Published in final edited form as:

Methods Enzymol. 2017 ; 583: 119–141. doi:10.1016/bs.mie.2016.09.001.

Analyses of Calcium-Independent Phospholipase A₂beta (iPLA₂β) in Biological Systems

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Abstract

The Ca²⁺-independent phospholipases A₂ (iPLA₂s) are part of a diverse family of PLA₂s, manifest activity in the absence of Ca²⁺, are ubiquitous, and participate in a variety of biological processes. Among the iPLA₂s, the cytosolic iPLA₂β has received considerable attention and ongoing studies from various laboratories suggest that dysregulation of iPLA₂β can have a profound impact on the onset and/or progression of many diseases (e.g., cardiovascular, neurological, metabolic, autoimmune). Therefore, appropriate approaches are warranted to gain a better understanding of the role of iPLA₂β *in vivo* and its contribution to pathophysiology. Given that iPLA₂β is very labile, its basal expression is low in a number of cell systems, and that crystal structure of iPLA₂β is not yet available, careful and efficient protocols are needed to appropriately assess iPLA₂β biochemistry, dynamics, and membrane association. Here, step-by-step details are provided to (a) measure iPLA₂β-specific activity in cell lines or tissue preparations (using a simple radiolabel-based assay) and assess the impact of stimuli and inhibitors on resting- and disease-state iPLA₂β activity, (b) purify the iPLA₂β to near homogeneity (via sequential chromatography) from cell line or tissue preparations, enabling concentration of the enzyme for subsequent analyses (e.g., proteomics), and (c) employ hydrogen/deuterium exchange mass spectrometry analyses to probe both the structure of iPLA₂β and dynamics of its association with the membranes, substrates, and inhibitors.

1. INTRODUCTION

The Ca²⁺-independent phospholipases A₂ (iPLA₂s) are members of a diverse family of PLA₂s that hydrolyze the *sn*-2 substituent from membrane phospholipids to release a free fatty acid and a lysophospholipid (Dennis, Cao, Hsu, Magrioti, & Kokotos, 2011; Gijon & Leslie, 1997). The iPLA₂s comprise Group VI PLA₂s and, in contrast to s(ecretory)PLA₂s and c(ytosolic)PLA₂s, do not require Ca²⁺ for either translocation to membrane or activity. Between 1994 and 2016, the Group VI PLA₂s have expanded to seven members: iPLA₂β (VIA-1 and 2), iPLA₂γ (VIB), iPLA₂δ (VIC), iPLA₂e (VID), iPLA₂ζ (VIE), and iPLA₂η (VIF). Due to their shared homology with patatin, the iPLA₂s are included in the patatin-like protein family and also referred to as PNPLAs. The iPLA₂s also share a consensus GX SXG

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catalytic motif contained within a patatin-like lipase domain. The iPLA₂s manifest a variety of activities in addition to PLA₂, are ubiquitously expressed, and participate in a multitude of biological processes including fat catabolism, cell differentiation, maintenance of mitochondrial integrity, phospholipid remodeling, cell proliferation, signal transduction, and cell death. As such, dysregulation of iPLA₂s can influence the metabolic state, CNS function, cardiovascular performance, and cell survival and therefore have a profound impact on onset/progression of many diseases. To date, the best-characterized iPLA₂s are iPLA₂β, localized predominantly in the cytosol, and membrane-associated iPLA₂γ (Mancuso, Jenkins, & Gross, 2000).

The iPLA₂β (PNPLA9) is the most widely described of the iPLA₂s and expression of its activity was first described in P388D1 macrophage-like cells in 1994 (Ackermann, Kempner, & Dennis, 1994) and later shown to be the same enzyme (Balboa, Balsinde, Jones, & Dennis, 1997) as that cloned from Chinese hamster ovary cells in 1997 (Balboa et al., 1997; Jones et al., 1996; Tang et al., 1997). Unlike cPLA₂, which exhibits preference for hydrolysis of arachidonic acid from the *sn*-2 position of glycerophospholipids (Ghosh, Tucker, Burchett, & Leslie, 2006), the iPLA₂s do not demonstrate *sn*-2 substrate specificity. The iPLA₂s manifest PLA₂/PLA₁, lysophospholipase (Lio & Dennis, 1998; Wolf & Gross, 1996), transacylase (Jenkins et al., 2004; Lio & Dennis, 1998), and thioesterase (Carper, Zhang, Turk, & Ramanadham, 2008; Jenkins, Yan, Mancuso, & Gross, 2006) activities.

1.1 iPLA₂β Characteristics

The iPLA₂β is an 84–88 kDa cytosolic protein with a serine lipase consensus sequence (GTSGT) in its catalytic domain that is preceded by eight N-terminal ankyrin repeats (Gross, Ramanadham, Kruszka, Han, & Turk, 1993; Ma et al., 1997; Tang et al., 1997). The 88 kDa isoform is a product of a mRNA species that arises from an exon-skipping mechanism of alternative pre-mRNA splicing (Larsson, Claesson, & Kennedy, 1998) and contains a 54-amino acid sequence that interrupts the eighth ankyrin repeat. The iPLA₂β protein contains a caspase-3 cleavage site (DVTD), a putative bipartite nuclear localization sequence (KREFGEHTKMTDVKKPK), and, upon stimulation, can associate with multiple subcellular-localized proteins and mobilize into various subcellular organelles (Golgi, endoplasmic reticulum [ER], mitochondria, and nucleus).

To date, there are two recognized catalytic activators of iPLA₂β (ATP and calmodulin kinase IIβ) and one of its transcription (sterol regulatory element-binding protein). Inhibitors of iPLA₂β include arachidonyl trifluoromethyl ketone (AACOCF₃), methyl arachidonyl fluorophosphonate (MAFP), and palmitoyl trifluoromethyl ketone (PACOCF₃), which are sometimes used for “selective” inhibition of cPLA₂. In contrast, the bromoenol lactone (BEL) suicide inhibitor has been demonstrated to be a more selective inhibitor of iPLA₂ with little or no effect on cPLA₂ or sPLA₂ (Hazen, Zupan, Weiss, Getman, & Gross, 1991; Jenkins et al., 2004; Ma, Ramanadham, Hu, & Turk, 1998). Further, the *S*- and *R*-enantiomers of BEL exhibit selective potency for iPLA₂β and iPLA₂γ, respectively (Jenkins, Han, Mancuso, & Gross, 2002), and they have been used to distinguish biological processes impacted by the two isoforms. Fluoroketone- and oxadiazole-based compounds currently under development are proving to be just as potent as BEL, while being more

specific for iPLA₂β and exhibiting reversible inhibition, without discernible toxicity (Ali et al., 2013; Dennis et al., 2011; Kalyvas et al., 2009; Kokotos et al., 2010; Li et al., 2011; Lopez-Vales et al., 2011, 2008; Mouchlis, Limnios, et al., 2016; Ong, Farooqui, Kokotos, & Farooqui, 2015).

2. EXPERIMENTAL PROCEDURES

2.1 iPLA₂β Activity Assay

Described is a facile assay that will provide rapid, selective, and quantifiable measurement of iPLA₂β-specific activity in a given preparation, without requiring purification of the enzyme.

Solutions needed

1. Homogenization buffer (HB, 250 mM sucrose, 40 mM Tris-HCl, pH 7.1 @ 4°C)
2. Assay buffer (AB, 200 mM Tris-HCl, pH 7.5 @ 37°C, 10 mM EGTA)
3. EGTA (10 mM)
4. ATP (Sigma, St. Louis, MO, A-5394; 1 or 10 mM)
5. *S*-BEL inhibitor (Cayman 10006801, prepare 10 mM stock in DMSO and store at -20°C in small aliquots. Do not reuse thawed aliquot. Final concentrations [0.10–10.0 μM] should be contained in less than 1% by volume of DMSO.)
6. Radiolabeled substrate Perkin Elmer (NEC765010UC, L-α-1-palmitoyl-2-arachidonyl-*sn*-phosphatidylcholine [arachidonyl-1-¹⁴C], or 16:0a/20:4-PC or PAPC, 2.5–5 μM in 5 μL EtOH)
7. Oleate standard (Sigma 143-19-1; 5 mg/mL CHCl₃)
8. TLC solvent (petroleum ether/ethyl ether/acetic acid, 80/20/1 by volume). This solvent composition resolves fatty acids (*R_f* 0.58) from monoacylglycerol (*R_f* 0.24) and diacylglycerol (*R_f* 0.21)
9. 100% ethanol
10. 1-Butanol
11. Scintillation cocktail
12. Glycerol
13. β-Mercaptoethanol

Specific supplies/equipment needed

1. Centrifuge
2. Cold microfuge
3. Shaking water bath
4. Scintillation counter

5. Vortex
6. Stopwatch
7. Compressed nitrogen tank
8. Hair dryer
9. 10×75 mm glass assay tubes (best for viewing separation of organic and aqueous phases)
10. TLC tank
11. Silica gel G channeled/scored TLC plates (Analtech Uniplate 31711)
12. Iodine (Sigma 376558)
13. Glass Hamilton syringes (50 μ L for substrate stock, 10 μ L for diluted substrate for assay, 50 μ L for TLC spotting; keep these separate and do not interchange.)
14. Repeater pipet for reagent additions (i.e., HB, AB, EGTA, butanol)
15. Scraper blade
16. 3" × 5" weighing paper
17. Scintillation vials (7 mL)
18. Scintillation cocktail (RPI 3a70B or equivalent)

Basic assay concept: iPLA₂ β in a given sample will hydrolyze the *sn*-2 (radiolabeled) fatty acid substituent from the provided substrate under zero-Ca²⁺ (no added Ca²⁺+EGTA) conditions. The released fatty acid is then resolved by TLC, recovered for scintillation counting, and dpm used to determine specific enzyme activity.

Assay protocol (Fig. 1)

1. Using your established methods, prepare sample (cytosolic, mitochondrial, ER, or nuclear fractions) from cells or tissues in HB. If only Ca²⁺-independent PLA₂ activity is to be measured, EGTA can be included in the HB. Otherwise, it can be added to activity assay tubes. At all times, keep the sample preparation, assay reagents, and substrate on ice.
2. Measure protein concentration in the sample. Approximately 25–50 μ g protein in aliquots of less than 50 μ L should be the target and the volume of HB used in Step 1 volume should be titrated accordingly. For cells in 100 mm dish, use 200 μ L HB for harvesting. For tissues, use sufficient volume (<1 mL) to allow efficient sonication and adjust the volume as needed during assay development. It is best to prepare a concentrated sample, which can be diluted subsequent to [protein] determination.
3. During sample preparation
 - a. *Water shaking bath.* Turn on and equilibrate to 37°C.

- b.** *Set up the activity assay tubes.* An initial protocol to establish the presence of iPLA₂β activity in a sample will include assays in the absence and presence of ATP (1 or 10 mM, activator of iPLA₂β) or *S*-BEL (0.1–10.0 μM, selective irreversible inhibitor of iPLA₂β). A typical assay template is as follows (tubes 2–5 are singlicates for a given sample; 6 on are for additional sample sets):
- c.** *Prepare the substrate fresh each time.* PAPC is a common choice for the radiolabeled substrate, although 1-palmitoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-lauroyl-*sn*-glycero-3-phosphocholine (PLPC), and 1-*O*-(*Z*)-hexadec-1'-enyl-2-[9,10-³H₂]octadec-9'-enyl-*sn*-glycero-3-phosphocholine [(16:0p/18:1)-PC] are also suitable. The latter substrate is a plasmalogen phospholipid, containing a vinyl ether linkage in the *sn*-1 position, and is an extremely favored substrate by iPLA₂β. However, it can be more expensive (to buy or synthesize) and very labile. In initial experiments, one can use the final substrate concentration of 2.5–10 μM. For a [¹⁴C]-labeled substrate, 100,000 dpm in 5 μL is added to the assay tube and this is typically contained in 1 μL of the stock substrate. After determining the number (*N*) of assay tubes (including replicates), transfer (*N*+2)μL of stock substrate into a clean 12×75 mm glass tube using the Hamilton syringe designated for stock (highest counts). Commercial radiolabeled substrates are usually prepared in chloroform or methanol. Using a nitrogen stream, evaporate the solvent in the tube and then resuspend the lipid in 5× (*N*+2)μL of ethanol. This will provide an extra 10 μL of substrate. Count 2 μL prior to assay to ensure that it contains ~40,000 dpm (=100,000 in 5 μL) using a second Hamilton syringe (second most counts). At the end of the assay, during sample counting, count 5 μL of the substrate to confirm total added dpm to assay tubes and for calculation of % substrate hydrolyzed by iPLA₂β in the sample aliquot.
- d.** *Scintillation vials.* Set aside the required number (*N*) of scintillation vials with 5 mL scintillation cocktail. Number the caps. Scintillation cocktail splashing will wipe off markings on the tube!!
- e.** *TLC plates.* Number the TLC plates at the top of each lane with a pencil (Fig. 2, “x” at top of lanes). If the assay requires more than one plate, make sure to include a blank for each plate. If less than one plate is sufficient, snap off extra lanes and save.
- f.** *Oleate standard.* Place a drop (~10 μL) of oleic acid solution into the middle of the loading area in each lane (Fig. 2, lane 2). Set aside and let air-dry. Free fatty acid will comigrate with the oleic acid standard, which will be visible upon exposure to iodine vapor.
- g.** *TLC tank.* In a fume hood, prepare the TLC tank by inserting a U-shaped double-folded Whatman paper into the tank. Place solvent (100

mL) at bottom, cover the container, and apply a weight on top. Monitor solvent migration upward on the Whatman filter paper. This preparation is good for multiple runs (four plates) on the same day as long as the tank is kept air-tight. You can run TLC plates back to back or by using paper click separators, up to four plates at one time.

- h.** *Iodine vapor tank.* In a fume hood, add a few crystals of iodine to a separate tank and cover. This tank can be used repeatedly for the same purpose until the iodine is exhausted.

4. *Preparing the assay tubes* (done at room temperature [RT], unless otherwise noted)

- a.** To all tubes, first add AB and EGTA.
- b.** Second, add HB to all tubes. To the blank tubes, add 100 μ L. For sample tubes, HB=100 μ L minus protein volume. Initial experiments should determine optimal protein amount that allows for linear accumulation of product during the course of the assay (to be contained in less than 50 μ L).
 $\Rightarrow$ Subsequent steps need to be performed rapidly.
- c.** Third, add source of protein in HB.
- d.** If testing ATP, add prior to substrate.
- e.** If testing *S*-BEL, add after Step c and incubate at RT for 3 min. Include this in the time frame described in *Step 6*.
- f.** When adding ATP and/or *S*-BEL, reduce the dH₂O volume by same amount.

5. *Assay.* Start the assay by adding substrate to tube. Prior to first addition, remove dead space in the second Hamilton syringe by flushing it with substrate two to three times (do not discard the substrate, empty it back into tube). Now, take up 5 μ L into the syringe, immerse it to the bottom of assay tube, and deliver the substrate. Quickly vortex the tube and add it to the shaking water bath. Dip the syringe in a clean tube of EtOH to wash off the tip (do not flush the syringe again), prior to taking up the next aliquot of substrate.

6. *Stopping the reaction.* At a predetermined time (*Step 6a*), add 100 μ L butanol (RT) to the tube, remove it from water bath. Vortex for 5 s and set aside (RT) in a test tube rack.

- a.** Initial experiments should test the optimal assay time (from 1 to 10 min), during which substrate hydrolysis is linear with respect to time. The iPLA₂ β -catalyzed hydrolysis typically occurs rapidly, between 3 and 5 min.
- b.** The time elapsed between substrate and butanol additions should equal the chosen assay time. For example, if a 3-min assay duration is chosen,

start with substrate addition to tube 1 at 0 min, tube 2 at 15 s, tube 3 at 30 s, ..., and tube 12 at 2:45 min. Starting at 3 min, stop reaction at 15 s intervals. This interval can be lengthened or shortened depending on the individual. Keep in mind to include at least one blank to each separate set of assay runs.

7. *Separating the hydrolyzed FA.* After all assay tubes have been collected, centrifuge them (RT, 2000×g, 5 min) to separate the organic (upper) from the aqueous phase. The interface will be a thin protein band. Continue with the next step without delay.
8. *Spotting TLC lanes.* Tilt the tube toward you and using the third Hamilton syringe (lowest counts) and without piercing the interface, carefully remove 25 μL (a fourth) of the upper layer. Gently apply this aliquot onto the loading area (Fig. 2, lane 3). Dry the lanes with the hair dryer and place in the TLC tank.
9. *Identifying hydrolyzed FA region.* After the solvent migrates to the top of the TLC plate, remove the plate from tank and allow residual solvent to evaporate in a fume hood. Place the TLC plate in the iodine tank. When the oleic acid standard is visualized (by appearance of a yellow streak across the plate, Fig. 2, lane 8), remove the plate from tank and mark the boundaries of the standard (Fig. 2, lane 9). Allow the iodine to sublime under hood using a hair dryer. At this point, the plate can be set aside for later processing.
10. *Harvesting the hydrolyzed FA.* Using a blade, scrape the marked off region (Fig. 2, lane 11) into a counting vial (Fig. 2, lane 12). Add scintillation cocktail to vial, cap, and mix vigorously by hand.
11. *Counting.* Allow an O/N quenching period and then count the samples in a ¹⁴C channel and record decays/minute (dpm). If only counts/minute (cpm) are reported, calculate dpms using efficiency of the counter.
12. *Activity calculation.* Subtract averaged blank dpm from spotted sample (25% of total) dpm and use that value to determine Specific Enzyme Activity:

$$\frac{4 \times \text{DPM}}{\text{SA label}} \times \frac{1}{\text{mg prt} \times \text{assay time}(\text{min})} \times 1000 = \text{pmol/mg protein} \times \text{min}$$

Keys to achieving optimal results

1. iPLA₂β is very labile and sensitive to increases in temperature for pro-longed periods. Therefore, any preparation in which iPLA₂β activity is to be measured needs to be maintained on ice.
2. When using a sonicator to prepare protein sample, use 1 s ON and 0.5 s OFF pulse for 15–20 s (~20% power, once for cells/2–3 times for tissue homogenate). Keep the sample tube immersed in ice during sonication.
3. Due to lability of iPLA₂β, it is best to perform the assay soon after the sample has been generated.

4. Utilizing samples prepared from frozen cells or tissues will give activity measurements, but they will tend to be much lower than freshly prepared samples.
5. iPLA₂β is stabilized by ATP, even at 37°C. Thus, storing a sample with ATP addition will preserve the activity. However, ATP is an activator of iPLA₂β and the specific activity measured will be higher than in the absence of ATP. This will preclude comparisons between samples prepared and assayed right away in the absence of ATP.
6. Once the activity assay procedure has commenced, it is best to carry through until the TLC plate has been run. The scraping and counting can be done later.

2.2 iPLA₂β Purification

(A) Below are details on how to purify iPLA₂β starting from tissue (i.e., brain, skeletal muscle, heart, >25–50 g) or cultured cells (15–20×175 cm² flasks; Carper et al., 2008; Ramanadham, Wolf, Jett, Gross, & Turk, 1994; Wolf & Gross, 1996) when a homogeneous concentrated enzyme preparation is needed for subsequent analyses.

Buffers needed (adjust pH of buffers first before adding DTT)

1. HB (250 mM sucrose, 10 mM imidazole, 10 mM KCl, pH 7.8 @ 4°C).
2. Pre-DEAE (anion exchange) dialysis buffer (15 mM imidazole, 5 mM K₂HPO₄, 10% glycerol, pH 7.8 @ 4°C).
3. DEAE column elution buffer (10 mM imidazole, 10 mM KCl, 100 mM NaCl, 10% glycerol, 1 mM DTT, pH 8.0 @ 4°C).
4. Pre-CF (chromatofocusing) dialysis buffer (10 mM imidazole, 10 mM KCl, 25% glycerol, 1 mM DTT, pH 8.4 @ 4°C).
5. CF column elution buffer (10% polybuffer 96 [GE Healthcare Life Sciences, Pittsburgh, PA, 17-0714-01], 5% polybuffer 74 [BOC Sciences, Shirley, NY, 82658-85-3], 25% glycerol, 1 mM DTT [Calbiochem, San Diego, CA, 578517], pH 6.9 @ 4°C).
6. ATP column elution buffer (10 mM imidazole, 25% glycerol, 1 mM DTT, pH 8.3 @ 4°C).
7. Calmodulin column elution buffer (10 mM HEPES, 1 mM EDTA, 340 mM sucrose, 1 mM DTT, 1 mM Triton X-100, pH 7.5).

Equipment/supplies needed

1. Cold room
2. Buffer pump
3. UV monitor
4. Chromatography columns and reservoirs (GE Healthcare)

5. DEAE Sephacel (GE Healthcare 17-0500-01) and PBE-94 CF (Axsyn, Chesham, UK, AX-9NNB) resins
6. ATP Agarose (Sigma A-9264) and Calmodulin-Sepharose (GE Healthcare 17-0529-01) column resins
7. Glass wool (Fisher, Hampton, NH, 22-289336)
8. Spectra/Por 1 dialysis tube (Spectrum Laboratories Inc., Rancho Dominguez, CA, 23 mm × 100 ft [132650], 40 mm × 100 ft [132660], and 50 mm × 100 ft [132665]; 6–8 kDa molecular weight cutoff)
9. Filters for buffers (Millipore, Darmstadt, Germany, GSWP04700)
10. Nunc freezing vials (ThermoFisher Scientific, Waltham, NH, 374080 [0.50 mL] and 374081 [1 mL])
11. Activity assay needs, as described in Section 2.1.

Columns preparation prior to sample loading

1. DEAE-Sephacel column
 - i. On Day 1, place resin (160 mL for a 5 × 8 cm column) in a Buchner funnel with filter and wash with 1 M NaCl, 100 mM Na Acetate, pH 4 (1 L).
 - ii. Wash the resin with dH₂O (1 L).
 - iii. Wash with Buffer 2 (1.5 L).
 - ⇒ During each wash/rinse, gently swirl the resin using a glass rod; apply gentle vacuum to keep filter in place, allow resin to settle for 5 min; apply vacuum to remove wash but do not let the resin dry.
 - iv. Place washed resin in 500 mL Buffer 2 in a filtering flask with side-arm. Degas and allow resin to settle at 4°C.
 - v. Remove floating particles by aspiration and pour resin mixture into column in the cold room and equilibrate with Buffer 2 (1.5–2 L, O/N, 1.5–2 mL/min).
 - ⇒ While loading the DEAE column, prepare remaining buffers.
 - ⇒ Cannot reuse this column, prepare fresh each time.
2. CF Column (can be prepared at the same time and similarly to the DEAE column)
 - i. Wash CF resin (60 mL) with 1 M NaCl, 100 mM Na Acetate, pH 4 (500 mL).
 - ii. Rinse resin with dH₂O (1 L).
 - iii. Rinse resin with Buffer 4 (1 L).

- iv. Degas resin in Buffer 4 (200 mL) and allow resin to settle.
- v. Aspirate floating particles and load slurry into column.
- vi. Level the column and apply air pressure to generate flow rate of 1.6 mL/min.
- vii. After column has packed, pump Buffer 4 at 1.6 mL/min for 15 min to pack the column and then at 1.0 mL/mL O/N through Day 2.
- viii. Allow Buffer 4 containing 1 mM DTT to flow through column (2 L @ 1.6 mL/min O/N into Day 3).
 - ⇒ Can reuse the column after washing with buffer containing 1 M NaCl, 100 mM Na Acetate, pH 4 (2 L, O/N). Then, wash with dH₂O and store column in 30% EtOH.

3. ATP column

- i. Apply ATP-Agarose affinity resin to column (1 × 1 cm).
- ii. Rinse with dH₂O to remove EtOH.
- iii. Wash with 1 M NaCl (30–50 mL, 4°C).
- iv. Rinse column from top to bottom exhaustively with dH₂O.
- v. Equilibrate with Buffer 6 (20 mL) and plug bottom until sample loading. Make sure to leave some buffer in the column so the resin does not dry.
 - ⇒ Can reuse column after washing with 1 M NaCl (30 mL) followed by dH₂O. Store column in 30% EtOH.

4. Calmodulin column

- i. Apply to column 1 mL of the Calmodulin-Sepharose slurry.
- ii. Wash column with 25 mL of 25 mM imidazole (pH 8.0).
- iii. Wash column with 25 mL of 25 mM imidazole (pH 8.0) containing 5 mM CaCl₂.
 - ⇒ Do not reuse this column—prepare it fresh each time.

Purification protocol (all purification columns to be performed at 4°C)

Day 1

1. Prepare crude cytosolic fraction in *Buffer 1*, using your established protocol.
2. Dialyze the fraction overnight against *Buffer 2*, using dialysis tubing previously equilibrated in the same buffer at 4°C.

Day 2

3. Load dialyzed fraction onto DEAE column (1.5 × 8 cm). Pass the eluent by gravity through a UV monitor to record the absorbance.

4. Wash DEAE columns with *Buffer 2* containing 1 mM DTT, until the A280 returns to baseline.
5. Elute iPLA₂β activity (in 3–5 mL fractions) using *Buffer 3*.
6. Measure iPLA₂β activity, as a reflection of activity present in each fraction. This can be achieved by immediate counting (@ 0.5 min) and will allow proceeding to the next step without delay. The fractions can be recounted after overnight quenching.
7. Pool fractions with activity and dialyze overnight against *Buffer 4*, at 4°C.

Day 3

8. Apply the dialyzed pool to PBE-94 CF column (1×8 cm, 1.8 mL/min).
9. Wash the CF column with *Buffer 4*.
10. Elute iPLA₂β activity (in 3–5 mL fractions) using *Buffer 4*.
11. Measure iPLA₂β activity in the fractions and pool fractions with activity.
12. Immediately load pool onto a 12×1 cm N6-[(6-aminohexyl) carbamomethyl]-ATP-agarose column. Reapply void one additional time.
13. Wash column with *Buffer 6* (25–50 mL).
14. Wash the column with *Buffer 6*, *Buffer 6* containing 10 mM AMP (Sigma A-2252; 5 × 5 mL fractions).
15. Wash the column exhaustively with *Buffer 6* to remove the AMP.
16. Elute iPLA₂β with *Buffer 6* containing 1 mM ATP (Sigma A-5394; 0.5–1.0 mL fractions).
17. The AMP and ATP fractions can be saved at 4°C and assayed for iPLA₂β activity the following day.

Day 4

18. Pool the fractions with activity and increase [Ca²⁺] in pool to 5 mM.
19. Load pool onto a Calmodulin-Sepharose column (1 × 1 cm).
20. Collect the void and reload onto the column.
21. Wash column with *Buffer 7* containing 0.5 mM CaCl₂.
22. Elute iPLA₂β with *Buffer 7* containing 8 mM EGTA (0.5–1.0 mL fractions).
23. Measure activity and pool fractions with activity.
24. Concentrate pool to dryness in the presence of 10% SDS by lyophilization.
25. Resuspend in small volume (based on subsequent assay protocols) of buffer containing 25 mM Tris-HCl (pH 6.8), 5% glycerol, 0.5% β-mercaptoethanol.
26. Store sample in Nunc cryotubes at –80°C in small aliquots and before use, thaw aliquots on ice. Use aliquot only once; do not refreeze.

(B) An abbreviated alternative protocol can be employed if less starting material is available or if low basal activity in the sample is predicted, as follows:

1. Sonicate sample in HB (10 mM HEPES, 1 M EDTA, 340 mM sucrose, 1 mM DTT, pH 7.5).
2. Centrifuge sonicate (1000×g, 10 min, 4°C).
3. Precipitate protein in the supernatant with the addition of ammonium sulfate (50%).
4. Centrifuge mixture (20,000×g, 30 min, 4°C).
5. Resuspend pellet in *Buffer 7*.
6. Proceed with ATP and Calmodulin Sepharose column elution steps as earlier.
7. Concentrate and store sample as described earlier.

Calculations

1. Prior to loading a column, save a small aliquot of the pool to determine total loaded protein/activity.
2. Record each column load volume.
3. Keep track of the fraction volumes collected.
4. These will allow subsequent determination of specific enzyme activity and fold purification following each step.

Keys to optimal purification yields

1. Perform all purification steps at 4°C (or cold room).
2. Do not let the columns dry.
3. Make sure to add DTT to elute activity from the DEAE column. Use fresh resin for each preparation.
4. If the DEAE Sephacel resin is mishandled, cracks will develop when poured into column or after sample is loaded. In that case, gently swirl the column section with crack.
5. The CF column is unforgiving and if the resin dries at any point after protein loading, fresh column and sample will need to be prepared!!
6. Do not delay in proceeding from the CF to ATP column steps.
7. Eluant from the ATP column is stable and can be assayed the following day. Store at 4°C until assay.
8. Do not use peristaltic pump to elute protein out of any column as iPLA₂β will stick to pump tubing and can be denatured by the pump action.

2.3 Membrane Association of iPLA₂β

Like many other members of the PLA₂ family, iPLA₂β is a water-soluble protein that must associate with lipid membranes to bind substrate. Indeed, the iPLA₂β catalytic cycle has been described as consisting of four steps: (1) membrane association, (2) extraction of a substrate molecule into the active site, (3) catalysis, and (4) diffusion of reaction products away from the active site, setting the stage for another round of catalysis (Batchu, Hokynar, Jeltsch, Mattonet, & Somerharju, 2015; Mouchlis, Bucher, McCammon, & Dennis, 2015; Mouchlis & Dennis, 2016). Although iPLA₂β-selective inhibitors have been identified (Ali et al., 2013; Dennis et al., 2011; Hazen et al., 1991; Jenkins et al., 2004; Kalyvas et al., 2009; Kokotos et al., 2010; Li et al., 2011; Lopez-Vales et al., 2011, 2008; Ma et al., 1998; Mouchlis, Limnios, et al., 2016; Ong et al., 2015), a more thorough understanding of the molecular mechanisms underlying the membrane association, substrate binding, and even catalytic mechanism could lead to development of more efficacious and potentially clinically relevant inhibitors.

Investigation of these mechanisms has been hampered because a high-resolution structure of iPLA₂β is not yet available. However, Dennis and coworkers have developed an elegant approach to gain insight into the structure of iPLA₂β and the molecular mechanisms underlying its association with membranes, substrates, and inhibitors that relies on the coordinated use of three approaches: selective enzyme assays (like the one described earlier), molecular dynamics, and hydrogen/deuterium exchange mass spectrometry (DXMS; Cao, Burke, & Dennis, 2013; Hamuro et al., 2004; Hsu et al., 2013; Hsu, Burke, Li, Woods, & Dennis, 2009; Mouchlis et al., 2015; Mouchlis & Dennis, 2016; Mouchlis, Limnios, et al., 2016; Mouchlis, Morisseau, et al., 2016). DXMS has been broadly used to study protein dynamics, protein–protein, and protein–ligand interactions (Hamuro et al., 2004; Percy, Rey, Burns, & Schriemer, 2012). In principle, DXMS measures solvent accessibility of amide protons by quantifying their exchange for deuterium when a protein is incubated in D₂O. Although there are other potential explanations for hydrogen/deuterium exchange (i.e., changes in hydrogen bonding, oligomerization, distal changes in protein conformation, and interaction with ligand), DXMS can be informative about local changes in protein conformation when coupled with other structural information (Cao et al., 2013; Gallagher & Hudgens, 2016; Percy et al., 2012). This approach has led to mapping of conformational changes in the group IVA phospholipase A₂ (cPLA₂α) and group IA phospholipase A₂ upon binding to membranes, substrates, inhibitors, and divalent cations (Burke et al., 2009, 2008; Cao et al., 2013; Hsu et al., 2008; Mouchlis et al., 2015; Mouchlis & Dennis, 2016). In all of these reports, the DXMS data were interpreted and confirmed through molecular dynamics simulations and comparisons to the high-resolution 3D structures of each enzyme.

As noted earlier, such data are not yet available for iPLA₂β. To circumvent this, homology modeling approaches were used to predict structures of the ankyrin repeat (residues 88–474) and catalytic (residues 475–806) domains of human iPLA₂β (Hsu et al., 2009). A BLAST homology search revealed that the ankyrin domain of iPLA₂β is 51% homologous to human ankyrin-R (PDB 1N11) and the catalytic domain is 34% homologous to the potato acyl hydrolase patatin (PDB 1OXW; Hsu et al., 2009; Mouchlis & Dennis, 2016). After applying

a variety of modeling approaches (Bennett-Lovsey, Herbert, Sternberg, & Kelley, 2008; Hsu et al., 2009; Rost, Yachdav, & Liu, 2004), homology models were developed that exhibited template modeling (TM, a measure of the similarity of two protein structures) scores of 0.77 (ankyrin domain) and 0.76 (catalytic domain; Hsu et al., 2009). The predicted structures were validated with DXMS, performed as described later.

2.3.1 Hydrogen/DXMS—The DXMS protocol essentially consists of four phases: (1) incubation of enzyme ($\pm$ substrate, inhibitor, source of “membrane” or a combination) in D₂O buffer for various periods of time (10–10,000 s); (2) quenching of the exchange (reduce to pH 2.5 and temperature to 0°C) and denaturation of the enzyme (guanidine hydrochloride); (3) online pepsin digestion followed by C18 reverse-phase HPLC of the resultant peptides; and (4) analyses of deuterated peptides through electrospray ionization tandem mass spectrometry (ESI-MS/MS). For additional detail on the theory, implementation, and optimization of DXMS, the reader is referred to two excellent reviews (Gallagher & Hudgens, 2016; Percy et al., 2012). The optimized protocol for analysis of iPLA₂ β is described in detail later:

Phase 1

Preparation of deuterated samples: DXMS has been used to assess changes in iPLA₂ β conformation induced by binding to substrate, inhibitor, and membranes (Hsu et al., 2013, 2009; Mouchlis et al., 2015; Mouchlis & Dennis, 2016). In general, 40 μ g of iPLA₂ β protein in 25 μ L of protein buffer (25 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM urea, 250 mM imidazole, 2 mM ATP, 30% glycerol) is added to 75 μ L of D₂O buffer (50 mM MOPS, pH 6.9, 100 mM NaCl, 2 mM DTT, 2 mM ATP; final D₂O concentration = 71%). Enzyme is preincubated with substrates, inhibitors, and membranes before addition of D₂O buffer. For membrane experiments, the ratio of lipid to iPLA₂ β is typically >60, to ensure that saturation of the enzyme (Cao et al., 2013) and enzyme is preincubated with inhibitor in such experiments to prevent extensive hydrolysis of the membranes (Cao et al., 2013; Hsu et al., 2009). Samples are then incubated at RT (23°C) for 10, 30, 100, 300, 1000, 3000, and 10,000 s.

Phase 2

Quenching and denaturation: As exchange of amide protons is negligible at pH 2.5 (Gallagher & Hudgens, 2016), the hydrogen/deuterium exchange reaction is quenched through addition of 100 μ L of 0.8% formic acid, 2 M guanidine hydrochloride. This solution also denatures the protein, in preparation for protease digestion in the next phase. The quenched reactions are immediately frozen on dry ice and then stored at –80°C.

Phase 3

Pepsin digestion and collection of peptides: All subsequent processing steps are performed at 0°C. Given its acid pH optimum, pepsin is among the most commonly used proteases to generate peptides for DXMS analyses (Gallagher & Hudgens, 2016; Percy et al., 2012). Porcine pepsin (Sigma, cat # P6887) is immobilized at 30 mg/mL on an Upchurch Scientific guard column (66 μ L bed volume; cat # C.130B) packed with Poros 20 AL-activated affinity medium (ThermoFisher, cat # 1602802). The quenched deuterium exchange reactions are

passed over the pepsin affinity column in 0.05% trifluoroacetic acid (TFA) at 100 $\mu\text{L}/\text{min}$ for 1 min (resulting digestion time = 13 s) before resultant peptides are passed onto a 1 $\times$ 50 mm reversed-phase C18 column (Vydac columns, Fisher; cat # 501120911). Peptides are eluted with a linear gradient of 0.046% TFA, 6.4% acetonitrile (v/v) to 0.03% TFA, 38.4% acetonitrile at 50 $\mu\text{L}/\text{min}$ for 30 min (Hsu et al., 2008). This online digestion approach minimizes back exchange (Cao et al., 2013; Gallagher & Hudgens, 2016; Percy et al., 2012). For reactions containing vesicles (typically at lipid to protein ratios of 60 or greater), it is advisable to pass the samples through a C8 precolumn to remove the lipids (Cao et al., 2013).

Phase 4

Mass spectrometry analysis of deuterated peptides: The peptides are analyzed by ESI-MS/MS. Specifically, iPLA₂ β analyses are performed using a Finnigan LCQ mass spectrometer, capillary temperature 200°C (Burke et al., 2008; Hamuro et al., 2004; Hsu et al., 2008).

Data analysis: The first step in data analysis is to identify and curate the deuterated peptides, using the SEQUEST software from Thermo Finnigan. Once overlapping peptides have been identified/aligned, a variety of software packages can be used to assess rate of hydrogen/deuterium exchange for each peptide (Gallagher & Hudgens, 2016). Solvent exchange rates are higher in solvent-exposed peptides than in peptides buried in the interior of the protein. Changes in the rate of exchange upon protein association with substrate, inhibitor, ligand, membranes, or oligomeric partners can provide mechanistic information about changes in conformation induced by these interactions (Cao et al., 2013; Gallagher & Hudgens, 2016; Percy et al., 2012). As noted earlier, there are alternative explanations for changes in the exchange rate of amide protons that can confound interpretation of DXMS data. As such, the iPLA₂ β DXMS experiments should be interpreted in the context of data generated in MD simulations, using the homology models described earlier (Hsu et al., 2013, 2009; Mouchlis et al., 2015; Mouchlis & Dennis, 2016). It is also important to include two control experiments to account for any back exchange that occurs during processing. In the first (“back exchange” control), a fully deuterated protein is generated (D₂O labeling of denatured protein) and then carried through the processing step to assess deuterium atoms lost during processing. The second control (“on exchange” control) is generated by adding D₂O to protein in quenching buffer to assess exchange induced during the processing steps (Cao et al., 2013). Back exchange can also be minimized by optimizing experimental conditions (Gallagher & Hudgens, 2016; Percy et al., 2012).

2.3.2 Outcomes of DXMS Analyses of iPLA₂ β —The protease digestion and processing phases have been optimized to yield 88% coverage of the iPLA₂ β primary sequence (Hsu et al., 2009, 2013). Several regions of the ankyrin repeat domain (205–211, 390–404, 439–450, 458–470) and two regions in the catalytic domain (617–629; 708–730) show rapid hydrogen/deuterium exchange on the order of >70% at 10 s. These observations suggest that these regions are highly solvent exposed, a supposition that is borne out in the homology model and in additional DXMS experiments which indicate that the amphipathic alpha helix formed by residues 708–730 becomes buried upon exposure to membrane

vesicles (Hsu et al., 2009). Binding of a fluoroketone inhibitor (1,1,1,3-tetrafluoro-7-phenylheptan-2-one) induces decreased hydrogen/deuterium exchange in five regions of iPLA₂β (residues 483–493, 516–525, 544–549, 631–655, 773–778) that are localized near the active-site serine (S519) and aspartic acid (D652) (Hsu et al., 2013). Comparable changes are observed in several regions of the catalytic domain when iPLA₂β is exposed to small unilamellar vesicles composed of 1-palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine, a membrane mimetic (Hsu et al., 2009). Upon binding to membrane, the catalytic domain of iPLA₂β exhibits reduced hydrogen/deuterium exchange in regions composed of residues 631–655, 658–664, 708–730, and 773–778. As noted earlier, the alpha helix in regions 708–730 exhibits the largest reduction (13.2 deuterons, 70% decrease), suggesting a dramatic shift in conformation/localization of this helix when iPLA₂β binds to membranes. When taken together and considered in light of molecular dynamics simulations, these observations lead to a model wherein the function of the 708–730 helix region is analogous to that of the “cap” region in group IVA PLA₂ (cPLA₂) (Hsu et al., 2009; Mouchlis et al., 2015). Upon membrane binding, this region is proposed to convert iPLA₂β from a “closed” to an “open” state, by penetrating the membrane and orienting such that its hydrophilic face (R710, K719) is positioned to interact with phospholipid head groups and its hydrophobic face (P711; P714; W715; L717; L721; W722) is positioned to interact with the fatty acid chains (Bucher, Hsu, Mouchlis, Dennis, & McCammon, 2013; Mouchlis et al., 2015). Based on these observations, an intriguing hypothesis has been proposed that the membrane is an allosteric regulator of iPLA₂β activity (Mouchlis et al., 2015).

In short, DXMS has provided novel insights into the molecular mechanisms governing iPLA₂β interaction with both substrate phospholipids and the membranes in which these molecules reside. A notable corollary to these studies is the potential to apply DXMS (together with other biophysical, structural, and biochemical approaches) to facilitate rational design of selective inhibitors of iPLA₂β (Mouchlis, Limnios, et al., 2016).

3. SUMMARY

iPLA₂β-derived lipids have critical roles in various biological processes and altered accumulation of these bioactive lipids, due to dysregulation of iPLA₂β expression or activation, can have profound consequences. Continued studies of this enzyme and its biology will therefore lead to a greater understanding of the roles that bioactive lipids play in the onset and progression of various disease states. They will also facilitate identification of novel pathways that can be targeted for drug therapy. The approaches described herein will facilitate measurement of iPLA₂β activity, purification of iPLA₂β from biological samples, and further delineation of conformational changes in iPLA₂β subsequent to membrane/substrate/inhibitor binding. Also provided are means to assess membrane dynamics of iPLA₂β. For a more comprehensive review of the iPLA₂s and related citations, the reader is kindly directed to the recent publication by Ramanadham et al. (2015).

Acknowledgments

Methods described here were developed in association with projects funded by the American Diabetes Association and NIH/NIDDK (R01-69455).

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Fig. 1.
iPLA₂β activity assay flow.

Fig. 2.
Recovery of hydrolyzed fatty acid for quantitation.

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Tube #	Protein (μL)	HB (μL)	dH ₂ O (μL)	AB (μL)	EGTA (μL)	ATP (μL)	S-BEL (μL)	Substrate (μL)	TV (μL)
1 (blank)	0	100	35	50	10	0	0	5	200
2	Total=100		35	50	10	0	0	5	200
3	Total=100		-ATP (μL)	50	10	10	0	5	200
4	Total=100		-BEL (μL)	50	10	0	10	5	200
5	Total=100		-ATP -BEL (μL)	50	10	10	10	5	200
6	Total=100		35	50	10	0	0	5	200
	Total=100		35	50	10				200
	Total=100		35	50	10				200
	Total=100		35	50	10				200
N (blank)	0	100	35	50	10	0	0	5	200