Kinetic Properties of a High Molecular Mass Arachidonoyl-hydrolyzing Phospholipase A₂ That Exhibits Lysophospholipase Activity*

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The first step in the production of eicosanoids and platelet-activating factor is the hydrolysis of arachidonic acid from membrane phospholipid by phospholipase A₂. We previously purified from the macrophage cell line RAW 264.7 an intracellular phospholipase A₂ that preferentially hydrolyzes sn-2-arachidonic acid. The enzyme exhibits a molecular mass of 100 kDa and an isoelectric point of 5.6. When assayed for other activities, the phospholipase A₂ was found to exhibit lysophospholipase activity against palmitoyllysoglycerophosphocholine, and both activities copurified to a single band on silver-stained sodium dodecyl sulfate-polyacrylamide gels. An antibody against the macrophage enzyme was found to quantitatively immunoprecipitate both phospholipase A₂ and lysophospholipase activities from a crude cytosolic fraction. When the immunoprecipitated material was analyzed on immunoblots, a single band at 100 kDa was evident, further suggesting that a single protein possessed both enzyme activities. When assayed as a function of palmitoyllysoglycerophosphocholine concentration and plotted as a double-reciprocal plot, two different slopes were apparent, corresponding to concentrations above and below the critical micellar concentration (7 μM) of the substrate. Above the critical micellar concentration, lysophospholipase exhibited an apparent Kₘ of 25 μM and a Vₘₐₓ of 1.5 μmol/min/mg. Calcium was not required for lysophospholipase activity, in contrast to phospholipase A₂ activity. The enzyme, when assayed as either a phospholipase A₂ or lysophospholipase, exhibited nonlinear kinetics beyond 1–2 min despite low substrate conversion. Readaptation to more substrate after the activity plateaued did not result in further enzyme activity, ruling out substrate depletion. Readaptation of enzyme, however, resulted in another burst of enzyme activity. The results are not consistent with product inhibition, but suggest that the enzyme may be subject to inactivation during catalysis.

Phospholipase A₂ is an important regulatory enzyme in the production of the potent lipid metabolites platelet-activating factor and the eicosanoids. This phospholipase A₂ becomes activated in response to receptor ligation by mechanisms that are poorly understood and catalyzes the hydrolysis of arachidonic acid from membrane phospholipid. When arachidonic acid is hydrolyzed from 1-O-alkyl-linked species of phosphatidylcholine, lyso-platelet-activating factor is also formed, which can then be acetylated to platelet-activating factor (1). Making the assumption that there may be substrate-specific phospholipase A₂ enzymes involved in the production of inflammatory lipid mediators, we set out to study these enzymes using a substrate relevant to both platelet-activating factor and eicosanoid production, namely, 1-O-hexadecyl-2-arachidonoyl-GPC. Using this substrate, Leslie et al. (2) have purified a high molecular mass calcium-requiring phospholipase A₂ from the macrophage cell line RAW 264.7. Mammalian cells contain several phospholipases A₂ enzymes including the low molecular mass forms (12–18 kDa) (3–6) and the more newly described higher molecular mass enzymes (30–110 kDa) (2, 7–10). Certain properties of the high molecular mass phospholipase A₂ enzymes make them likely candidates for being involved in lipid mediator production. Some of these enzymes exhibit preference for substrates containing sn-2-arachidonic acid (2, 7, 10), and they are active at physiologically relevant concentrations of calcium (7–11). Also, a high molecular mass phospholipase A₂ from rat mesangial cells appears to be subject to hormonal regulation (12). Leslie and Channon (11) have recently shown that the activity of the high molecular mass phospholipase A₂ from RAW 264.7 cells is stimulated by anionic phospholipids, which act to increase the affinity of the phospholipase A₂ for calcium. In addition, Channon and Leslie (13) have demonstrated that this phospholipase A₂ exhibits calcium-dependent association with membrane. These properties may be involved in the mechanism of phospholipase A₂ activation. In this study, evidence is presented that the RAW 264.7 phospholipase exhibits both phospholipase A₂ and lysophospholipase activities, which copurify and immunoprecipitate as a 100-kDa protein. The enzyme is also shown to exhibit unusual kinetic properties against both phospholipid and lysophospholipid substrates.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-3H]Arachidonic acid (100 Ci/mmol) and 1-[14C]palmitoyl-sn-glycero-3-phosphocholine (50 mCi/mmol) were from Du Pont-New England Nuclear. 1-O-Hexadecyl-2-

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1 The abbreviations used are: GPC, glycerophosphocholine; PBS, phosphate-buffered saline; EGTA, [ethylendia(zee)nitrilol] tetracetic acid; SDS, sodium dodecyl sulfate.
arachidonyl-GPC was from Biolog Research Laboratories (Philadelphia). 1-O-Hexadecyl-2-lyso-GPC was from Bachem Feinchemikalien (Bubendorf, Switzerland). Palmitoyllyso-GPC was from Avanti Polar Lipids, Inc. (Birmingham, AL). 1-O-Hexadecyl-2-[14C]arachidonyl-GPC was prepared by incubating 1-O-hexadecyl-lysophosphatidylcholine (Avanti) with 3H-arachidonic acid, ATP, MgCl₂, and lysophospholipase A₂ in the presence of rat liver microsomes as described in detail elsewhere (2, 14). 1-O-[1-14C]Palmitoyllyso-GPC was also prepared using rat liver microsomes, except that the reaction mixture contained 20 μCi of 1-14Cpalmitoyllyso-GPC, 50 μCi of [14C]arachidonic acid, and 200 μg of unlabeled arachidonic acid together with ATP, MgCl₂, and CoA at a final volume of 1 ml.

Cell Culture and Purification of Phospholipase A₂—The RAW 264.7 macrophage cell line (American Type Culture Collection, Rockville, MD) was used as a source for the arachidonyl-hydrolyzing phospholipase A₂. The cells were grown in suspension, and cell-free homogenates were prepared by N₂ cavitation in the presence of chelators and fresh protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin) as described in detail elsewhere (2, 14). The homogenate was centrifuged at 100,000 x g for 1 h, and the supernatant (cytosolic fraction), which contained the phospholipase A₂, was adjusted to 60% (NH₄)₂SO₄ and stirred overnight at 0 °C. The precipitate was recovered by centrifugation at 100,000 x g for 45 min and solubilized with 10 mM Tris-HCl, pH 8, containing 10% glycerol (Buffer A). The phospholipase A₂ was purified as previously described (2, 14) with minor modifications. Sepharose 6B (rather than Sephadex G-150) was used for the first gel filtration step as described (14). Active fractions were pooled, and chromatography was performed over DEAE Bio-Gel P-6, except that fractions were eluted in a linear salt gradient from 100 to 250 mM NaCl. The phospholipase A₂-containing fractions were pooled, adjusted to 25% ammonium sulfate, and chromatographed over a phenyl-Sepharose column (2). The next chromatographic step was over aMono Q column (HR 10/10) previously equilibrated with 10 mM Tris-HCl, pH 8, containing 10% glycerol. Active fractions were pooled and chromatographed over a Mono Q column, which was followed by the addition of 1.5 ml of heptane and the amount of radioactivity was determined by liquid scintillation spectrometry.

Antibody Production and Immunoblot Analysis—Polyclonal antibody was used for immunoprecipitating phospholipase A₂ from the RAW 264.7 cells for immunoblot analysis. Suspension cells were centrifuged at 250 x g for 10 min; the pellet was washed in Hanks' balanced salt solution, and the final pellet was resuspended in lysis buffer (1% Nonidet P-40 in PBS containing 1 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min, the lysate was centrifuged at 10,000 x g for 30 min. Aliquots of the supernatant were incubated with different dilutions of immune and preimmune sera overnight at 4 °C. After adding protein A-Sepharose beads, the mixture was incubated at 4 °C for 2 h with tumbling and then centrifuged at 250 x g for 1 min. The supernatant was removed, and the beads were washed with 15 min with tumbling in 0.5% Nonidet P-40 in PBS, pH 7.2. The beads were washed an additional five times. The supernatant was removed from the pellet, and 50 μl of 2 x Laemmli buffer (22) containing 5 μl of 2-mercaptoethanol was added followed by boiling for 1 min. After centrifugation at 250 x g for 1 min, the supernatants were electrophoresed on SDS-polyacrylamide gels (7.5% running gel, 4% stacking gel). The proteins in the gels were transferred onto nitrocellulose in a Bio-Rad Trans-Blot Cell overnight at 4 °C to 200 mA. The nitrocellulose was washed once with PBS and then incubated in 5% non-fat dry milk for 1 h containing a 1:200 dilution of antiserum for 2 h at room temperature with shaking. The antibody solution was decanted, and the nitrocellulose was incubated in PBS for 5 min with shaking. This step was repeated four times followed by incubation in 5% non-fat dry milk containing an alkaline phosphatase EIA substrate kit (Bio-Rad) for 2 h at room temperature with shaking. The nitrocellulose was then rinsed in PBS six times, and autoradiographs were prepared.

For some experiments, the amount of phospholipase A₂ and lyso-phospholipase activities that could be immunoprecipitated from the 100,000 x g cytosolic fraction of RAW 264.7 cells was determined. The protein A-Sepharose beads were first incubated in 5% bovine serum albumin and then washed with PBS. The beads were then incubated at 4 °C with various dilutions of preimmune or immune serum overnight with tumbling. The beads were washed two times with PBS and then incubated with the cytosolic fraction for 2 h at 4 °C. The beads were pelleted, and the supernatant was assayed for phospholipase A₂ activity.

RESULTS

Copurification of Phospholipase A₂ and Lyso-phospholipase—The phospholipase A₂ purified from the RAW 264.7 macrophage cell line was found to exhibit lyso-phospholipase activity. Both phospholipase A₂ and lyso-phospholipase activities copurified throughout the purification. Results from the last purification step, hydroxylapatite chromatography, are shown in Fig. 1. The phospholipase A₂ and lyso-phospholipase activities exhibited almost identical elution profiles and eluted at 0.15–0.17 M phosphate. The peaks of enzyme activity always
were loaded onto a hydroxylapatite column and eluted in a linear salt gradient from 10 to 500 mM potassium phosphate. Fractions were assayed for enzyme activity as described under "Experimental Procedures."

The peak of phospholipase A₂ and lysophospholipase activities from the hydroxylapatite column were analyzed on SDS-polyacrylamide gels (Fig. 1), whereas immunoprecipitation using preimmune serum did not result in a loss of phospholipase activity from the cytosol (Fig. 3). The dose response for precipitation with immune serum was very similar for both activities, and a 1:25 dilution of serum precipitated 98 and 95% of the phospholipase A₂ and lysophospholipase activities, respectively. When whole cell lysates were immunoprecipitated with immune serum and analyzed on immunoblots, a single band of 100 kDa was apparent, further suggesting that a single protein exhibited both enzyme activities (Fig. 4). There was no evidence of the 100-kDa phospholipase when preimmune serum was used. When the RAW 264.7 cells were homogenized by sonication in the presence of calcium chelators and 100,000 × g total membrane and cytosolic fractions were prepared, the 100-kDa phospholipase band was detected only in the cytosolic fraction on immunoblots (data not shown).

Properties of Lysophospholipase Activity—When assayed as

eluted slightly later than the major protein peak. Individual fractions around the peak of phospholipase activity from the hydroxylapatite column were boiled in Laemmli buffer (22) and analyzed on SDS-polyacrylamide gels followed by silver staining using a Pharmacia LKB Biotechnology Phast System. Fraction numbers correspond to the fractions from the hydroxylapatite column shown in Fig. 1. B, isoelectric focusing of the phospholipase (with protein standards with pI values in the pI 3-9 range) was carried out as described under "Experimental Procedures." stds, standards.

As previously described (13), the RAW 264.7 phospholipase A₂ activity is found in the 100,000 × g cytosolic fraction when cells are homogenized in the presence of calcium chelators. Using a polyclonal antibody against the 100-kDa phospholipase, both phospholipase A₂ and lysophospholipase activities could be quantitatively immunoprecipitated from the 100,000 × g cytosolic fraction, whereas immunoprecipitation using preimmune serum did not result in a loss of phospholipase activity from the cytosol (Fig. 3). The dose response for precipitation with immune serum was very similar for both activities, and a 1:25 dilution of serum precipitated 98 and 95% of the phospholipase A₂ and lysophospholipase activities, respectively. When whole cell lysates were immunoprecipitated with immune serum and analyzed on immunoblots, a single band of 100 kDa was apparent, further suggesting that a single protein exhibited both enzyme activities (Fig. 4). There was no evidence of the 100-kDa phospholipase when preimmune serum was used. When the RAW 264.7 cells were homogenized by sonication in the presence of calcium chelators and 100,000 × g total membrane and cytosolic fractions were prepared, the 100-kDa phospholipase band was detected only in the cytosolic fraction on immunoblots (data not shown).

Properties of Lysophospholipase Activity—When assayed as

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a lysophospholipase against 1-[¹⁴C]palmitoyllyso-GPC, the enzyme was fully active in the absence of calcium (5 mM EGTA, 5 mM EDTA), and the activity was neither inhibited nor stimulated by calcium concentrations up to 10 mM (data not shown). This is in contrast to the phospholipase A₂ activity of this enzyme, which requires calcium as previously reported (2, 11). Both lysophospholipase and phospholipase A₂ activities exhibited similar pH profiles (Fig. 5). There was an increase in activity at pH 6, followed by a plateau up to pH 8, followed by another increase in activity up to pH 9–10. In Fig. 5, enzyme activity was measured using Tris at pH 9; however, when glycine buffer was used at pH 9, the activity was higher at pH 9 (110 pmol/min for phospholipase A₂ and 245 pmol/min for lysophospholipase) than at pH 10. All the experiments described thus far measuring lysophospholipase activity were conducted with a substrate concentration of 50 μM, which is well above the critical micellar concentration for 1-palmitoyl-2-lyso-GPC, which is 7 μM (17). Experiments were conducted to determine lysophospholipase activity as a function of substrate concentration (Fig. 6). Lineweaver-Burk analysis of the data revealed a discontinuous curve with two different slopes corresponding to concentrations of substrate above and below the critical micellar concentration. The break in the curve occurred at ~10 μM. The Vₘₐₓ and Kₑₜₐ values for the lysophospholipase were 1.5 μmol/min/mg and 25 μM, respectively.

Kinetic Properties of Phospholipase A₂ and Lysophospholipase Activities—The phospholipase was assayed as a function of time using the double-labeled substrate 1-[¹⁴C]palmitoyl-2-[³H]arachidonoyl-GPC to determine if the enzyme exhibited phospholipase A₂ activity and to determine the extent of lysophospholipase activity when the lysophospholipid substrate was generated in a bilayer (Fig. 7). Hydrolysis of sn-2-[³H]arachidonic acid preceded and was ~10 times greater than hydrolysis of sn-1-[¹⁴C]palmitic acid. These data indicated that the phospholipase did not possess significant phospholipase A₂ activity. They also demonstrated that lysophospholipase activity was low compared to phospholipase A₂ activity when relatively low levels of lysophospholipid substrate were generated in a bilayer as a result of phospholipase AS action.

As shown in Fig. 7, the phospholipase A₂ exhibited nonlinear kinetics after short incubation times despite relatively low substrate conversion (4% at 1 min). Experiments were performed to explore the potential mechanisms involved in the nonlinear kinetics exhibited by the phospholipase. One possibility was that the phospholipase was subject to end product inhibition by arachidonic acid. When the products of phos-
Phospholipase A₂ action (arachidonic acid and lysophospholipid) were codispersed either individually or together at various concentrations with the alkyl-[³H]arachidonoyl-GPC substrate, no inhibitory effect on phospholipase A₂ activity was observed (data not shown), suggesting that end product inhibition did not play a role in the nonlinear kinetics. To further investigate the basis for the nonlinear kinetics, the experiments shown in Fig. 8A and B were carried out. In Fig. 8A, phospholipase A₂ was assayed against hexadecyl-2-[³H]arachidonoyl-GPC for increasing incubation times; and when activity had plateaued, either more substrate or more enzyme was added to the incubation mixture and further assayed. The readdition of substrate did not result in a significant increase in enzyme activity, suggesting that substrate depletion was not the cause of the diminished phospholipase A₂ activity. This is verified by the observation that readdition of enzyme resulted in another burst of enzyme activity with kinetics similar to the first reaction. Consequently, adequate substrate was available for hydrolysis upon readdition of enzyme. The results showing phospholipase A₂ activity with the readdition of enzyme also further verified that end product inhibition was not involved. To ensure that the phospholipase A₂ was not labile at 37 °C in the presence of Ca²⁺, the enzyme was preincubated in the absence of substrate for 8 min at 37 °C in assay buffer containing calcium. Upon addition of alkyl-2-[³H]arachidonoyl-GPC, the phospholipase was found to exhibit full activity, indicating that the enzyme was not labile under the assay conditions employed. When the same experiment was carried out using [¹⁴C]palmitoyllyso-GPC as substrate, essentially identical results were obtained (Fig. 8B).

**FIG. 8.** Phospholipase kinetics against 1-O-hexadecyl-2-[³H]arachidonoyl-GPC (A) and 1-[¹⁴C]palmitoyllyso-GPC (B). Phospholipase A₂ and lysophospholipase kinetics were assayed as described under “Experimental Procedures.” When enzyme activity had plateaued, an equivalent amount of substrate (Δ) or enzyme (A) was readded to the reactions, and enzyme activity was compared to reactions with no additions (O). In some reactions, the enzyme was first preincubated for 8 min in the presence of assay buffer, calcium, and albumin, and the reaction was started by the addition of substrate (O). The data represent the mean ± S.D. of triplicate samples of a representative experiment.

Readdition of enzyme resulted in a further burst of lysophospholipase activity, whereas there was no further enzyme activity upon readdition of substrate. These results indicate that the nonlinear kinetics were not unique to the arachidonic acid-containing substrate.

**DISCUSSION**

Mammalian cells contain multiple forms of phospholipase A₂ that can be classified into low (12-18 kDa) and high (30-110 kDa) molecular mass forms. In general, the low molecular mass phospholipases do not appear to exhibit specificity for sn-2-fatty acids and are very active against disaturated phospholipids and membranes derived from *Escherichia coli*. The high molecular mass intracellular phospholipase A₂ enzymes have only recently been described, in part because defined phospholipid substrates other than disaturated phospholipids or *E. coli* membranes have been used for their purification. Some of these high molecular mass phospholipases (7, 10), including the RAW 264.7 enzyme (2), prefer substrates containing sn-2-arachidonic acid and would be nearly undetectable in *in vitro* assays using the more saturated phospholipid substrates. The immunoprecipitation experiments demonstrate that, under our assay conditions using 1-O-alkyl-2-arachidonoyl-GPC as substrate, >98% of the phospholipase A₂ activity in the crude cytosolic fraction is due to the 100-kDa phospholipase. Leslie et al. (2) had previously reported that the molecular mass of the RAW 264.7 phospholipase was 60/70 kDa on reduced/nonreduced SDS-polyacrylamide gels. Since the modifications in the purification protocol have been made (described under “Experimental Procedures”), the phospholipase routinely exhibits a molecular mass of 100 kDa, with no evidence of the 60-kDa species, despite identical chromatographic properties as previously reported (2). The 60-kDa protein may have been a proteolytic fragment, which is similar to the results observed for the phospholipase A₂/l-lysophospholipase from guinea pig intestine (18). The intestinal enzyme displayed a main band at 97 kDa and a minor band at 67 kDa, which exhibited low phospholipase activity and was postulated to be a proteolytic fragment. Alternatively, the 60-kDa protein may have been a contaminant, as suggested by results of other recent studies with high molecular mass phospholipases from U937 cells (7) and rat kidney (9). Both of these studies provide evidence that the phospholipase A₂ (which could be eluted from gels and shown to have phospholipase A₂ activity) has a molecular mass of 110 kDa and that a 55-kDa protein that copurifies with the phospholipase A₂ is a contaminant. In the report (9) describing the rat kidney phospholipase A₂, the 55-kDa protein was sequenced and shown to be disulfide isomerase. Interestingly, another group (10) has recently described the purification from U937 cells of a similar calcium-dependent phospholipase A₂ that exhibits preference for sn-2-arachidonic acid and has a molecular mass of 56 kDa. Our data strongly suggest that the 100-kDa protein is a phospholipase A₂, since antibody against this protein quantitatively immunoprecipitates phospholipase A₂ activity from the crude cytosolic fraction of the RAW 264.7 cells and reveals a single protein of 100 kDa in immunoblots.

The results of this study provide strong evidence that the 100-kDa phospholipase from the RAW 264.7 cells possesses both phospholipase A₂ and lysophospholipase activities based on the copurification and immunoprecipitation data. Two other high molecular mass phospholipase A₂ enzymes recently purified from canine myocardium (40 kDa) (19) and guinea pig intestinal brush-border membrane (97 kDa) (18) also exhibit lysophospholipase activity. In contrast to the RAW...
264.7 enzyme, the phospholipase A₂ activities of the myocardial and intestinal enzymes were calcium-independent. This dual activity for the intracellular macrophage enzyme would represent an efficient mechanism whereby a single enzyme could function as a phospholipase A₂ and then rapidly degrade potentially toxic 1-acyl-linked lysophospholipids. However, 1-
O-alkyllyso-GPC would be resistant to lysophospholipase action and would be available for platelet-activating factor production. The macrophage enzyme exhibited ~2-fold greater lysophospholipase activity than phospholipase A₂ activity when saturating concentrations of substrate were used. For lysophospholipase activity, this required substrate concentrations well above the critical micellar concentrations (7 µM) of palmitoyllyso-GPC. These highly toxic concentrations of lysophospholipids would not be expected to be achieved in vivo, suggesting that the 100-kDa phospholipase functions primarily as a phospholipase A₂ in vivo. This is supported by the results with the double-labeled substrate (1-['4C]palmito-
yl-2-['H]arachidonoyl-PC), which demonstrated that phos-
pholipase A₂ activity was ~10 times greater than lysophos-
pholipase activity when only low levels of lysophospholipid were generated in a bilayer.

The macrophage phospholipase was found to exhibit un-
usual kinetics, becoming nonlinear when >2-4 and >5-7% of the phospholipid or lysophospholipid substrate had been hy-
drolized. The results from experiments adding back enzyme or substrate to reactions that had plateaued rule out the possibilities of end product inhibition or substrate depletion as reasons for the nonlinear kinetics. A low molecular mass phospholipase A₂ from the macrophage cell line P388D₁ has been reported (20) to exhibit similar nonlinear kinetics when hydrolyzing substrates containing sn-2-arachidonic acid, but not substrates containing more saturated sn-2-fatty acids. The P388D₁ enzyme was shown to be subject to end product inhibition by arachidonic acid. We found that arachidonic acid or lysophospholipid, when codispersed with the phospholipid substrate, had no effect on the initial reaction rates of the RAW 264.7 enzyme, indicating that a different mechanism was involved. Also, the kinetics of the RAW 264.7 enzyme were not unique to substrates containing arachidonic acid since they also occurred with 1-palmitoyllyso-GPC as sub-
strate. The data also suggest that the physical form of the substrate is not a factor since the nonlinear kinetics occurred with either a phospholipid substrate in a bilayer form or with lysophospholipid in the form of micelles. Finally, the nonlinear kinetics were not due to inherent instability of the phospholipase or degradation by any possible contaminating pro-
teases since preincubation of the phospholipase in the pres-
ence of all reaction components except substrate did not result in significant inhibition of the enzyme. Although the basis of the nonlinear kinetics is not known, the results suggest that inactivation of the phospholipase occurs during catalysis. The kinetics of the phospholipase inactivation are not unlike those described for certain enzymes in the eicosanoid biosynthetic pathway that are subject to suicide substrate inactivation (21). However, it should be emphasized that the nonlinear kinetics observed with the RAW 264.7 phospholipase occurred with a purified enzyme against a defined phospholipid sub-
strate. Mechanisms involving down-regulation of phospho-
lipase A₂ activity may exist in stimulated cells to limit the levels of arachidonic acid liberated; however, the regulatory mechanisms involved in both phospholipase activation and inactivation in the cell remain to be elucidated.

The newly described class of high molecular mass phospho-
lipase A₂ enzymes, such as the arachidonoyl-hydrolyzing phospholipase A₂ from the RAW 264.7 macrophages, exhibits characteristics that make the enzymes likely candidates for catalyzing the release of arachidonic acid from membrane phospholipid that occurs in many cells in response to receptor ligation. Elucidating the biochemical and kinetic properties is important in understanding the mechanism of action of these potentially important regulatory enzymes that initiate the production of inflammatory lipid mediators.

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