

Assaying Phospholipase A₂ Activity

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Summary

Mammalian cells contain many structurally and functionally diverse phospholipases A₂ (PLA₂) that catalyze the hydrolysis of *sn*-2 fatty acid from membrane phospholipid. Assays are described for measuring the activity of Group IVA cytosolic PLA₂α (cPLA₂α) and for secreted PLA₂s (sPLA₂) that are suitable for purified enzymes and for measuring activity in crude cell lysates and culture medium. The assay for cPLA₂α involves measuring the calcium-dependent release of radiolabeled *sn*-2 arachidonic acid from small unilamellar vesicles of phosphatidylcholine. Methods are described for distinguishing cPLA₂α activity in cell lysates from other PLA₂s. sPLA₂ activity is monitored using a fluorimetric assay that measures the continuous calcium-dependent formation of albumin-bound pyrene fatty acid from the *sn*-2 position of phosphatidylglycerol.

Key Words: Phospholipase A₂; phospholipid; fatty acid; arachidonic acid; calcium; small unilamellar vesicles; phosphatidylcholine; phosphatidylglycerol; liposome; pyrene fatty acid.

1. Introduction

Mammalian cells contain multiple structurally diverse forms of phospholipases A₂ (PLA₂) that catalyze the hydrolysis of the *sn*-2 fatty acid from membrane phospholipid. PLA₂s function in dietary lipid breakdown, in phospholipid acyl-chain remodeling, in lipid-mediator production and in host defense against microorganisms. There are at least 15 genes in mammals encoding PLA₂ enzymes that comprise three main types: the secreted PLA₂s (sPLA₂), the Group IV cytosolic PLA₂s (cPLA₂), and the Group VI calcium-independent PLA₂s

(iPLA₂) (1–3). All cells contain members of each of these types of enzymes, suggesting that they play distinct functional roles. This chapter describes assays for measuring the activity of the well-characterized, ubiquitous cPLA₂α (group IVA) that mediates agonist-induced arachidonic-acid release (4), and for measuring the activity of sPLA₂s that are secreted into cell culture medium. The assays can be used for purified enzymes or for measuring their activity in crude cell fractions. The cPLA₂α assay involves measuring the hydrolysis of radiolabeled arachidonic acid from the *sn*-2 position of phosphatidylcholine, in the form of small unilamellar vesicles (5). Procedures are described for processing the cells, preparing the substrate, incubating cell fractions with substrate, terminating the enzymatic reaction, and separating product. Approaches for distinguishing cPLA₂α activity from other PLA₂ enzymes in cell lysates are outlined. The assay for measuring sPLA₂ activity involves monitoring the calcium-dependent formation of albumin-bound pyrene fatty acid from the *sn*-2 position of phosphatidylglycerol. This continuous fluorimetric assay is carried out in a cuvet or a microtiter plate.

2. Materials

2.1. cPLA₂α Assay

1. Homogenization buffer: 10 mM HEPES, pH, 7.4, 0.34 M sucrose, 10% glycerol, 1 mM EGTA, 1 mM phenylmethylsulfonylchloride, 1 μg/mL leupeptin, 10 μg/mL aprotinin.
2. Dithiothreitol (DTT).
3. 1-Palmitoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine (Perkin Elmer Life Sciences).
4. 1-Palmitoyl-2-arachidonyl-phosphatidylcholine (Avanti Polar Lipids).
5. CaCl₂.
6. Dioleoylglycerol.
7. Sodium chloride.
8. Bovine serum albumin (BSA; fatty acid free).
9. Dole Reagent: 2-propanol/heptane/0.5 M H₂SO₄, 20/5/1 (v/v/v).
10. Silica Gel LC-SI solid-phase extraction (SPE) tubes (Supelco, Bellefonte, PA).
11. Visiprep soldi-Phase extraction manifold (Supelco).
12. Oleic acid.
13. Probe sonicator with microprobe tip (Braun Instruments).
14. Arachidonyl trifluoromethylketone (Caymen Chemicals).
15. Bromoenol lactone (Caymen Chemicals).
16. Heptane.

2.2. Materials for sPLA₂ Assay

1. BSA (fatty acid free).
2. CaCl₂.
3. EGTA.
4. 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol ammonium salt (Molecular Probes, Inc., Eugene, OR).
5. Isopropanol.
6. 1-Pyrenedecanoic acid (Molecular Probes, Inc.).
7. Sodium chloride.
8. Toluene.
9. TRIS.

3. Methods

3.1. Assay for Measuring cPLA₂α Activity

cPLA₂α is present in most cells at sufficient amounts to detect its enzymatic activity in cell lysates. cPLA₂α can also be over-expressed in mammalian cells or in insect cells using baculovirus (6,7). The vesicle assay described here is suitable for measuring activity of the purified enzyme or for measuring activity of cPLA₂α in crude cell lysates.

3.1.1. Preparation of Cell Lysates

1. Wash cells twice with Homogenization Buffer (*see Note 1*).
2. Resuspend the cells in a small amount of Homogenization Buffer that will result in a protein concentration of approx 2 μg/μL after homogenization.
3. Sonicate the cell suspension on ice using a microprobe tip for 10 s, 2–4 times. Monitor the extent of cell disruption, which is variable depending on the cell type, after each 10-s burst of sonication by light microscopy.
4. Centrifuge the lysate at 100,000g for 1 h at 4°C to separate the soluble (cytosolic) and particulate (membrane) fractions using a Beckman TL100 tabletop ultracentrifuge. Transfer the supernatant to another tube and maintain at 0°C. Gently rinse the pellet and sides of the tube with homogenization buffer to remove the small amount of remaining cytosol (discard the rinse), and then resuspend the pellet in a small volume of homogenization buffer (*see Note 2*).
5. Determine the protein concentration in the cytosol and membrane fractions, and then add DTT to a final concentration of 1 mM to protect cPLA₂ from oxidative inactivation (*see Note 3*).

3.1.2. Concentration of Assay Components

The final concentration of components in the assay (50 μL final volume/reaction) are as follows.

1. 30 μM 1-palmitoyl-2-[1- ^{14}C]arachidonyl-phosphatidylcholine (100,000 dpm/reaction) (see **Notes 4** and **5**).
2. 9 μM dioleoylglycerol (see **Note 6**).
3. 150 mM NaCl (see **Note 6**).
4. 1 mg/mL BSA (see **Note 6**).
5. 50 mM HEPES, pH 7.4.
6. 1 mM CaCl_2 (see **Note 7**)

3.1.3. Preparation of Substrate

1. Prepare a concentrated stock of substrate by mixing in a glass tube (12 mm \times 75 mm) 1-palmitoyl-2[1- ^{14}C]arachidonyl-phosphatidylcholine and unlabeled 1-palmitoyl-2-arachidonyl-phosphatidylcholine to achieve a ratio of 100,000 dpm/1.5 nmol. Mix enough for several reactions and then add dioleoylglycerol at 30 mol% (30% of lipid is dioleoylglycerol and 70% is phosphatidylcholine) of the phosphatidylcholine concentration (this results in 0.45 nmol dioleoylglycerol/reaction).
2. Evaporate the solvents from the lipid mixture under a stream of nitrogen. Add 50 mM HEPES buffer, pH 7.4, to make a concentrated substrate solution that is 5–10 times the final assay concentration.
3. Sonicate the substrate mixture for 4 s on ice using a microprobe (Braun Instruments) to form small unilamellar vesicles. Count an aliquot of the solution after sonication to determine the efficiency of liposome formation. Usually >90% of the radioactivity is solubilized. Prepare liposomes fresh (if they are not used immediately, then store on ice for no more than 4 h).

3.1.4. Incubation Conditions

1. Aliquot the substrate mixture into assay tubes (round bottom disposable glass screw cap tubes, 13 mm \times 100 mm).
2. Add the NaCl, albumin and calcium.
3. Start the reaction by adding the cytosol or membrane fractions (approx 20 μg protein or more depending on the amount of cPLA $_2\alpha$ present in the cells). The final reaction volume after adding all the components is 50 μL . Incubate at 37°C with shaking for five min.
4. Terminate the reaction by adding 2.5 mL of Dole reagent (2-propanol:heptane: 0.5 M H_2SO_4 , 20:5:1, v/v/v) to each tube. After each reaction is terminated with Dole reagent, add 1.5 mL heptane and 1 mL water to each tube.

5. Add unlabeled oleic acid (20 µg of a 2 mg/mL stock in chloroform) to each tube to aid in the extraction efficiency.
6. Vortex each tube for approx 20 s and then centrifuge the tubes at 1000g at room temperature for 5 min to cleanly separate the two phases. The radiolabeled fatty acid product is extracted into the heptane phase. The reactions do not need to be immediately processed at this point but can be refrigerated and processed the next day.
7. Remove the upper heptane phase with a pasteur pipet being careful not to remove any of the lower phase. Pass the heptane phase through an SPE tube using a Visiprep SPE manifold. Elute the radiolabeled fatty acid with 2.5 mL of chloroform and collect the eluent in scintillation vials. Contaminating radiolabeled phospholipid substrate in the heptane phase is not eluted by chloroform and is retained on the silica gel.
8. Dry the eluent thoroughly under a stream of nitrogen to remove chloroform, which is a strong quenching agent. Add 0.5 mL heptane, vortex the vials, and let the vials stand at room temperature for about 10 min to solubilize the dried radiolabeled fatty acid. Add scintillation fluid, vortex briefly, and count by liquid-scintillation spectrometry.
9. Control reactions that contain substrate but no enzyme are similarly incubated and processed, and the dpms are subtracted from the experimental results.

3.2. Methods for-Distinguishing cPLA₂α Activity from Other PLA₂ Enzymes

3.2.1. Group VI Calcium-Independent PLA₂s (iPLA₂)

iPLA₂s are widely distributed in tissues and are found in both the soluble and membrane fractions of cells (8). They are commonly-assayed using micelles of phospholipid mixed with Triton-X-100, however, the vesicle assay described for measuring cPLA₂α activity will also detect iPLA₂s (9). To determine if some of the activity measured using the vesicle assay in crude cell fractions is owing to iPLA₂, omit calcium from the assay and include excess EGTA (1 mM). cPLA₂α and sPLA₂s are inactive in the absence of calcium and remaining activity is likely owing to iPLA₂. Additionally, bromoenol lactone (Cayman Chemical) selectively inhibits iPLA₂s in the 0.5–5.0 µM range without affecting cPLA₂α activity (10,11) (see Note 8).

3.2.1. Secreted PLA₂ Enzymes

There are 10 distinct mammalian sPLA₂ enzymes that are synthesized and secreted by cells or stored in intracellular granules of secretory cells (2). An assay for measuring the activity of purified sPLA₂, or sPLA₂ secreted into cell-

culture media, is described in **Subheading 3.3**. The vesicle assay described for measuring cPLA₂ α activity in crude cell fractions is not optimal for measuring sPLA₂ enzymes, especially if the vesicle assay is carried out in the presence of 1–2 μ M free calcium with phosphatidylcholine substrate (*see below*), which is optimal for cPLA₂ α but suboptimal for sPLA₂ enzymes. Many sPLA₂s (Groups IB, IIA, IID, IIE, IIF) preferentially hydrolyze anionic phospholipids, however, Group V and X sPLA₂s hydrolyze phosphatidylcholine vesicles and may be present in crude cell fractions (**12**).

Although the sPLA₂s are rich in disulfide linkages, the susceptibility of these enzymes to inactivation by treatment with DTT varies widely (**12**). For example, groups IIE, IIF, and X sPLA₂ retain significant activity even when treated for 30 min at 50°C with 10 mM DTT, whereas some sPLA₂s like Group IIA are fully inactivated after a 10-min treatment at 37°C (**12**). DTT sensitivity is a commonly reported method for evaluating whether the PLA₂ activity in a cell lysate is owing to an sPLA₂, but caution is now advised. However, treating cell fractions with the commercially available inhibitor, arachidonyl trifluoromethylketone at a concentration of 10 μ M will inactivate cPLA₂ α but not sPLA₂s (**13**).

3.2.2. Other Group IV cPLA₂ Enzymes

Group IVC cPLA₂ γ is constitutively associated with membrane and is calcium-independent because it lacks the C2 domain (**14–16**). Consequently, cPLA₂ α can be distinguished from cPLA₂ γ by omitting calcium from the vesicle assay. Group IVB cPLA₂ β is calcium-dependent because it contains a C2 domain, but preliminary characterization of its enzymatic properties suggests that it does not exhibit specificity for *sn*-2 arachidonic acid, a characteristic of cPLA₂ α (**17**). Therefore cPLA₂ α can be distinguished from cPLA₂ β by demonstrating that activity in cell fractions is greater (10- to 20-fold) using vesicles composed of 1-palmitoyl-2-arachidonyl-phosphatidylcholine compared to dipalmitoyl-phosphatidylcholine.

3.3. Assay for Measuring sPLA₂ Activity

The sPLA₂ assay described here is a modification of the original procedure first described by Radvanyi et al. (**18**). The assay makes use of a phosphatidylglycerol substrate (*see Note 9*) with a pyrene fluorophore on the terminal end of the *sn*-2 fatty acyl chain. When these phospholipids pack into the membrane bilayer, the close proximity of the pyrenes, from neighboring phospholipids, causes the spectral properties to change relative to that of monomeric pyrene (owing to exciplex formation). BSA is present in the aqueous phase and captures the pyrene fatty acid when it is liberated from the glycerol backbone owing to the sPLA₂-cat-

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alyzed reaction. These features allow for a sensitive sPLA₂ assay by monitoring the fluorescence of monomeric, albumin-bound pyrene fatty acid. This fluorimetric assay monitors product formation continuously in the presence of calcium (see **Note 10**), and the sensitivity of the assay approaches that of conventional fixed time-point assays with radiolabeled phospholipids.

3.3.1. Preparation of Assay Solutions and Substrate

1. Dissolve 1 mg of 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol (PPyrPG) in 1 mL of toluene:isopropanol (1:1) and vortex until all solid dissolves (may require slight warming).
2. Measure the concentration of PPyrPG in the stock solution by determining the absorbance at 342 nm in methanol using the extinction coefficient of 40,000 M⁻¹ cm⁻¹. Store the stock solution at -20°C in a vial with a teflon-lined screw cap (tightly secure the cap in place with parafilm).
3. Pipet 200 μL of PPyrPM stock solution into an Eppendorf tube, and completely remove the solvent with a stream of nitrogen. Add ethanol (1 mL), and vortex the sample until the lipid dissolves (may require slight warming). Centrifuge the sample (full speed in a microfuge for 2 min) to remove a small amount of particulate that may be present. Transfer the supernatant to a new tube and determine the absorbance of the solution at 342 nm (as above). The concentration of PPyrPM should be approx 100–200 μM. Store the ethanol stock solution at -20°C.
4. Prepare assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EGTA) and pass the solution through a 0.2–0.4 micron syringe filter to remove dust particles. Use high quality water to make the buffer (i.e., Milli-Q, Millipore Inc.). Store assay buffer at 4°C.
5. Prepare a solution of BSA (fatty acid-free, Sigma) in purified water at a concentration of 100 mg/mL and filter through a 0.2–0.4 micron syringe filter. Store in small aliquots at -20°C.
6. Prepare 1 M CaCl₂ in purified water and store at 4°C.
7. To prepare a working solution of assay cocktail for 30 assays (1 mL each), add 300 μL of 100 μM PPyrPG in ethanol (or the appropriate volume of a more concentrated substrate stock) (warm the stock solution in your hands and vortex it before taking the aliquot) to 29.7 mL of assay buffer. Add the PPyrPG drop-wise over approx 1 min to the continuously vortexing solution of assay buffer. This gives a working solution of 1 μM PPyrPG as unilamellar liposomes. This solution is stable at room temperature for up to 24 h.

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3.3.2. sPLA₂ Assay Procedure

1. Into a quartz fluorescence cuvet fitted with a small magnetic stir bar, add 0.98 mL of assay cocktail and then add 10 μL of 10% BSA. Finally add the

desired amount of sPLA₂ (see **Notes 11** and **12**) to the cuvet. Place the cuvet in the fluorimeter. For most accurate kinetics, the cuvet holder should be thermostatted at 37°C, but reasonably reliable data can be obtained at ambient temperature. Set the fluorimeter excitation wavelength to 342 nm and the emission wavelength to 395 nm. The fluorescence is recorded for approx 1 min in the absence of calcium to obtain the background rate. The reaction is initiated by adding 10 μL of 1 M CaCl₂ stock solution. The initial reaction velocity is recorded for approx 2–4 min with magnetic stirring (see **Note 13**).

3.3.3. Assay Calibration

In order to determine the specific activity from the observed assay slope, the assay has to be calibrated by adding a known amount of product, 1-pyrenedecanoic acid, to the complete assay cocktail in the absence of sPLA₂.

1. Prepare a stock solution of 1-pyrenedecanoic acid (Molecular Probes Inc.) in absolute ethanol at a concentration of 50 μM. Store at –20°C in a glass vial with a teflon-lined screw cap.
2. Prepare an assay reaction as above with all components except enzyme. Record the initial fluorescence and the increase in fluorescence after adding 250 pmole of 1-pyrenedecanoic acid to the cuvet. Use the measured increase in fluorescence per pmole to convert the observed slope measured in the sPLA₂ assay to μmol product produced per min per mg of sPLA₂.

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3.3.4. Microtiter Plate Assay of sPLA₂

The aforementioned assay with PPyrPG can also be carried out in a 96-well microtiter-plate using a microtiter plate fluorimeter.

1. Prepare a stock solution of 3% (w/v) BSA in assay buffer and filter as above. Store in aliquots at –20°C.
2. Prepare working solution A (60 μL of 3% BSA plus 940 μL of assay buffer).
3. Prepare solution B (1 mL of solution A plus an aliquot of sPLA₂, typically 5–2000 ng depending on the specific activity of the sPLA₂). Solution B should be prepared immediately before use to minimize loss owing to absorption of sPLA₂ to the tube.
4. Prepare solution C (4.2 μM PPyrPG in assay buffer, prepared as above for the 1 μM solution).
5. Add 100 μL solution A to each well followed by 100 μL solution B to each well. With a multichannel pipettor, deliver 100 μL of solution C to each

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well to start the reaction (*see* **Note 14**). Place the plate in the plate reader to monitor the fluorescence in each well (*see* **Note 15**).

4. Notes

1. Protease inhibitor-cocktail tablets are commercially available (Roche Diagnostics) and can be used in place of the individually added protease inhibitors (phenylmethanesulfonylfluoride [PMSF], leupeptin, aprotinin). However, if the PLA₂ assay is carried out at calcium concentrations in the nM– μ M range, which requires the use of calcium/EGTA buffers, use protease inhibitor cocktails without EDTA.
2. Small plastic Pellet Pestles (Kontes Glass Company) are very useful for resuspending the compact membrane pellet into a homogeneous suspension. It is important not to vortex the cell fractions, which will subject cPLA₂ to oxidation.
3. cPLA₂ α exhibits calcium-dependent translocation from cytosol to membrane in cells treated with agonists that increase intracellular calcium (**19,20**). However, we have found that this membrane association is not stable and can be reversed if cells are homogenized in the presence of calcium chelators (**21**). However, some reports have suggested that there is stable association of cPLA₂ α with membrane, although the basis for this is not understood (**22,23**). The relative amount of cPLA₂ α in the soluble and particulate fraction can be determined by Western blotting using commercially available antibodies (Santa Cruz Biotechnology, Inc.). cPLA₂ α activity associated with membrane can be measured using the vesicle assay, but it is important to note that the specific activity can not be directly compared to the specific activity in the soluble fraction. Because cPLA₂ α will hydrolyze the unlabeled phospholipid in the membrane, this effectively dilutes the labeled substrate in the vesicles, resulting in a lower specific activity.
4. 1-stearoyl-2[1-¹⁴C]arachidonyl-phosphatidylcholine (Amersham Biosciences) or the sn-1 ether-linked substrate 1-O-hexadecyl-2[1-¹⁴C]arachidonyl-phosphatidylcholine (American Radiolabeled Chemicals) can also be used as substrates to measure cPLA₂ α activity. When using a diacyl-phospholipid substrate, cPLA₂ α will sequentially cleave the sn-2 and then the sn-1 fatty acid because it has both PLA₂ and lysophospholipase activity (**24,25**). However, cPLA₂ α cannot hydrolyze the sn-1 aliphatic group from the 1-O-alkyl-linked substrate, 1-O-hexadecyl-2-arachidonyl-phosphatidylcholine.
5. Arachidonic acid is highly susceptible to oxidation and the arachidonyl-containing substrates (labeled and unlabeled) should be stored under nitrogen. Make a concentrated stock solution of unlabeled 1-palmitoyl-2-arachi-

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donyl-phosphatidylcholine in chloroform:methanol (90:10) and store aliquots at -80°C in glass vials with Teflon-lined caps and with the caps additionally sealed with teflon tape. Chloroform is very volatile and any evaporation will affect the concentration of the stock solution.

6. cPLA₂ α has unusual kinetic properties on sonicated vesicles, exhibiting an initial burst of activity that ceases abruptly in about 10–20 min (24,26,27). This is not owing to depletion of substrate, product inhibition, or irreversible inactivation of cPLA₂ α . Rather cPLA₂ α becomes “trapped” on the vesicles as product accumulates. The addition of NaCl and albumin to the reaction helps to prevent trapping and leads to more linear reaction-progress curves. The presence of dioleolyglycerol in the vesicle also enhances activity and improves the reaction progress (26–28). Dioleolyglycerol acts to perturb the packing density of the phosphatidylcholine bilayer, thereby allowing greater ability of cPLA₂ α to penetrate the bilayer and access the substrate. Dioleolyglycerol may also help prevent trapping on the vesicle.
7. Calcium is required in the assay to promote binding of cPLA₂ α to phospholipid vesicles (6,29,30). A final concentration of 1 mM free calcium in the assay is saturating. To achieve this final calcium concentration, the amount of EGTA in the homogenization buffer added to the assay (from the cell fractions) must be taken into account. At 37°C , 1 mol of EGTA chelates 1 mol of calcium. A characteristic of cPLA₂ α is that concentrations of calcium in the low micromolar range promote its binding to phospholipid vesicles (29). To evaluate the effect of physiological concentrations of free calcium in the vesicle assay, calcium/EGTA buffers are used. A computer program (available at <http://www.stanford.edu/~cpatton/webmaxcSR.htm>) is used to determine the amount of CaCl₂ to add to the reaction mixture, containing a known final concentration of EGTA, which will result in free calcium in the range of 0.1–5 μM . Do not include albumin in the vesicle assay for measuring cPLA₂ activity at low micromolar concentrations of free calcium because it can bind calcium.
8. The inhibitors, arachidonyl trifluoromethylketone and methylarachidonyl fluorophosphonate, cannot be used to distinguish cPLA₂ α and iPLA₂ because both enzymes are inhibited at similar concentrations of these compounds (10,31,32).
9. A detailed study of the interfacial kinetic properties of the full set of mammalian sPLA₂s has revealed that all of these enzymes display relatively high activity on anionic phosphatidylglycerol vesicles, whereas only the group V and X sPLA₂s also display high activity on phosphatidylcholine vesicles (12). The use of phosphatidylcholine vesicles is not recommended for detecting sPLA₂s in general, because many enzymes such as the human and

mouse group IIA sPLA₂s display very low activity on these vesicles owing to poor interfacial binding to the zwitterionic interface. However, even using phosphatidylglycerol, which is the most preferred sPLA₂ substrate, the specific activity of the mammalian sPLA₂s varies considerably. For example, the specific activity of human group IB sPLA₂ is approx 2000-fold higher than the specific activity of human group IIE sPLA₂ (12). The physiological significance of this variation in specific activity for the various mammalian sPLA₂s remains to be understood, and it raises the possibility that glycerophospholipids may not always be the physiological substrates for all of the enzymes. At the present time, phosphatidylglycerol remains the most generally preferred substrate for mammalian sPLA₂s.

10. It is often stated that sPLA₂s require millimolar concentrations of calcium for maximal activity. This is not the case (12). The concentration of calcium required for optimal enzymatic activity of an sPLA₂ depends on the enzyme species and the type of phospholipid substrate used in the assay. This is because calcium is required for the binding of a single phospholipid molecule in the active site of the sPLA₂ at the membrane interface and substrate binding cannot occur if the enzyme is in the water layer. Thus, interfacial binding of sPLA₂ to the membrane surface and calcium binding in the active site are coupled. For example, human group V sPLA₂ binds tighter to phosphatidylglycerol vesicles than to phosphatidylcholine vesicles, and the observed apparent K_{Ca} for this enzyme in the presence of the anionic vesicles is 1 μ M, whereas the apparent K_{Ca} increases to 225 μ M in the presence of zwitterionic vesicles (12). The statement made in **Subheading 3.2.1.** that cPLA₂ α but not sPLA₂ is maximally active in the presence of 1–2 μ M calcium holds as long as phosphatidylcholine is the substrate. In the presence of phosphatidylglycerol vesicles, the apparent K_{Ca} varies from about 1 μ M (group V sPLA₂s) to about 100 μ M (group IIE sPLA₂s) (12).
11. Purified sPLA₂s readily adsorb on the walls of containers at concentrations below about 10 μ g/mL. Highly dilute sPLA₂ stock solutions, appropriate for adding nanogram amounts to the PPyPG assay are best prepared by fresh dilution into buffer with approx 1 mg/mL BSA. For the most active sPLA₂s (i.e., group IB and IIA), as little as 0.2–0.5 ng of enzyme gives a readily measured initial velocity. The specific activities of the full set of human and mouse sPLA₂s acting on phosphatidylglycerol vesicles has been measured (12).
12. It is also possible to add crude samples containing sPLA₂ to the PPyPG assay. For example, we have been able to add up to 50 μ L of tissue culture medium containing sPLA₂ (from transfected cells) to the assay
13. If the amount of sPLA₂ added to the PPyPG assay is too high, the reaction-progress curve will display significant curvature, and all of the sub-

strate will be consumed in less than a few minutes. A sufficient amount of enzyme should be added to give a progress curve that remains linear for the first few minutes. It is best to confirm that the initial velocity is proportional to the amount of enzyme.

14. The use of a multichannel pipettor allows the reaction to be initiated in all wells at the same time.
15. Typically 8 wells are assayed at a time (more wells can be assayed if the plate reader is capable of measuring the fluorescence from multiple wells several times per minute). At least one well should be reserved for a minus enzyme control. The plate reader needs to be equipped with the appropriate filters to deliver the desired excitation and emission wavelengths (see the cuvet assay for wavelengths). The microtiter plate assay can be calibrated with 1-pyrenedecanoic acid as for the cuvet assay.

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