a specific activity of 8.1 μmol/min per mg protein when assayed on pure phosphatidylethanolamine. Comparison of hydrolysis of membranous substrates compared favorably with the specificity found when pure phospholipids were used (45). In this case it is possible that the aggregate state of the substrate has minimal effect on activity.

It is clear that the activity of this enzyme is coupled to the energy state of the mitochondria. Parce, Cunningham, and Waite (46) demonstrated that no hydrolysis of mitochondrial phospholipid occurred until respiratory control was lost and the ATP concentration within the mitochondria dropped. Although there is continual turnover of the acyl groups of phospholipids within the mitochondria, the integrity of the membrane is maintained so long as reacylation occurs (47).

Considerable interest surrounds the platelet phospholipase A_2 since regulation of deacylation controls the "arachidonate cascade." Trugnan et al. (48) demonstrated that a phospholipase A_2 is membrane-associated, being recovered in the plasma membrane and a granular fraction. It is possible that the plasma membrane enzyme is that involved in the "arachidonate cascade" and responds to thrombin stimulation; however, this speculation is not yet verified. The enzyme appears to be an extrinsic protein since salt extraction solubilizes it from membranes (49). A preparation partially purified from a salt extract of rabbit platelet membranes had an apparent molecular weight of 12,000, close to the size of the phospholipase A_2 made from an H_2SO_4 extract of rabbit platelets. It is interesting to note that Jesse and Franson (50) found that non-steroid anti-inflammatory compounds such as indomethacin caused a 50% inhibition at a concentration of 5×10^{-5} M, similar to the results of Kaplan, Weiss, and Elsbach (51) who studied the leukocyte phospholipase A_2. While is it known that indomethacin directly inhibits the prostaglandin H_2 synthase, the finding that indomethacin inhibits phospholipase A_2 activity shows that the use of indomethacin as a specific inhibitor of cyclooxygenase should be viewed with caution.

Phospholipase C

Mammalian phospholipase C was first recognized by Sloane Stanley (52) over three decades ago (1953); he found that guinea pig brain preparations released inositol and phosphate from phosphatidylinositol phosphate. Shortly thereafter considerable interest in phospholipase C action was generated by the observation that phosphatidylinositol turnover was stimulated by a number of cell agonists, the "phosphatidylinositol cycle" (53) (Scheme 3). It is now thought that in some systems phosphatidylinositol phosphate and bisphosphate are substrates for this cycle as well (54).



Scheme 3

Recent work has shown an interplay between this cycle and the "arachidonate cascade" leading to the formation of prostaglandins, thromboxanes, hydroxytetraenoates, and leukotrienes (eicosanoids). As a result, considerable emphasis has been placed on isolating the phospholipase C from cells that produce the eicosanoids. It is clear that phospholipases C are widely distributed and their activity is intimately associated with the receptor-response characteristic of that cell (55). While most, if not all, purified phospholipases C studied thus far require Ca^{2+}, this has not been shown uniformly with intact cells.

Evidence obtained during purification of the ram seminal vesicles (56) and by isoelectric focusing of the cytosol of rat brain (18) shows that phospholipase C activity is actually catalyzed by a family of proteins of unknown relationship. Two immunologically distinct phospholipases C were purified from ram seminal vesicles, one (phospholipase C_I) to homogeneity. Both subfractions had a specific activity of about 25 μmol/min per mg of protein but differed in their molecular weights; C_I was 65,000 whereas C_{II} was 85,000. The phospholipase C purified from platelets was considerably larger (molecular weight = 143,000) (57). The purified phospholipase C_I had a rather high percentage of hydrophilic amino acids, as would be expected of a cytosolic protein. Likewise, it appeared to be devoid of carbohydrate.

Crude brain cytosolic preparations had two major types of phospholipase C activity, one active at pH 5.5 and the other at pH 7.0–8.0 (18). The isoelectric points for the brain enzyme(s) active at pH 5.5 ranged from 3.8 to 7.4, while the enzyme active at 7.0 focused sharply with a pI of 4.6–4.8. This contrasted with the pI for ram seminal vesicle phospholipase C_I, 5.5. It appears that the phospholipase C_I corresponds to a minor form separated by isoelectric focusing from rat brain. Indeed, the antibody against phospholipase C_I precipitated only about 15% of the total phospholipase C activity in sheep brain cytosol, suggesting that the other 85% of the activity in sheep brain could be the other three forms identified in rat brain cytosol. On the other hand, about 80% of the phospholipase C activity in the liver cytosol was enzyme I.

Both phospholipases C_I and C_{II} were specific for phosphatidylinositols and absolutely required Ca^{2+} for activity, 4–5 mM being required for optimal activity on phosphatidylinositol. The pH optimum was dependent upon the conditions employed. The enzyme, like that purified from muscle (58), could degrade all three phosphatidylinositols. When pure phosphatidylinositol was used, the optimal pH for hydrolysis was 5.3–5.4. However, the addition of deoxycholate stimulated activity some 10-fold at pH 7.0 at a ratio of 3:1 (detergent–substrate). Similar results have now been obtained with a crude phospholipase C preparation (18) from brain and with more highly purified preparations from platelets (57). As might be expected, the relative ratios of Ca^{2+}, substrate, detergent, and $[H^+]$ influenced the rate of reaction. The Ka for Ca^{2+} increased from 5 μM to 180 μM when phosphatidylinositol bisphosphate was added to phosphatidylinositol undergoing hydrolysis by the platelet phospholipase C (59). It was suggested that proteolysis of the platelet phospholipase C might be involved in its activation (57).

The product of the reaction, diacylglycerol, plus compounds that are known to be metabolically derived from diacylglycerol, unsaturated fatty acid and phosphatidate, stimulated hydrolysis of phosphatidylinositol (60–62). This led to the postulate that the system could be self-stimulatory. This is an appealing concept since the triggering of activity in response to a stimulant should be rapid but under tight regulation. A 7:1 ratio of phosphatidylinositol to 1,2-dioleoylglycerol produced marked stimulation of hydrolysis (63). The acyl composition of the diacylglycerol was important in this stimulation and correlated with the ability of the diacylglycerol to disrupt the bilayer structure. Phosphatidylcholine was found to inhibit the phospholipase C from seminal vesicles, although this inhibition could be relieved by the addition of phosphatidylserine (60). The enzyme did not bind to vesicles of a mixture of phosphatidylcholine/phosphatidylethanolamine/phosphatidylinositol, leading to the conclusion that phosphatidylcholine blocks the physical interaction with the aggregate rather than at the binding of the sub-

strate to the active site. This was demonstrated by the stimulation rather than inhibition of hydrolysis by dihexanoyl phosphatidylcholine that disrupts bilayers (63).

More precise information on the interactions of activators and substrates was obtained from studies on monomolecular films of phosphatidylinositol. Phosphatidic acid showed two stimulatory effects in this system: *1*) it increased the maximal surface pressure allowing hydrolysis from 33 to about 40 dynes/cm; and *2*) it decreased the lag period prior to degradation by about 50% at 30–33 dynes/cm. Phosphatidylcholine both decreased the maximal surface pressure permitting hydrolysis and increased the lag period (64).

Quite recently a novel phospholipase C was purified from the cytosol of canine hearts (65). This neutral active enzyme hydrolyzed both 1-O-alkyl and 1-O-acyl phosphatidylcholine and, to a lesser extent, phosphatidylethanolamine. This enzyme is distinguished from other neutral active phospholipases C by its apparent failure to hydrolyze phosphatidylinositol. Also, this enzyme did not exhibit an absolute requirement for Ca^{2+}. Apparently, an endogenous inhibitor was present in the cytosol that almost completely blocked activity; in the first step of purification there was a 30- to 40-fold increase in total activity recovered. This phospholipase C is distinguished from that in rat liver lysosomes by its substrate specificity, pH optimum, subcellular localization, and inhibition by EDTA (66).

Phospholipase D

There are relatively few reports of cellular phospholipases D. Brain and lung have been shown to have such activity and to be similar to the plant phospholipases D that act via a transphosphatidylation mechanism (67). It has been shown with the plant phospholipases D that the phosphatidate group is transferred from phosphatidylcholine and becomes covalently linked to the enzyme during catalysis (68). A number of hydroxyl-containing compounds can accept the phosphatidate group, in addition to water. In the brain the synaptic vesicle was reported to have the highest specific activity, roughly 2 nmol of product/min^{-1} per mg^{-1} protein (69). Either free fatty acid or bile salt was required for activity. On the other hand, the reaction was insensitive to EDTA, unlike the enzymes that exchange the base group of phospholipids and require Mg^{2+}. It was suggested by Hattori and Kanfer (69) that this reaction could serve as a mechanism by which choline can be provided in the brain for acetylcholine synthesis.

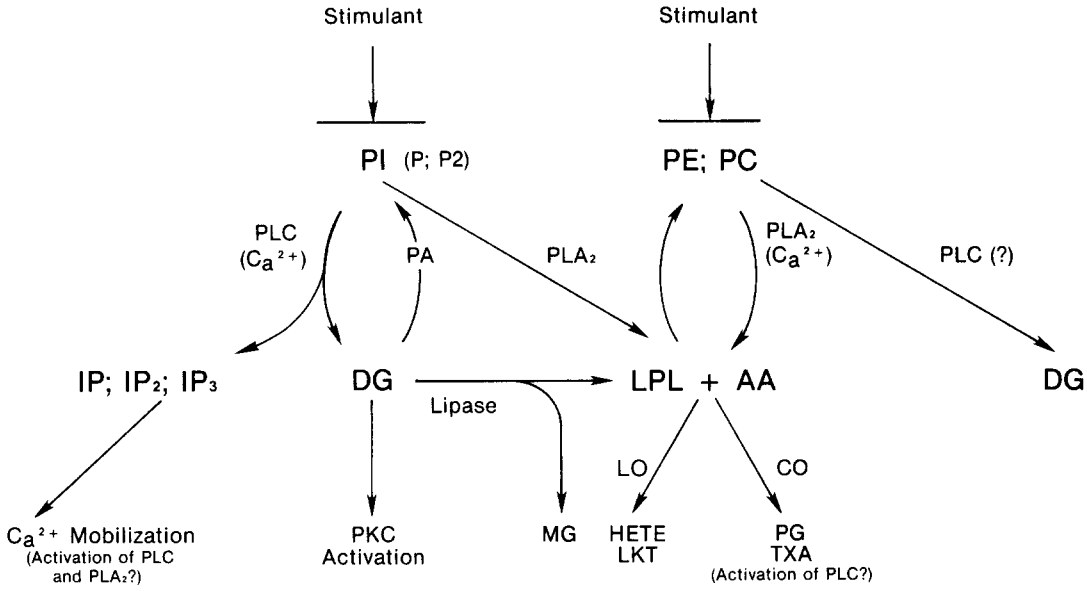
A phospholipase D with stringent substrate specificity was reported by Wykle, Kraemer, and Schremmer (70). They found that a number of rat tissues were capable of removing the choline from 1-alkyl lysophosphatidylcholine. The hydroxyl group in position two was absolutely required and substitution of the alkyl by an acyl moiety eliminated most activity. This enzyme, unlike the phospholipase D reported by Hattori and Kanfer (69), required Mg^{2+}. This enzyme could be involved in platelet activating factor metabolism via a transphosphatidylation reaction. Since it does act on the ethanolamine lysophospholipid, it could convert the 1-alkyl lysophosphatidylethanolamine ether to 1-alkyl lysophosphatidylcholine.

The role of phospholipases in the "arachidonate cascade"

The role of phospholipases in the "arachidonate cascade" has been the subject of intense interest over the past decade. No attempt will be made here to review this area, but mention will be made of possible interplay between the two phospholipases involved, phospholipase A_2 and phospholipase C (**Scheme 4**).

At present, the published evidence available indicates that phospholipase C is specific for phosphatidylinositols or one of its phosphate derivatives, although some recent preliminary data suggest that phorbol myristoyl acetate can stimulate a phospholipase C that acts on phosphatidylcholine (Daniel, L., Mueller, H. and Vance, D., personal communication). In order for arachidoante to be derived from the phospholipase C pathway, a lipase must act on the diacylglycerol (71, 72). It is now known that the products of phospholipase C action, diacylglycerol and inositol trisphosphate (when phosphatidylinositol bisphosphate is the substrate), have important actions without directly entering the "arachidonate cascade." It has been proposed that the diacylglycerol that is produced by phospholipase C acting on the plasma membrane can activate the lipid-requiring protein kinase C leading to the phosphorylation of a number of proteins (13). The inositol trisphosphate is thought to mobilize cellular stores of Ca^{2+} that in turn activate a number of enzymes (73). Some workers have suggested that one of these phospholipase C-initiated events can activate the phospholipase A_2 to release arachidonate for various phospholipids, depending on the cell studies (54). Lapetina, Billah, and Cuatrecasas (74) proposed that phosphatidate can play a significant role in the release of arachidonate, either by activating the phospholipase A_2 or as the major substrate for hydrolysis. While the latter could account for the release of arachidonate originating from phosphatidylinositol, it would not account for its release from the major sources of arachidonate from other phospholipids (54).

On the other hand, Rittenhouse (75) has presented evidence that the phospholipase A_2 is the first phospholipase activated when platelets are stimulated by calcium ionophore A23187 and that the prostaglandin E_2 produced from the released arachidonate is responsible for the activation of the phospholipase C. Although these differences are yet to be resolved, it does appear clear that the two phospholipases have a distinct function in agonist



Scheme 4. This scheme depicts two interdependent pathways emphasizing the roles phospholipases may play. The scheme does not represent any particular cell but reflects activities that may occur with different importance in a variety of cells. Briefly, phosphatidylinositol (PI) can be attacked by phospholipase C (PLC) or phospholipase A_2 (PLA_2) to yield diacylglycerol (DG) plus an inositol phosphate (IP; IP_2; IP_3), or arachidonate (AA), respectively. The IP_3 is thought to mobilize Ca^{2+}, while the DG activates protein kinase C. Alternatively, the DG can be degraded by a lipase to release AA or phosphorylate to form phosphatidate (PA) that is reconverted to PI. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are degraded by PLA_2 to release AA and the lysophospholipid (LPL) that is reacylated by an acyl CoA or another phospholipid. Some evidence suggests that PC may be degraded by PLC. Arachidonate is then metabolized to prostaglandin (PG) and thromboxane (TXA) by cyclooxygenase (CO) or hydroxyeicosatetraenoate (HETE) and leukotriene (LKT) by lipoxygenase (LO). Interactive activation between the PLA_2 and PLC degradative pathways may be mediated by Ca^{2+} mobilization, by PKC phosphorylation of proteins or by cyclooxygenase products.

response: the phospholipase A_2 to release arachidonate, and the phospholipase C to produce the "second messengers," diacylglycerol and inositol trisphosphate.

The regulation of these events remains obscure. Undoubtedly Ca^{2+} plays a significant role in regulation since both phospholipases require Ca^{2+}. A more specific regulator of the phospholipases has been proposed, lipocortin (lipomodulin, macrocortin) (15, 76, 77) that is produced in response to steroidal anti-inflammatory drugs. A third potential regulator has been suggested by Ballou and Cheung (78), a lipid (fatty acid) that is removed during purification of the phospholipase A_2. Together these results suggest that there might be multiple mechanisms of regulation. This area of study has proved to be extremely complex yet presents an interesting and important challenge to the investigator of cellular phospholipases.

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