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MEASUREMENTS OF PHOSPHOLIPASES A_2, C AND D (PLA_2, PLC AND PLD): IN VITRO MICROASSAYS, ANALYSIS OF ENZYME ISOFORMS, AND INTACT-CELL ASSAYS

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1. Introduction

Phospholipases have been studied in great detail and their role in cell signaling has been established in the last 20 years. A role for phospholipases in mitogenesis has been described in terms of the products of their enzymatic reactions. Lysophosphatidic acid (LPA) is produced by the action of a phosphatidic acid (PA)-specific phospholipase A_2 (PLA_2). LPA is a potent mitogen for quiescent fibroblasts and triggers GTP-dependent phosphoinositide breakdown (1) through a membrane receptor (2). A phosphatidylcholine-specific PLA_2 is responsible for the release of arachidonic acid in response to cell stimulation. Arachidonic acid is the precursor of biologically-active eicosanoids such as prostaglandins, thromboxanes and leukotrienes of key importance in the body's inflammatory response (3).

Phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C, associated to membrane G proteins (PLCβ), hydrolyzes PIP_2 to form 1,2-diacylglycerol (DAG) and inositol 1,2,5-trisphosphate (IP_3). DAG and IP_3-generated Ca^{2+} are well known agonists of protein kinase C (PKC) activation (4,5), and this phospholipid breakdown is one of the earliest key events in the regulation of various cell functions, including cell growth. A phosphatidylcholine-specific PLC, associated to tyrosine kinases, also releases DAG as well as phosphorylcholine. The latter compound is known to be accumulated in the cell during the activation of Raf-1 and mitogen-activated protein (MAP) kinase that follows to growth factor stimulation and mitogenesis (6,7). Phosphocholine in conjunction with sphingosine-1-phosphate can greatly stimulate MAP kinase activity (8).

Phosphatidic acid (PA) is synthesized by the combined action of PLC and a DAG kinase, as well as by a direct action of phospholipase D (PLD) on membrane phospholipids. PA induces a transient intracellular Ca^{2+} rise and has growth-factor like effects on cultured cells (9). The formation of substantial amounts of PA immediately before entry into mitosis is important for establishing a delay in the cell cycle at the G_2/M border brought about by exogenous ligand (10). There are alternative pathways for PLC and PLD in regulatory events of intracellular mitogen signaling (11). In order to be properly divisible, internal membranes must be converted into a vesiculated state prior to mitosis. Due to its membrane-

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perturbing and fusogenic properties, an increased level of PA in specific membrane compartments may counteract this process and the cell is delayed in the G_2 phase (12). Activation of PLD by serum is observed during *Ras*-induced transformation in NIH3T3 fibroblasts (13). Finally, the other product of PLD action, choline or ethanolamine (depending upon the nature of the membrane phospholipid can also have mitogenic effects. Particularly, ethanolamine analogues stimulate DNA synthesis by a mechanism not involving phosphatidylethanolamine synthesis (14).

2. Materials

2.1. Preparation of the biological sample

1. Hanks' Buffer: 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4; 150 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM dextrose.
2. PLA_2 buffer: 250 mM Tris HCl, pH 8.5 (3.93 g/100 ml ddH_2O).
3. PLC buffer: 20 mM Tris-HCl pH 7.4, 60 mM NaCl, 3 mM $CaCl_2$ (0.32 g Tris-HCl, 0.35 g NaCl, 0.044 g $CaCl_2$ in 100 ml ddH_2O).
4. PLD buffer: 5 mM HEPES, pH 6.6. Prepare fresh each time from stock (200 mM HEPES, pH 6.6) as follows: 50 μl stock + 1.95 ml ddH_2O).
5. Protease/phosphatase inhibitors (recipe for 5 ml): 5 μg/ml aprotinin (12.5 μl from 1 mg/0.5 ml stock); 5 μg/ml pepstatin A (12.5 μl from 1 mg/0.5 ml stock); 5 μg/ml leupeptin (12.5 μl from 1 mg/0.5 ml stock); 100 μM sodium orthovanadate (4 μl from 10 mg/0.5 ml stock); 40 μM PMSF (1 μl from 10 mg/0.5 ml stock). Stocks are kept at −20 °C.
6. Right before the experiment add the protease/phosphatase inhibitors to 5 ml each of the PLA_2, PLC or PLD buffers and keep on ice.

2.2. PLA_2 *in vitro* microassay

1. Calcium solution: 100 mM $CaCl_2$ (1.47 g/100 ml ddH_2O).
2. Dilution buffer: 5 mM Tris HCl, pH 8.5 (0.04 g/50 ml ddH_2O).
3. Radiolabeled PC (*PC): phosphatidylcholine, L-α-1-stearoyl-2-arachidonyl (specific activity 200 Ci/mmol; 1mCi/ml) from American Radiolabeled Chemicals (ARC).
4. Cold PC: L-α-phosphatidylcholine-β-arachidonyl-γ-stearoyl (C20:4/C18), dissolved at the concentration of 10 mg/ml in chloroform:methanol (9:1, vol/vol).
5. Positive controls: PLA_2 from bovine pancreas (purified enzyme), 5.7 Units/mg (stock: 10 mg dissolved in 0.3 ml dilution buffer, aliquot in 50 μl and freeze (activity is 3 Units/50 μl)). PLA_2 from bee venom (purified enzyme), 1225 units/mg (stock: 1 mg dissolved in 1 ml dilution buffer aliquot in 50 μl and freeze (activity is 60 units/50 μl)).
6. 'Dole's extraction medium': isopropanol, n-heptane, H_2SO_4 (80:20:0.06, vol/vol).

2.3. PLC *in vitro* microassay

1. DOC solution: dissolve 20 mg deoxycholate (DOC) in 1 ml of PLC buffer.
2. Radio labeled PC (*PC): L-3-Phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl (specific activity 55 mCi/mmol; 25 μCi/ml) from Amersham.

3. Cold PC: L-α-phosphatidylcholine.
4. Positive controls: PLC from *B. cereus* (purified enzyme); stock: dissolve 25 Units in 100 μl PLC buffer; aliquot into 50 μl and freeze (12 Units/50 μl).
5. Stopping solution: methanol:chloroform:10N hydrochloric acid (20:10:0.2, vol/vol/vol).
6. Authentic standards: Three species of diacylglycerol (DAG): C14:0/C14:0-DAG; C16:0/C18:0-DAG; C18:0/C20:4-DAG, dissolved separately in chloroform/methanol (9:1, vol/vol) at the concentration of 10 mg/ml (*see* Note 1).
7. Solvent system for TLC chamber ('hydrophobic solvent system'): 208.2 ml benzene, 39 ml Cl_3CH and 8.5 ml methanol (*see* Note 2).

2.4. PLD *in vitro* microassay

1. 200 mM HEPES
2. 5 mM HEPES, pH 6.6 (50 μl of 200 mM HEPES + 1.95 ml ddH_20).
3. PC8: 20 mg dioctanoyl phosphatidylcholine (PC8)/ml chloroform.
4. Radiolabelel butanol: [^{3}H]butan-1-ol (0.6 mCi/ml) from American Radiolabeled Chemicals (ARC).
5. Positive controls: PLD from cabbage (purified enzyme); 0.25 Units/μl (dissolve 500 Units in 2 ml PLD buffer. Store aliquots at -20 °C).
6. Stopping solutions and related solvents: chloroform/methanol (1:2), chloroform/methanol (95:5, vol/vol), ethyl acetate, iso-octane, acetic acid, 1% perchloric acid.
7. Authentic standard for TLC: dissolve PBut in chloroform:methanol (9:1, vol/vol) at the concentration of 10 mg/ml.
8. Solvent system for TLC chamber ('PLD solvent system'): In a separatory funnel, shake 130 ml ethyl acetate, 20 ml iso-octane, 30 ml acetic acid, and 100 ml ddH_2O. Allow the biphase to form and settle. Next, discard the lower phase, pour upper phase into TLC chamber lined with #3 Whatman paper (18 cm × 21 cm).

2.5. Immunoprecipitatioin and immunocomplex enzyme assays

1. 1 M HEPES 23.8 g/100 ml H_2O and pH to 7.3.
2. '10XLB' (stock lysis buffer): 100 mM HEPES, pH 7.3, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2.1 μM sodium orthovanadate, 1 mM dithiothreitol (DTT), 10 μM ammonium molybdate, 12 mM diisopropyl fluorophosphate (DFP), 100 mM p-nitro-phenyl-phosphate, 10 mM β-glycerophosphate, 5.5% Triton X-100, and 5 μg/ml each of leupeptin, aprotinin, and pepstatin A (*see* Note 3).
3. LB: (1 ml '10XLB' + 7 ml Hank's + 2 ml glycerol) prepared right before the experiment; keep on ice.

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[1]Authentic standards are always kept under N_2 in a sealed tube, wrapped up in aluminum foil to avoid direct light, and placed in a desiccator inside a deep (−70 °C) freezer.
[2]The hydrophobic solvent system for TLC separation must be prepared fresh for each use.
[3]The HEPES, $MgCl_2$ and EGTA solutions are prepared in small plastic bottles and kept at 4 °C for up to 6 months. The protease/phosphatase inhibitors are prepared from lyophilized samples, immediately aliquoted down and stored at −70 °C protected from light and with a desiccant (in this way they are still effective up to three months). At the time of the experiment, thaw them out and add to the base buffer. Discard aliquots after using, never re-freeze.

4. LB/BSA: Dissolve 10 mg of fatty acid free-BSA in 1 ml of LB.
5. Anti-rabbit IgG secondary Antibody ('2ry') linked to agarose beads (this comes as a slurry with 1μl packed beads per each 2 μl of slurry) (*see* Note 4).
6. Primary ('1ry') Antibody (polyclonal anti-PLD2) (*see* Note 4).
7. LiCl wash: 100 mM Tris-HCl, pH 7.4, 400 mM LiCl.
8. NaCl wash: 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA.

2.6. Intact-cell (*in vivo*) assays

2.6.1. Measurement of cPLA$_2$—

1. Hanks' Buffer: 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4; 150 mM NaCl, 5 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM Dextrose.
2. Lysis Buffer: 50 mM HEPES, pH 7.4; 250 mM sucrose, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, 5 mM dithiothreitol (DTT), 20 μM each of aprotinin, leupeptin and pepstatin.
3. [^{3}H]arachidonic acid (usually dissolved in ethanol).
4. Stopping solution: Hexane/isopropanol/0.1 N HCl (300:200:4; vol/vol).

2.6.2. Measurement of PLCβ—

1. Carrier-free $H_2[^{32}P]O_3$ (specific activity 1 mCi/ml).
2. Hanks' Buffer (RPMI or any tissue culture media of choice).
3. Lipid extraction mixture: hexane/isopropanol/0.1 N HCl (300:200:4 by vol).
4. Hexane/isopropanol (ratio 3:2).
5. X-ray films (Kodak O-mat).

2.6.3. Measurement of PLD—

1. [^{3}H]palmitic acid (specific activity 5 mCi/ml).
2. Earl's Balanced Solution (EBS), RPMI (or tissue culture media of choice).
3. Authentic standard: Phosphatidylpropanol (PProp) (25 mg/ml stock) from Avanti Polar Lipids (Alabaster, AL). Before applying to TLC plate prepare the following 'standard mix': 280 μl PProp + 970 μl chloroform.
4. Materials listed in points 6-8 under **Subheading 2.4.**

3. Methods

Here we describe comprehensive methods for assaying phospholipases A_2, C and D (PLA_2, PLC and PLD). These assays are divided into three major sections: [1] *in vitro* microassays (**Subheading 3.1**), in which a radiolabeled substrate is exogenously added to cell lysates or sonicates to measure the enzyme activity(ies); [2] immunocomplex assays (**Subheading 3.2**), in which immunoprecipitation with a specific antibody is performed (that is

[4]It is crucial that the primary and secondary antibodies are of the same species. For a monoclonal (mouse) primary antibody always use anti-mouse IgG secondary antibody linked to agarose beads (available from Sigma). For a polyclonal (rabbit) primary antibody always use anti-rabbit IgG secondary antibody linked to agarose beads (also available from Sigma).

immediately followed by the *in vitro* microassays alluded to before) in order to study the contribution of a particular isoform within a family of enzymes; and [3] intact-cell or *in vivo* assays (**Subheading 3.3**), in which cells are labeled with a radioactive substrate until steady state is reached and then the intact cells are challenged with appropriate stimuli.

3.1. *In vitro* microassays

The uniqueness of the method described here is that it allows the measurement of, in parallel, the activities of three phospholipases utilizing aliquots derived from the same biological sample (FIGURE 1).

The biological sample is prepared as described in detail in **Subheading 3.1.1**. The cell sonicates generated are then split in three equal aliquots and are utilized for the *in vitro* microassay methods. These are tailored to the measuring of total activity for: PLA_2 (**Subheading 3.1.2**), PLC (**Subheading 3.1.3**) and PLD (**Subheading 3.1.4**). The specificity in the method for each phospholipase is conferred by the proper use of a specific substrate, reaction assay buffers and methods of isolation of enzymatic products. All the assays described below are "microassays", i.e., they can be performed in the laboratory using 1.5-ml eppendorf tubes for all the reactions (instead of the traditional large glass test tubes). The small reaction volumes allow for a saving in reagent expenses and processing time. The inclusion of positive controls (like purified enzymes), with known specific activities, is necessary for completeness (15).

3.1.1. Preparation of the biological sample, or 'source of enzyme'—The very first step in the in vitro microassay protocols is the preparation of the sample from which the three enzyme activities (PLA_2, PLC and PLD) will be measured.

1. Resuspend cells in fresh RPMI (culture cells) or Hanks' buffer (neutrophils) in 50 ml conical tubes (blue cap) at a concentration of $0.8\text{-}1\times10^7$ cells/ml (lower range for cultured cells; higher for neutrophils or any other small size cell).
2. Preincubate with stimuli if needed (*see* Note 5).
3. Take 1 ml aliquots in triplicate and transfer to pre-chilled eppendorf tubes kept on ice.
4. Spin down tubes at 1,744xg for 30 seconds and decant supernatant.
5. Resuspend pellets in 1 ml of each buffer (PLA_2, PLC or PLD) supplemented with the protease/phosphatase inhibitors (the volume should equate to approximately 500 μl buffer/mg protein).
6. Sonicate samples on ice (set sonicator at mid-high setting, 2 cycles, 10 seconds per cycle) (*see* Note 6).

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[5]Add appropriate cell stimuli (at variable concentration or time of incubation) whose action on the enzyme activity is to be tested. Some phospholipases (like PLA_2) will benefit from the presence of calcium in the media. To this end, pre-incubate cells with 1.6 M $CaCl_2$ at 37 °C for 5 minutes. Since cells tend to sediment, make sure the tube is being rotated properly in the water bath. To stimulate cells, use 1 μl stimulus stock per 1 ml of cells. In this way the stimulus is diluted 1,000 times. Some stimuli might require an intermediate dilution. For example, when using PMA, we usually prepare a μg/ml (in DMSO) stock from which we make a 1:10 dilution in Hanks' buffer right before the experiment. We then add 1 μl per ml of cell suspension (final concentration is 50 ng/ml). All intermediate and final dilutions are discarded after use and never re-frozen. Whenever adding the stimulus to the cell suspension, hold conical tube in hand while swirling it, to facilitate immediate dispersion; add the stimulus very slowly to the suspension going back and forth several times with the pipette to completely release the contents. Place conical tube on bath.

[6]As an alternative to sonication, cell lysis can be performed in 'lysis buffer': 12 mM Tris-HCl, pH 7.2; 14 mM NaCl; 0.75 mM EDTA; protease and phosphatase inhibitors as described, and 0.25% Triton X-100. Mix cell pellets with lysis buffer and carry out lysis on ice for 20 minutes with intermittent (every 3-5 min) vortexing. Spin lysates at 13,000 rpm and collect supernatant. We have found (27) that Triton X-100 is inhibitory for PLD, thus, a different detergent must be used. β-octyl-glucopyranoside, in our hands, is appropriate, as long as it is kept at a final concentration of 0.05%, well below its critical micelle concentration (CMC).

7. Do a quick (30 sec) spin (14,000xg) to pull down unbroken cells and other debris. Transfer supernatants to clear eppendorfs labeled 'cell sonicates' and leave on ice until ready to assay. Measure protein concentration (the yield is usually 1-2 mg/ml). Fifty μl of these sonicates will be needed (per condition) for the *in vitro* microassays.

3.1.2. PLA_2 *in vitro* microassay—The first enzymatic assay we will consider is the measurement of PLA_2 activity in cell sonicates. The rate limiting step in eicosanoids synthesis is the release of arachidonic acid. This release involves PLA_2 activation and translocation to the compartments where the substrate (e.g. phosphatidylcholine), is located (16-23). The PLA_2 enzyme system is also crucial in the generation of the ether-lipid platelet-activating factor (PAF), a mediator of inflammation. The cytosolic form of PLA_2 ($cPLA_2$), mediates the production of agonist-induced arachidonic acid release, and activation of $cPLA_2$ requires the phosphorylation of the enzyme, an increase in the concentration of intracellular free calcium, and the translocation to the compartments where the substrates are localized (FIGURE 2).

For *in vitro* measurements of PLA_2, the exogenous substrate is a phosphatidylcholine (PC) radiolabeled in the *sn*-2 position (arachidonic acid). The radiolabeled arachidonic acid (free fatty acid) released after the enzymatic cleavage is purified by TLC. The method indicated below will detect total PLA_2 activity present in the biological sample. For a specific analysis of cytosolic PLA_2 ($cPLA_2$) (24) (*see* Note 7).

3.1.2.1. Preparation of liposomes:

1. Label a small unsiliconized glass test tube 'liposomes'; rinse with ddH_2O and dry.
2. Add 0.2 μl (0.2 μCi) of *PC per condition to the tube.
3. Add 1 μl (12 nmol) of cold PC per condition to the tube.
4. Dry lipids under Nitrogen.
5. Once dry, add 45 μl of PLA_2 buffer and 5 μl calcium solution per condition (final reaction concentration in a 100 μl volume is: 112.5 mM Tris HCl, 5 mM $CaCl_2$).
6. Vortex vigorously; let sit for approximately 10 minutes at room temperature.
7. Vortex again for 10 seconds; sonicate briefly (sonicator in mid-low setting, 3 cycles of 3 seconds each) on ice. Keep liposomes on ice until needed.

3.1.2.2. Reaction and lipid separation: At this point the enzyme (in cell sonicates) is ready to be mixed with the radiolabeled substrate in the optimal conditions of co-factors, ionic strength, pH, etc.

1. To 1.5 ml screw cap tubes, add 50 μl of 'liposomes' (from **3.1.2.1**) and 50 μl of 'cell sonicates' (**Subheading 3.1.1**).

[7]The Measurement of cytosolic $cPLA_2$ activity *in vitro* can be determined by measuring the amount of arachidonic acid released using 1-stearoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine as a substrate (24). The substrate is dried under a stream of nitrogen and resuspended in DMSO with vigorous vortex-mixing. A known volume of the substrate (2 μl) (final concentration 15 μM) and 5 μl of 16 mM $CaCl_2$ (final concentration 2 mM) are added to eppendorf microcentrifuge tubes. The reaction is initiated by adding a known volume (33 μl, 30-40 μg protein)) of the cytosolic fraction of cell homogenate. This is obtained by suspending a known number of cells ($1\text{-}5\times10^7$ cells) in 1.2 ml lysis buffer. The mixture is sonicated and then centrifuged at 150,000 g for 20 minutes. The supernatant obtained is used to measure $cPLA_2$ activity. The reaction is carried out for a preset time (5-40 minutes), and it is stopped by adding a known volume (40 μl) of ice-cold quench solution, comprising 40 μg/ml arachidonic acid in ethanol containing 2% (vol/vol) acetic acid. A 40 μl of the solution is spotted on LK5DF silica-gel TLC plates and developed in organic phase of ethyl acetate/iso-octane/acetic acid/water (55:75:8:100, by volume). The area corresponding to arachidonic acid is revealed by brief exposure to iodine vapor, scraped and the radioactivity is counted.

2. Include positive controls (pancreas and bee venom PLA_2 pure enzymes) and negative controls (PLA_2 buffer instead of cell sonicates).
3. Incubate 25-30 minutes at 30 °C in a orbital shaking incubator at 60 rpm.
4. Stop the reaction by adding 500 μl 'Dole's extraction medium' to each tube with a repeater pipette.
5. Add 400 μl heptane.
6. Add 300 μl ddH_2O.
7. Vortex tubes and spin down using micro-centrifuge (14,000xg, 1 min).
8. Carefully remove upper layer with a pipette and transfer to a clean eppendorf tube.

3.1.2.3. Isolation of the reaction product (radiolabeled arachidonic acid): This is based in the method by Van den Bosh *et al*. (25) and involves the use of column chromatography for the separation of radiolabeled arachidonic acid (also *see* Note 8).

1. Prepare a glass wool plugged Pasteur pipet containing 240 mg silica gel (70-230 mesh pore size) per condition.
2. Prepare a rack of scintillation vials each containing 4 ml scintillation cocktail.
3. Secure Pasteur pipet with a clamp and position tip of pipet directly over scintillation vial.
4. Apply 400 μl of sample upper phase (from 2.1.2.3, last point) to the pipet and wait until it becomes wet.
5. Next, add 800 μl of diethyl ether (via a 1 ml 'tuberculin' syringe) and collect eluate ([^{3}H]arachidonic acid) directly in scintillation vial containing (4 ml) scintillation cocktail.
6. Repeat procedure for each sample, using a new Pasteur pipet each time.
7. Cap and vortex scintillation vials and count by scintillation spectrometry.

3.1.3. PLC *in vitro* microassay—For PLC the substrate is a radiolabeled PC (either *sn*-1 or *sn*-2 positions) and the product of the reaction (radiolabeled diacyl-glycerol, DAG) is separated by TLC developed in a 'hydrophobic' solvent system.

3.1.3.1. Preparation of liposomes: This is based in the method by Waite and Smith (26) tailored for polymorphonuclear neutrophils, but can be applied to a variety of cultured cells.

1. Label a small unsiliconized glass test tube 'liposomes'; rinse with ddH_2O and dry.
2. Dry the tube.
3. Add 2.5 μl cold PC (320 μM final) and 2 μl *PC (50 nCi or 8 μM final) per condition.
4. Dry lipids under nitrogen.
5. Once dry, add 48 μl of PLC buffer per condition to the tube.

[8]An alternative procedure for separation of the [^{3}H]arachidonic acid product, is to apply upper phase (from **Subheading 3.1.2.2**, last point) to a silica gel plate and TLC and develop it in the TLC solvent system hexane:diethylether:formic acid (60:40:2, vol/vol/vol) (R_f is ~0.7). This system allows the quantification of total [^{3}H]PC that should remain in, or very close to, the application spot after running the plate (R_f is ~0-0.05).

6. Next, add 2 μl of diluted DOC per condition to the tube (1 mM final).
7. Vortex vigorously; sonicate briefly (low-mid setting) on ice and keep liposomes on ice until needed.

3.1.3.2. Reaction and lipid separation: At this point the enzyme (in cell sonicates) is ready to be mixed with the radiolabeled substrate in the optimal conditions of co-factors, ionic strength, pH, etc.

1. To 1.5 ml screw cap tubes, mix 50 μl of 'liposomes' (**Subheading 3.1.3.1**) and 50 μl of 'cell sonicates' (**Subheading 3.1.1**).
2. Include positive controls (*B. cereus* PLC pure enzyme) and negative controls (PLC buffer instead of cell sonicates).
3. Incubate 10 minutes at 30 °C in a orbital shaking incubator at 60 rpm.
4. Stop the reaction by adding 200 μl of stopping solution to each tube with repeater pipette.
5. Add 75 μl of Cl_3CH.
6. Add 25 μl ddH_2O.
7. Cap and vortex each tube vigorously.
8. Centrifuge at 5,000 rpm for 2 minutes.
9. Transfer 120 μl of the lower layer (organic phase containing the lipids) to a new set of transfer tubes.

3.1.3.3. Isolation of the reaction product (radiolabeled DAG):

1. Prepare a "hydrophobic solvent system" and dump it in TLC chamber, cover with lid, and let it sit for a minimum of 1 hour before using it.
2. Spot 30 μl of each lower, organic phase sample (from **3.1.3.2**) onto a TLC plate; repeat in rounds until each sample is spotted.
3. Spot 5 μl of each DAG standard onto each plate.
4. Place the plate in the TLC chamber and run it for about 40 minutes (*see* Note 9).
5. Remove plate from the chamber and let it dry.
6. Place plate in iodine chamber to expose standards.
7. Mark standards (R_f of C18:0/C20:4-DAG in this solvent system is ~0.5) and draw lines 1 cm above top standard and 1 cm below standard.
8. Scrape each lane, place scraped silica into scintillation vial containing 4 ml scintillation cocktail and count samples by scintillation spectrometry (*see* Note 10).

3.1.4. PLD *in vitro* microassay—PLD catalyzes the hydrolysis of membrane phospholipids (such as PC) releasing phosphatidic acid (PA) and a polar head group. In addition to this, in the presence of a primary alcohol (usually ethanol or 1-butanol), PLD can generate a phosphatidylalcohol (phosphatidylethanol (PEt) or phosphatidylbutanol (PBut))

[9] To run a plate in this 'nonpolar' solvent mixture takes approximately 40 minutes, much shorter than other chromatography system, thus it must be watched closely or it will overrun.

[10] After plate has run, a zone around (0.5 cm above and below) the application spot (R_f ~0) could also be scraped to analyze total [^{14}C] PC. As PC is broken down by PLC to form PA, the amount of PC should *decrease*.

end-product by what is termed a 'transphosphatidylation' reaction. This reaction is unique to PLD and, as a result, is a commonly used method for measuring PLD activity without the possibility of contamination from other phospholipase activities (chiefly the PLC/DAG kinase system that can also generate PA). For the description of the measurement of PLD in this chapter, the substrates or the reaction are: [a] a special, short-chain PC (PC8) and [b] radiolabeled butanol. The reaction product is phosphatidyl-butanol (PBut) that is separated by TLC in a 'polar' solvent. This method was originally described in (27).

3.1.4.1. Preparation of liposomes:

1. Label a small unsiliconized glass test tube 'liposomes'; rinse with ddH_2O and dry.
2. Add 13.5 μl (final concentration 24 mM) of PC8 per condition to the tube.
3. Dry PC8 lipid under Nitrogen.
4. Once dry, add 22.2 μl of 5 mM HEPES per condition to the tube.
5. Vortex vigorously.
6. Let sit for one hour at room temperature.
7. Sonicate for 30 seconds in a bath sonicator; keep liposomes on ice until needed.

3.1.4.2. Preparation of the assay mix:

1. To a 2 ml eppendorf tube labeled 'assay mix', add the following per condition: 30 μl of 200 mM HEPES; 7.89 μl of [^{3}H]butanol and 12.1 μl of ddH_2O.

3.1.4.3. Reaction and lipid separation: At this point the enzyme (in cell sonicates) is ready to be mixed with the radiolabeled substrate in the optimal conditions of co-factors, ionic strength, pH, etc.

1. To a 1.5 ml polypropylene tube, add 20 μl of 'liposomes' (**Subheading 3.1.4.1**), 50 μl of 'assay mix' (**Subheading 3.1.4.2**), and 50 μl of 'cell sonicates' (**Subheading 3.1.1**).
2. Include positive controls (cabbage PLD pure enzyme) and negative controls (PLD buffer instead of cell sonicates).
3. Incubate 30 minutes at 30 °C in a slowly shaking incubator, agitate lightly during the hour.
4. Stop the reaction by adding 3 ml of ice-cold chloroform/methanol (1:2, vol/vol) and 0.7 ml of 1% perchloric acid.
5. Vortex for 1 minute and let sit for 10 minutes.
6. Add 1 ml of chloroform and 1 ml of 1% perchloric acid.
7. Vortex for 1 minute, centrifuge and aspirate upper phase.
8. Wash lower phase with 2 ml of 1% perchloric acid.
9. Vortex, centrifuge, and aspirate upper phase.
10. Repeat wash if necessary.
11. Collect lower phase for TLC (should be approximately 1 ml).

3.1.4.4. Isolation of reaction product (radiolabeled PBut):

1. Dip a TLC plate in 1.3% potassium oxalate for 1 minute.

2. Let dry at room temperature.
3. Heat plate at 115 °C for 1 hour; let it cool down.
4. Dry samples under nitrogen.
5. Add 25 μl of chloroform/methanol (95:5, vol/vol) to dried down sample and then immediately spot 25 μl on the TLC plate—repeat until all samples are spotted.
6. Spot 20 μl of PBut standard on each plate.
7. Place the plate in the TLC chamber containing the 'PLD solvent system' (run takes approximately 55 minutes).
8. Remove plate and let dry.
9. Place plate in iodine chamber to visualize authentic standards.
10. Scrape 0.5 cm above and 2 cm below PBut (R_f in this solvent system is ~ 0.45) (*see* Note 11).
11. Count radioactivity.

3.2. Immunoprecipitation and immunocomplex enzyme assays

The immunocomplex assay is an extra step that can be added prior to the *in vitro* microassays alluded above (**Subheadings 3.1.2-3.1.4**). This extra step relies on the inclusion of a specific antibody that will ensure that a particular isoform out of a collection of enzymes with similar reaction activities will be measured (Figure 3).

In this chapter, a specific example is given for PLD2 isoform as we have described previously (28,29). The *in vitro* microassay described in 2.1.4 will measure total PLD in a sample, while immunoprecipitation with PLD2 antibody, as described below, will target that particular activity. This approach can be extrapolated to the study of PLA_2 or PLC isoforms, just by using the appropriate antibody.

3.2.1. Preparation of antibody conjugates—With this step the primary antibody will be bound to the secondary antibody (linked to agarose beads) forming the tertiary complex: [1ry/2ry/agarose] or 'antibody conjugate'. The following amounts are given for one experimental condition. For the experiment, multiply the amounts by the number of conditions and plan for one extra condition for negative controls, i.e., all reagents except the primary antibody. Note that the protocol given below is for PLD2, but it can be easily extrapolated to analyze isoforms of the other phospholipases (PLA_2 and PLC) simply by using the appropriate specific antibody against the isoform intended to be studied. All steps below must be performed at 4 °C.

1. Take 1.5 ml screw cap eppendorf tube labeled 'Ab conjugate' and place on ice; add 40 μl anti-mouse beads slurry.
2. Add 100 μl LB to the tube, vortex gently and spin down at 12-13,000 rpm for 15 sec and discard supernatant (*see* Note 12).

[11]We routinely use a long-chain analog of PC8-PBut since, as far as we can tell, there are not commercially available standards for PBut with the short, dioctanoyl chain (PC8). In the TLC system considered, the R_f for PC8-PBut is located approximately one cm below the long-chain PBut (and actually close to phosphatidylethanol (PEt) that can be used as an alternative standard).

[12]During all steps that require washes during the immunoprecipitation procedure, always pull off supernatant very carefully, leaving a small amount of liquid on the precipitate so as to avoid touching and unsettling the beads. Alternatively, the tube can be decanted, positioning it at a 45 degree angle with the mouth of the tube against a paper napkin/kimwipe. Never overdo it (it is better to leave some liquid on top of the beads pellet, forming a meniscus), since it is vital that beads remain at the bottom of the eppendorf and none are not lost.

3. Add 40 μl of LB and 60 μl LB/BSA to the beads and vortex gently.
4. Add 1-3 μg of primary antibody (anti-rabbit PLD2); vortex again.
5. Place the tube in a orbital shaker immediately to rotate upside down overnight at 4 °C (a cold box is best suited for this).

3.2.2. Formation of immunocomplexes—With this step the tertiary complex: [1ry/2ry/agarose] or 'antibody conjugate' (**Subheading 3.2.1**) will be mixed with the cell sonicates. Here, the primary antibody will bind to its antigen (i.e., PLD protein) forming the immunocomplexes. These can be used in the in vitro microassays for measuring phospholipase activity as indicated earlier.

1. Pellet down live cells (whose phospholipase activity is to be measured) and subject them to lysis. For this, resuspend pellet in 400 μl of LB in an eppendorf tube labeled 'lysates'. Incubate on ice for 20 min, with one gentle vortexing at 10 min and another 2 or 3 min before the end (*see* Note 13).
2. Spin down at 14,000xg for 1 minute, decant and resuspend the pellet in 400 μl LB (*see* Note 14).
3. Take the overnight 'antibody conjugate' out and spin down at 14,000 rpm at 4 °C; carefully remove supernatant, add 100 μl of fresh LB and mix gently (*see* Note 15).
4. Add the whole 100 μl of 'antibody conjugate' in fresh LB to the 'cell lysates' kept on ice per each condition. For negative controls, use just LB instead of cell lysates.
5. Cap tubes, lightly vortex and place in a shaker to rotate at 4 °C for 3 hours.
6. Spin down at 4 °C for 15 sec. at 14,000 rpm after the incubation time is up.
7. Dump out supernatant and wash once with 500 μl of pre-chilled LiCl wash solution and once with 500 μl of pre-chilled NaCl wash solution.
8. Remove as much of remaining liquid from all tubes without disturbing the pellet and resuspend in 110 μl of LB (*see* Note 12). These are the 'immunocomplexes' that contain the isoform of the enzyme of interest (PLD2) in a highly enriched form. They can be split into two 50 μl tubes for a PLD *in vitro* assay in duplicate as indicated above (**Subheading 3.1.4.3**, first point). The 50 μl 'immunocomplexes' are to be added in lieu of the 'cell sonicates' to a 1.5 ml polypropylene tube, along with 20 μl of 'liposomes' and 50 μl of 'assay mix'. Follow the rest of the protocol for the measurement of activity (agarose beads do not interfere with the enzymatic reaction, only make sure that they do not settle down during the incubations).

3.3. Intact-cell (*in vivo*) assays

We now describe the intact-cell or *in vivo* assays (**Subheadings 3.3.1** for PLA_2 ($cPLA_2$); **3.3.2** for PLC (PLCβ); and **3.3.3** for PLD) in which living cells are labeled with a radioactive substrate until steady-state is reached. Then the intact cells are challenged with an appropriate stimulus and the product of the reaction is isolated from the cells (FIGURE 4).

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[13] In order to increase the yield of protein, sometimes it helps to sonicate the lysates briefly (this should always be done on ice): two cycles (on mid-high setting of sonicator), one for 8 sec. then for 6 sec. and let settle on ice for 10-20 min.

[14] Since imunoprecipitation requires only 200 μl of sample, the rest of the lysates could be stored frozen at −70 °C for future use. We routinely save 20 μl aliquots for protein determination the same day of the experiment.

[15] Cut a small amount from the end of the yellow tips and resuspend 1-2 times to ensure that the beads will not clog the end of the tip. Vortex tube of immunocomplex beads often to ensure that beads remain in suspension all the time.

The advantages of assaying phospholipases in intact cells are: [a] One can assay receptor-mediated activation. It is sometimes difficult to maintain the coupling in broken-cell preparations (enriched membrane fractions, whole lysates or sonicates); [b] if the conditions of the *in vitro* assays are set at optimal (as described previously), they certainly will detect all activity there is there, but it might not necessarily reflect what is happening in the intact cell. The phospholipase under study might be down-regulated in the intact cell, but not in the *in vitro* assay.

3.3.1. Measurement of cPLA$_2$ in intact cells or *in vivo*—The activity of cytosolic phospholipase A$_2$ (cPLA$_2$) *in vivo* is determined by measuring the amount of arachidonic acid released from cells prelabeled with [^{3}H]arachidonic acid.

3.3.1.1. Cell labeling with [^{3}H]arachidonic acid:

1. Sample [^{3}H]arachidonic acid into a round bottom flask at the final concentration of 1-3 μCi/ml; evaporate the ethanol under a Nitrogen stream (30).
2. Add one ml per experimental condition of a cell suspension (at a 1-5×10^7 cells/ml density).
3. Incubated at 37 °C for an hour or until steady-state is reached (*see* Note 16).
4. Pellet cells (2,000xg, 5 min) and wash twice with Hanks' buffer to eliminate non-incorporated [^{3}H]arachidonic acid (*see* Note 17).
5. Resuspend cells in Hanks' buffer at a 1×10^7 cells/ml density. Place cell suspension in a 50-ml conical tube in a reciprocating water bath at 37 °C. Initiate the reaction by the addition of the stimulus (e.g. PMA, 50 ng/ml) to the suspended cells (31).

3.3.1.2. Measurement of total arachidonic acid to the incubation media: This method measures the radioactivity in both arachidonic acid and any labeled metabolites of arachidonic acid. The main advantage of this method is its simplicity. The disadvantage is that it measures only the radioactivity that is released into the suspending media. Accordingly, it underestimates the amount of arachidonic acid released.

1. Start with a steady state-labeled, agonist-stimulated cell suspension from **Subheading 3.3.1.1.**
2. Remove 1 ml-aliquots at a preset time (e.g., 5-10 min for PMA stimulation); pellet down cells (14,000xg, 30 sec, 4 °C). Keep tubes on ice (repeat this procedure for each time point in a time-course experiment).
3. Transfer 100 μl of the supernatant to scintillation vials and count radioactivity in a β-scintillation counter.

3.3.1.3. Measurement of cell-released arachidonic acid: The advantage of this method over the preceding one is that it measures total arachidonic acid released. There are two main disadvantages. First, it is time consuming and not simple. Secondly, it does not account for any possible metabolism of arachidonic acid. This is very minor if the reaction is carried out rapidly (32,33).

[16]This period of incubation varies, but the goal is to achieve steady-state. This is accomplished when cell-associated radioactivity does not change with time. The time needed to reach steady-state varies among different cells.

[17]Hanks buffer is ideal for neutrophils, but any desired buffered solution can be used. For cultured cells, fresh media is fine. The goal of the washes is to remove any non-incorporated labeled arachidonic acid. Thus, the washing must be thorough. A sample of the supernatant of the last wash can be quickly counted for any residual radioactivity. If necessary, additional washing may be required.

1. Start with a steady state-labeled, agonist-stimulated cell suspension from **Subheading 3.3.1.1**.
2. Pipette down 1 ml-aliquots and transfer to glass tubes.
3. Stop the reaction after a preset time by the addition of 100 μl hexane/isopropanol/ concentrated 0.1 N HCl 300:200:4 (vol/vol) (5 volumes of stopping solution to 1 volume of suspended cells).
4. For lipid isolation, first vortex mixture and keep overnight at 4 °C (32-35).
5. The next day, vortex again for 1 minute; centrifuge at low speed (2,000x*g*, 5 minutes) to separate the two phases.
6. Transfer the upper phase (‘organic’) phase to a new set of clean tubes. Add 2 ml of *n*-hexane to the remaining lower (‘aqueous’) phase in old tubes; vortex and centrifuge as above. Transfer the upper phase and combine with the previous extraction.
7. Dry the combined organic phase under a Nitrogen stream; resuspend the lipids in a small volume (50-100 μl) of hexane/isopropanol, 3:2 vol/vol.
8. Spot aliquots (20 μl) on silica gel 60-precoated TLC plates.
9. Add an aliquot (10 μl of a 10 mg/ml solution) of authentic, non-labeled, arachidonic acid at the origin with the experimental samples to help detection of arachidonic acid spots after the plate is developed.
10. Develop plates in the solvent system chloroform/acetone, 96:4 (vol/vol).
11. Dry plate and expose to I_2 vapors to visualized lipids; circle the spots of authentic arachidonic acid with a pencil; spray plates lightly with water; scrape the identified silica zones with a razor blade and count for radioactivity.

3.3.2 Measurement of PLC_β in intact cells or *in vivo*—The activity of PLC_β *in vivo* is determined either by measuring the amount of IP_3 released or the hydrolysis of PIP_2 from cells prelabeled with radioactive inorganic phosphate (32-35). The procedure described here measures the latter and the extraction protocol assures the recovery of ~80% of PIP_2 and phosphatidylinositol 4-monophosphate. Differently from the protocols studied before, it makes use of X-ray films to expose radioactivity.

3.3.2.1. Hydrolysis of PIP_2:

1. Add a known volume of carrier-free $H_2[^{32}P]O_3$ (final concentration 33 μCi/ml) to a cell suspension (at a 1-5×10^7 cells/ml density).
2. Incubate cells at 37 °C for one hour or until steady-state is achieved (*see* Note 16).
3. Pellet cells (2,000xg, 5 min) and wash twice with Hanks’ buffer to eliminate non-incorporated $H_2[^{32}P]O_3$ (*see* Note 17).
4. Resuspend cells in Hanks’ buffer at a 1×10^7 cells/ml density. Initiate the reaction by the addition of the stimulus of choice to the cell suspension.
5. Extract lipids and apply TLC plates as indicated in steps 1-8, **Subheading 3.3.1.3**.
6. Develop plates in one dimension in chloroform/methanol/20% methylamine (60:36:10 vol/vol).
7. Dry plates at room temperature and place them in a Kodak exposure cassette with an X-ray film overnight to visualize the radioactive lipids.

8. The following day, develop the film; carefully realign film and plate to equate the exposure position; locate lipids and puncture the film and the silica layer under it with a needle. Next, scrape lipids of interests with a razor blade and count radioactivity (*see* Note 18).

3.3.3. Measurement of PLD in intact cells or *in vivo*—The activity of PLD *in vivo* is determined by measuring the amount of radiolabeled phosphatidylpropanol (PProp) synthesized in cells prelabeled with [^{3}H]palmitic acid and in the presence of a primary alcohol (1-propanol) (36). As indicated in **Subheading 3.1.4**, PLD can generate a phosphatidylalcohol (in this case PProp) by the 'transphosphatidylation' reaction.

1. Plate out cells in 12-well plates at the density of 1×10^6/per well.
2. Prepare tissue culture media (e.g., RPMI1640) containing 15 μCi/ml [^{3}H]palmitic acid (final concentration).
3. Add 0.8 ml per well and incubate cells for 24 hours or until steady-state is achieved (*see* Note 16).
4. Remove all the media from the wells, one well at a time (as to keep the wells from drying out) and add 0.8 ml of fresh, non-radioactive, EBS media (serum free).
5. Remove the media over the cells and replace with 500 μl of EBS. Preincubate 10 min at 30 °C; change the media to 500 μl of EBS containing the stimulus to be tested (e.g., PMA) plus 1-propanol (1% final concentration).
6. At the end of the incubation, transfer media to a 15 ml tube, leave it on ice.
7. Add 1 ml of methanol to the wells, and put the plate on ice. Centrifuge the 15 ml tube, remove the supernatant. Scrape the cells from the well and transfer to the corresponding 15 ml tube. Add an additional 1 ml of methanol to the well, scrape and transfer to the corresponding tube. Vortex and then let the tube sit on ice while you complete the other extractions.
8. Add 1 ml of chloroform and 0.7 ml of 1% perchloric acid to each tube, vortex for 1 min. Let stand for 10 min at room temperature.
9. Add 1 ml of chloroform, 1 ml of 1% perchloric acid, vortex 1 min and aspirate the upper phase.
10. Wash lower phase twice with 2 ml of 1% perchloric acid
11. Remove two 500-μl aliquots from lower phase for chromatography, store in freezer. Count a 50 μl aliquot to determine the total amount of labeled phospholipids.
12. Run TLC as indicated in **Subheading 3.1.4.4**. Spot 5 μl of the 'standard mix' over each sample and 5 μl on an extra lane by itself; dry plate, visualize lipids with I_2 vapors and scrape the spots that co-migrate with PProp (R_f = 0.5-0.6), and count.

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[18] To help locate the position of inositol-containing phospholipids, it is useful to label an aliquot of the cells with [^{3}H]inositol instead of $H_2[^{32}P]O_3$ (this can be done at point 4). A sample is then taken for lipid extraction and TLC application along with the experimental samples.

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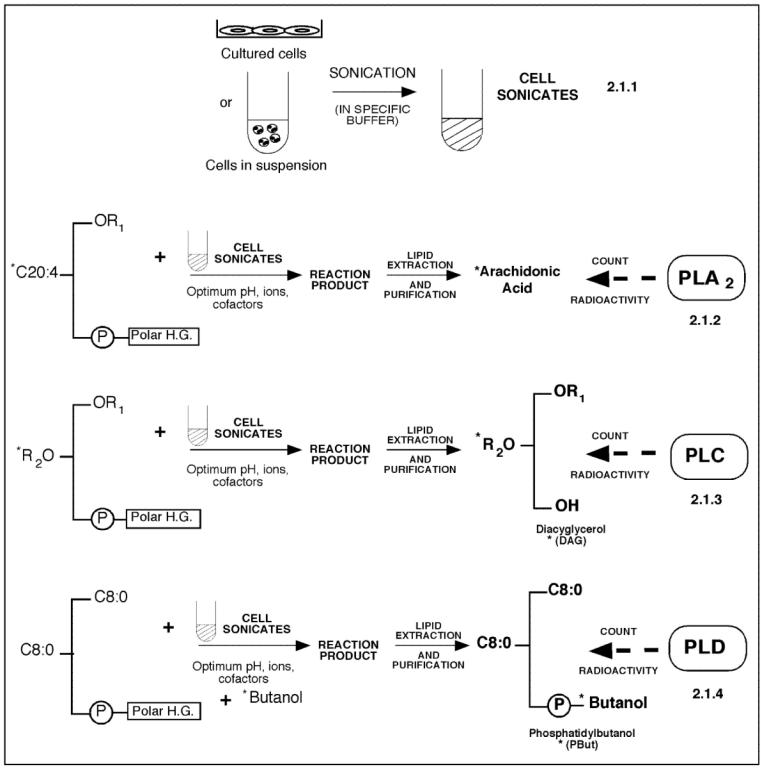

Figure 1.

In vitro microassays. From a common step that generates a 'cell sonicate' mixture the three phospholipases (PLA_2, PLC and PLD) can be measured. The cell sonicates are incubated with the appropriate substrate (radiolabeled) and the product of the reaction is purified (normally by thin layer chromatography, TLC). Counting radioactivity at the end of the experiment provides cpm, that are a direct index of the phospholipase activity studied. (R1 and R2, generic fatty acid moieties in *sn*-1 and *sn*-2 positions; C20:4, arachidonoyl; C8:0, octanoyl; polar H. G., polar head group).

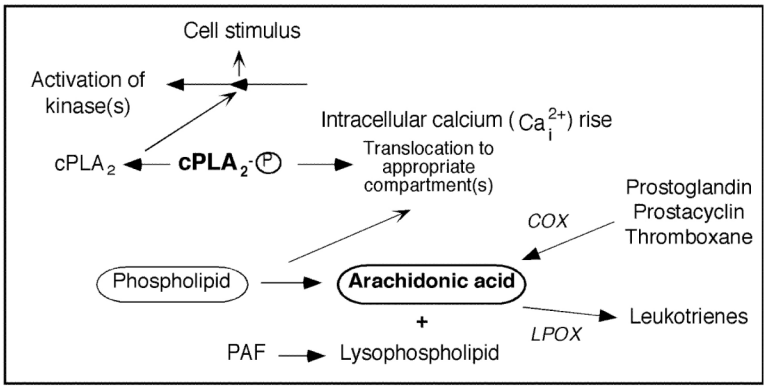

Figure 2.

Schematic representation of activation of cytosolic PLA_2 ($cPLA_2$) and the metabolism of arachidonic acid. Kinase(s) include mitogen-activated protein kinase (p42/44), p38 MAP kinase and calcium/calmodulin-dependent protein kinase II. ($cPLA_2$-P, phosphorylated, active form of $cPLA_2$; PAF, platelet-activating factor; COX, cycloxygenase(s); LPOX, lipoxoxygenase).

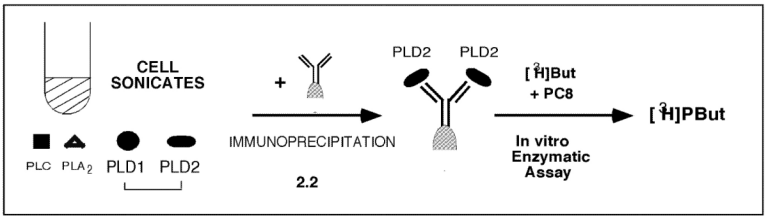

Figure 3.

Immunoprecipitation and immunocomplex enzyme assays. A specific antibody (linked to agarose beads) effectively isolates a particular phospholipase isoform (shown here PLD2) from a cell mix. The activity of the immunoprecipitated enzyme can be assayed then *in vitro*.

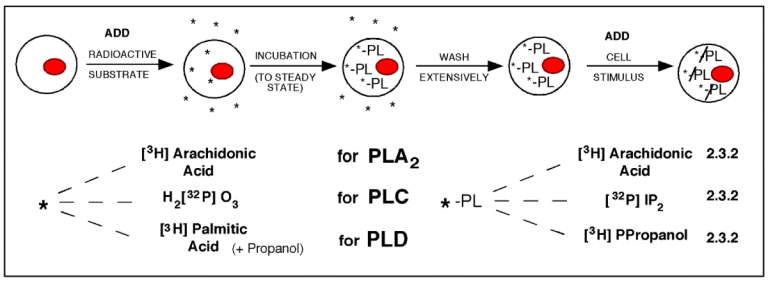

Figure 4.

Intact-cell or *in vivo* assays. Cultured or suspended cells are incubated with a radioactive precursor: either arachidonic acid, inorganic phosphate or myristic acid, for the measurement of PLA_2, PLCβ or PLD, respectively. Once a steady state has been reached and the cellular lipids become labeled, cells are washed and stimulated with the agonist under study. This can cause the breakdown of the radiolabeled phospholipids and the release of either arachidonic acid, PIP_2 or phosphatidylpropanol, that are isolated and purified. (*, radiolabeled substrate; PL, phospholipid).