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Assaying Phospholipase A_2 Activity

Christina C. Leslie and Michael H. Gelb

Summary

Mammalian cells contain many structurally and functionally diverse phospholipases A_2 (PLA_2) that catalyze the hydrolysis of *sn-2* fatty acid from membrane phospholipid. Assays are described for measuring the activity of Group IVA cytosolic PLA_2alpha (cPLAα) and for secreted PLA_2s ($sPLA_2$) that are suitable for purified enzymes and for measuring activity in crude cell lysates and culture medium. The assay for $cPLA_2α$ involves measuring the calcium-dependent release of radiolabeled *sn-2* arachidonic acid from small unilamellar vesicles of phosphatidylcholine. Methods are described for distinguishing $cPLA_2α$ activity in cell lysates from other PLA_2s. $sPLA_2$ 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 $A_{2;}$ 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 A_2 (PLA_2) that catalyze the hydrolysis of the *sn*-2 fatty acid from membrane phospholipid. PLA_2s 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 PLA_2 enzymes that comprise three main types: the secreted PLA_2s ($sPLA_2$), the Group IV cytosolic PLA_2s ($cPLA_2$), and the Group VI calcium-independent PLA_2s

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($iPLA_2$) ***(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 $cPLA_2\alpha$ (group IVA) that mediates agonist-induced arachidonic-acid release ***(4)***, and for measuring the activity of $sPLA_2$s 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$ activity from other PLA_2 enzymes in cell lysates are outlined. The assay for measuring $sPLA_2$ 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. $cPLA_2\alpha$ Assay

1. Homogenization buffer: 10 m*M* HEPES, pH, 7.4, 0.34 *M* sucrose, 10% glycerol, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonylchloride, 1 μg/mL leupeptin, 10 μg/mL aprotinin.
2. Dithiothreitol (DTT).
3. 1-Palmitoyl-2[1-^{14}C]arachidonyl-phosphatidylcholine (Perkin Elmer Life Sciences).
4. 1-Palmitoyl-2-arachidonyl-phosphatidylcholine (Avanti Polar Lipids).
5. $CaCl_2$.
6. Dioleoylglycerol.
7. Sodium chloride.
8. Bovine serum albumin (BSA; fatty acid free).
9. Dole Reagent: 2-propanol/heptane/0.5 *M* H_2SO_4, 20/5/1 (v/v/v).
10. Silica Gel LC-SI solid-phase extraction (SPE) tubes (Sulpelco, 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 sPLA$_2$ Assay

1. BSA (fatty acid free).
2. $CaCl_2$.
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 cPLA$_2$α Activity

cPLA$_2$α is present in most cells at sufficient amounts to detect its enzymatic activity in cell lysates. cPLA$_2$α 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 cPLA$_2$α 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 100,000*g* 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 m*M* to protect cPLA$_2$ 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 µ*M* 1-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine (100,000 dpm/reaction) (*see* **Notes 4** and **5**).
2. 9µ*M* dioleoylglycerol (*see* **Note 6**).
3. 150 m*M* NaCl (*see* **Note 6**).
4. 1 mg/mL BSA (*see* **Note 6**).
5. 50 m*M* HEPES, pH 7.4.
6. 1 m*M* $CaCl_2$ (*see* **Note 7**)

3.1.3. Preparation of Substrate

1. Prepare a concentrated stock of substrate by mixing in a glass tube (12 mm × 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 phosphotidylcholine) of the phosphatidylcholine concentration (this results in 0.45 nmol dioleolyglycerol/reaction).
2. Evaporate the solvents from the lipid mixture under a stream of nitrogen. Add 50 m*M* 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 × 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 1000*g* 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-Distinquishing cPLA$_2\alpha$ Activity from Other PLA$_2$ Enzymes

3.2.1. Group VI Calcium-Independent PLA$_2$s (iPLA$_2$)

iPLA$_2$s 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 cPLA$_2\alpha$ activity will also detect iPLA$_2$s ***(9)***. To determine if some of the activity measured using the vesicle assay in crude cell fractions is owing to iPLA$_2$, omit calcium from the assay and include excess EGTA (1 m*M*). cPLA$_2\alpha$ and sPLA$_2$s are inactive in the absence of calcium and remaining activity is likely owing to iPLA$_2$. Additionally, bromoenol lactone (Cayman Chemical) selectively inhibits iPLA$_2$s in the 0.5–5.0 µ*M* range without affecting cPLA$_2\alpha$ activity ***(10,11)*** (*see* **Note 8**).

3.2.1. Secreted PLA$_2$ Enzymes

There are 10 distinct mammalian sPLA$_2$ 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 sPLA$_2$, or sPLA$_2$ secreted into cell-

culture media, is described in **Subheading 3.3.** The vesicle assay described for measuring cPLA$_2\alpha$ activity in crude cell fractions is not optimal for measuring sPLA$_2$ 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 cPLA$_2\alpha$ but suboptimal for sPLA$_2$ enzymes. Many sPLA$_2$s (Groups IB, IIA, IID, IIE, IIF) preferentially hydrolyze anionic phospholipids, however, Group V and X sPLA$_2$s hydrolyze phosphatidylcholine vesicles and may be present in crude cell fractions ***(12)***.

Au: where below?

Although the sPLA$_2$s 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 sPLA$_2$ retain significant activity even when treated for 30 min at 50°C with 10 m*M* DTT, whereas some sPLA$_2$s 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 PLA$_2$ activity in a cell lysate is owing to an sPLA$_2$, 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 cPLA$_2\alpha$ but not sPLA$_2$s ***(13)***.

3.2.2. Other Group IV cPLA$_2$ Enzymes

Group IVC cPLA$_2\gamma$ is constitutively associated with membrane and is calcium-independent because it lacks the C2 domain ***(14–16)***. Consequently, cPLA$_2\alpha$ can be distinguished from cPLA$_2\gamma$ by omitting calcium from the vesicle assay. Group IVB cPLA$_2\beta$ 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 cPLA$_2\alpha$ ***(17)***. Therefore cPLA$_2\alpha$ can be distinguished from cPLA$_2\beta$ 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 sPLA$_2$ Activity

The sPLA$_2$ 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 sPLA$_2$-cat-

alyzed reaction. These features allow for a sensitive $sPLA_2$ 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 Substate

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 µ*M*. Store the ethanol stock solution at −20°C.

Au: Where above?

4. Prepare assay buffer (50 m*M* Tris-HCl, pH 7.4, 100 m*M* NaCl, 1 m*M* 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* $CaCl_2$ 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 µ*M* 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 µ*M* PPyrPG as unilamellar liposomes. This solution is stable at room temperature for up to 24 h.

3.3.2. $sPLA_2$ 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 $sPLA_2$ (*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* $CaCl_2$ 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 callibrated by adding a known amount of product, 1-pyrenedecanoic acid, to the complete assay cocktail in the absence of $sPLA_2$.

1. Prepare a stock solution of 1-pyrenedecanoic acid (Molecular Probes Inc.) in absolute ethanol at a concentration of 50 µ*M*. Store at −20°C in a glass vial with a teflon-lined screw cap.

Au: Where above?

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 $sPLA_2$ assay to µmol product produced per min per mg of $sPLA_2$.

3.3.4. Microtiter Plate Assay of $sPLA_2$

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

Au: Where above?

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 $sPLA_2$, typically 5–2000 ng depending on the specific activity of the $sPLA_2$). Solution B should be prepared immediately before use to minimize loss owing to absorption of $sPLA_2$ to the tube.

Au: where above? Step #?

4. Prepare solution C (4.2 µ*M* PPyrPG in assay buffer, prepared as above for the 1 µ*M* 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

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 (phenylmethysulfonylfluoride [PMSF], leupeptin, aprotinin). However, if the PLA_2 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.

Au: location of Roche Diag. & Kontes glass. (#2)

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 $cPLA_2$ to oxidation.
3. $cPLA_2\alpha$ 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 $cPLA_2\alpha$ with membrane, although the basis for this is not understood ***(22,23)***. The relative amount of $cPLA_2\alpha$ in the soluble and particulate fraction can be determined by Western blotting using commercially available antibodies (Santa Cruz Biotechnology, Inc.). $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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-^{14}C]arachidonyl-phosphatidylcholine (Amersham Biosciences) or the sn-1 ether-linked substrate 1-O-hexadecyl-2[1-^{14}C]arachidonyl-phosphatidylcholine (American Radiolabeled Chemicals) can also be used as substrates to measure $cPLA_2\alpha$ activity. When using a diacylphospholipid substrate, $cPLA_2\alpha$ will sequentially cleave the sn-2 and then the sn-1 fatty acid because it has both PLA_2 and lysophospholipase activity ***(24,25)***. However, $cPLA_2\alpha$ cannot hydrolyze the sn-1 aliphatic group from the 1-O-alkyl-linked substrate, 1-O-hexadecyl-2-arachidonyl-phosphatidylcholine.

Au: location of Amersham

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-

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. $cPLA_2\alpha$ 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 $cPLA_2\alpha$. Rather $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$ to phospholipid vesicles ***(6,29,30)***. A final concentration of 1 m*M* 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 $cPLA_2\alpha$ 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 $CaCl_2$ 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 μ*M*. Do not include albumin in the vesicle assay for measuring $cPLA_2$ 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 $cPLA_2\alpha$ and $iPLA_2$ 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 $sPLA_2$s has revealed that all of these enzyme display relatively high activity on anionic phosphatidylglycerol vesicles, whereas only the group V and X $sPLA_2$s also display high activity on phosphatidylcholine vesicles ***(12)***. The use of phosphatidylcholine vesicles is not recommended for detecting $sPLA_2$s in general, because many enzymes such as the human and

mouse group IIA $sPLA_2$s 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 $sPLA_2$ substrate, the specific activity of the mammalian $sPLA_2$s varies considerably. For example, the specific activity of human group IB $sPLA_2$ is approx 2000-fold higher than the specific activity of human group IIE $sPLA_2$ ***(12)***. The physiological significance of this variation in specific activity for the various mammalian $sPLA_2$s remains to be understood, and it raises the possibility that glycero-phospholipids 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 $sPLA_2$s.

10. It is often stated that $sPLA_2$s 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 $sPLA_2$ 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 $sPLA_2$ at the membrane interface and substrate binding cannot occur if the enzyme is in the water layer. Thus, interfacial binding of $sPLA_2$ to the membrane surface and calcium binding in the active site are coupled. For example, human group V $sPLA_2$ 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 μM, whereas the apparent K_{Ca} increases to 225 μM in the presence of zwitterionic vesicles ***(12)***. The statement made in **Subheading 3.2.1.** that $cPLA_2\alpha$ but not $sPLA_2$ is maximally active in the presence of 1–2 μM calcium holds as long as phosphatidylcholine is the substrate. In the presence of phosphatidylglycerol vesicles, the apparent K_{Ca} varies from about 1 μM (group V $sPLA_2$s) to about 100 μM (group IIE $sPLA_2$s) ***(12)***.
11. Purified $sPLA_2$s readily absorb on the walls of containers at concentrations below about 10 µg/mL. Highly dilute $sPLA_2$ stock solutions, appropriate for adding nanogram amounts to the PPyrPG assay are best prepared by fresh dilution into buffer with approx 1 mg/mL BSA. For the most active $sPLA_2$s (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 $sPLA_2$s acting on phosphatidylglycerol vesicles has been measured ***(12)***.
12. It is also possible to add crude samples containing $sPLA_2$ to the PPyrPG assay. For example, we have been able to add up to 50 µL of tissue culture medium containing $sPLA_2$ (from transfected cells) to the assay
13. If the amount of $sPLA_2$ added to the PPyrPG 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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