Chapter 16
Measuring Phospholipase D Activity in Insulin-Secreting Pancreatic β-Cells and Insulin-Responsive Muscle Cells and Adipocytes

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Abstract Phospholipase D (PLD) is an enzyme producing phosphatidic acid and choline through hydrolysis of phosphatidylcholine. The enzyme has been identified as a member of a variety of signal transduction cascades and as a key regulator of numerous intracellular vesicle trafficking processes. A role for PLD in regulating glucose homeostasis is emerging as the enzyme has recently been identified in events regulating exocytosis of insulin from pancreatic β-cells and also in insulin-stimulated glucose uptake through controlling GLUT4 vesicle exocytosis in muscle and adipose tissue. We present methodologies for assessing cellular PLD activity in secretagogue-stimulated insulin-secreting pancreatic β-cells and also insulin-stimulated adipocyte and muscle cells, two of the principal insulin-responsive cell types controlling blood glucose levels.

Keywords Phospholipase D · insulin · GLUT4 · adipocyte 3T3-L1 · myotube · L6 · MIN6 · pancreatic β-cell

16.1 Introduction

Phospholipase D (PLD) was first described as a lecithinase in the 1940 s [1] and is now recognized as a phosphodiesterase that catalyzes the hydrolysis of the most abundant cellular membrane phospholipid, phosphatidylcholine (lecithin). In mammalian cells, phosphatidylcholine hydrolysis by PLD produces soluble choline and phosphatidic acid. In the 1980 s, this activity, which in unstimulated cells is generally quite low, was seen to be increased by a number of agonists including a variety of growth factors, cytokines, and hormones [2]. PLD has consequently often been thought of as a signaling enzyme; indeed, its activity is associated with a number of signaling cascades (reviewed by Jenkins and Frohman [3]). In addition, PLD activity has also been associated with a

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number of intracellular vesicular trafficking processes (also reviewed by Jenkins and Frohman), including those associated with the Golgi [4], endocytosis [5], and exocytosis from neurons [6] and endocrine cells [7, 8]. Phospholipase D activity has also been associated with increases in the uptake of glucose triggered by the hormone insulin [9, 10]. However, in many of these vesicle trafficking processes, the direct role for the activity of the enzyme is only partially understood.

A primary function of the hormone insulin in mammalian organisms is to control glucose homeostasis. This is achieved through appropriately regulated exocytosis of insulin from pancreatic β-cells, which, in turn, regulates major metabolic processes, including the promotion of glucose uptake into adipocytes and myotubes and the inhibition of gluconeogenesis in the liver. Insulin exocytosis and its metabolic effects are mediated by a complex series of signaling cascades, defects in which can cause diabetes. In the case of insulin secretion from pancreatic β-cells, signaling cascades are triggered by a variety of stimuli, such as glucose, hormones, or neurotransmitters. Subsequent metabolic and receptor-mediated signaling cascades generally result in an increase in cytosolic calcium, which then triggers insulin vesicle/granule exocytosis [11–13]. In the case of muscle and adipose cells, signaling is initiated when insulin binds to its receptor(s). Activated receptor(s) phosphorylate and recruit downstream effector proteins, in two principal signaling cascades involving phosphatidylinositol 3-kinase (PI3 K) and protein kinase B (PKB/Akt) or a Ras-mitogen-activated protein kinase (MAPK) pathway [14]. Glucose uptake into muscle and adipose tissues is regulated by insulin via the PI3 K-PKB-dependent arm of the insulin signaling pathway, resulting in the exocytosis of vesicles containing the glucose transporter, GLUT4, from intracellular compartments to the plasma membrane. Fusion of these vesicles with the plasma membrane allows glucose to enter the cell via facilitated transport through GLUT4 [15].

Phospholipase D activity has been associated with both stimulated insulin exocytosis from pancreatic β-cells [7] and insulin-dependent fusion of GLUT4-containing vesicles with the plasma membrane in adipocytes [10] and muscle cells (Cazzolli and Hughes, unpublished data).

Studies into the role of cellular PLD activity are normally relatively straightforward, with good and specific assays having been in use for a number of years [16]. However, studies particularly with respect to the enzyme's role in insulin-signaling cascades and insulin-regulated vesicular trafficking are complicated by the requirements for maintaining and differentiating insulin-sensitive cell lines. Specifically, measuring cellular PLD activity, without attenuating insulin's effect on its signaling cascade, is a major concern if meaningful insight into the role of this enzyme is to be achieved. Here we present detailed protocols for culturing and differentiating insulin-secreting and insulin-responsive cell lines, with particular emphasis on ensuring that the responsiveness of the cells is maintained. We provide protocols to radioactively label cellular phospholipids within appropriately differentiated cells and to produce a metabolically stable

and unique labeled phospholipid PLD product. We also detail phospholipid extraction protocols and a chromatographic separation technique to allow quantitation of the PLD product, providing an indication of relative cellular PLD activity.

16.2 Materials

16.2.1 Cell Culture for MIN6 Pancreatic β-Cells, L6 Myoblasts, and 3T3-L1 Fibroblasts

1. MIN6 murine pancreatic β-cells (*see* Note 1) are grown in high-glucose (25 mM) Dulbecco's Modified Eagle Medium (DMEM; Gibco/BRL) supplemented with 10% fetal calf serum (FCS; Trace, Melbourne, Australia), 15 mM HEPES, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin (Gibco/BRL). Similarly supplemented low-glucose (5 mM) DMEM (Gibco/BRL) is also used.
2. L6 rat skeletal myoblasts (*see* Note 1) are grown in minimal essential media-α (αMEM; Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Trace, Melbourne, Australia) and 1% antibiotic/antimycotic (Gibco/BRL; equivalent to 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin sulfate, and 0.25 μg/ml pf amphotericin B).
3. 3T3-L1 fibroblasts (*see* Note 1) are grown in DMEM (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM glutamine (Gibco/BRL), 100 IU/ml of penicillin, and 100 μg/ml of streptomycin (Gibco/BRL).
4. Modified Krebs-Ringer bicarbonate buffer (KRB; *see* Note 2): 136 mM NaCl, 4.7 mM KCl, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$ (7 H_2O), 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES (pH 7.4), glucose 2.8 or 25 mM, 0.5% albumin (bovine serum; BSA, Sigma, A7030).
5. Insulin (Sigma; St Louis, MO) or, alternatively, the human insulin analog Actrapid^{TM} from Novo Nordisk (Copenhagen, Denmark) was used. For 3T3-L1 fibroblast differentiation, prepare a 2-mg/ml insulin stock (Sigma) in 3 mM HCl and store at 4°C. To use, dilute 1:500 in complete media.
6. Dexamethasone (Sigma): Prepare a stock solution at 0.39 mg/ml in 100% ethanol, store at −20°C. Dilute 1:4,000 in media for differentiation.
7. 3-Isobutyl-1-methylxanthine (IBMX, Sigma): Prepare fresh each time. Make a stock of 57.5 mg/ml in dimethyl sulfoxide (DMSO) and dilute 1:500 in complete media for differentiation.
8. Trypsin (JRH Biosciences): equivalent to 0.012% trypsin in Dulbecco's PBS with 0.02% EDTA and 0.04% glucose.
9. 10 mM phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7\,H_2O$, 1.4 mM KH_2PO_4.

16.2.2 In vivo *Phospholipase D Assay*

1. Palmitic acid [9,10-^{3}H(N)] (American Radiolabeled Chemicals, St Louis, MO, or Perkin Elmer, Boston, MA); myristic acid [9,10-^{3}H(N)] is from Perkin Elmer.
2. Phorbol 12-myristate 13-acetate (PMA, Sigma) is dissolved at 0.1 mM in DMSO and stored in aliquots at −80°C.
3. A 10 μM working solution of insulin for PLD assay is made fresh by diluting neat insulin (Actrapid^{TM}) 1:60 into water. This working solution can then be diluted 1:100 directly into culture medium to give a final concentration of insulin of 100 nM.

16.2.3 Phospholipid Extraction, Separation, and Quantification

1. Chloroform and methanol, analytical grade (Sigma).
2. 0.88% KCl (Sigma) in water.
3. Thin layer chromatography plates: LK5D Silica Gel 150 A (Whatman, UK).
4. 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphobutanol (PtdBut) is from Avanti Polar Lipids (Alabaster, AL).
5. 1% oxalic acid (Sigma); 2 mM EDTA (Sigma) w/v in methanol:water 2:3 (*see* Note 3).
6. Butan-1-ol; ethyl acetate; 2,2,4-trimethyl pentane; acetic acid (glacial); all analytical grade; and iodine (Sigma).
7. EN^{3}Hance (Perkin Elmer).
8. Teflon cell scrapers (Fisher).
9. Scintillation fluid, Ultima Gold XR (Perkin Elmer).

16.3 Methods

16.3.1 Cell Culture and Differentiation

16.3.1.1 Culture of MIN6 Pancreatic β-Cells

MIN6 pancreatic β-cells are maintained at 37°C and 5% CO_2 in supplemented DMEM (as described above). Stock flasks are usually split every 3 to 4 days, when the cells are approximately 60–70% confluent. MIN6 cells are well differentiated and do not grow well when split too hard, growing best when allowed to readhere in small clumps of two to three cells rather than as single cells. MIN6 cells secrete insulin in response to glucose as a secretory stimulus but lose this capacity after approximately passage 42. Consequently, cells are routinely tested for secretory competence (by insulin RIA, protocols not detailed here) and are generally only used between passages 25–42. For

experimentation cells, $\sim 5 \times 10^6$ cells/well of a 6-well plate are incubated overnight in high-glucose DMEM (as described above) before incubation for 24–48 h in low-glucose DMEM (as described above).

16.3.1.2 Culture and Differentiation of L6 Myoblasts

L6 myoblasts are maintained at 37°C and 5% CO_2 in supplemented αMEM (as described above). Stock 10-cm dishes (Falcon) are usually split every 3 to 4 days, when the cells are approximately 60–70% confluent. L6 myotubes will align and spontaneously start to differentiate if they reach full confluency. Hence, to avoid selecting for myoblasts with a nonfusing phenotype, it is imperative that stock dishes of L6 myoblasts are not allowed to exceed 60–70% confluency. Furthermore, stocks should not be kept beyond 12 to 15 passages, as their ability to differentiate and their response to insulin will be diminished. For splitting, dishes are washed twice in 5 ml of warm PBS and then rinsed in 1 ml of warm trypsin, most of which is then removed. The dish is incubated for approximately 3 minutes at 37°C, and then 5 ml of warm supplemented α-MEM (10% FCS plus antimicrobials) is added to harvest the cells. Usually, the cells are split 1:6–1:10 into new 10-cm stock dishes.

In order to differentiate the myoblasts into myotubes for experiments, the cells are seeded in supplemented αMEM, with one 10-cm dish at approximately 80–90% confluence usually being sufficient to seed two 12-well plates (2×10^4 cells/well) or one 6-well plate (6×10^4 cells/well). At confluence, usually 3 to 4 days after seeding, the media is changed to αMEM supplemented with only 2% FCS and antimicrobials, to initiate differentiation. This media is changed daily for the first 3 to 4 days of the differentiation process and every 2 days thereafter. The cells are used after approximately 5 to 7 days of differentiation.

16.3.1.3 Culture and Differentiation of 3T3-L1 Fibroblasts

Murine 3T3-L1 fibroblasts are cultured in DMEM (as above) at 37°C in 8% CO_2 (*see* Note 4). These cells can be passaged at least 10 times (and probably longer) provided the fibroblasts are maintained rigorously. Differentiation is based on established protocols [17]. Plates/flasks are usually split every 4 days and seeded at a lower cell density to prevent cells from becoming confluent. Normally, a 15-cm plate or T150 flask can be seeded with $6–10 \times 10^4$ cells. For differentiation, 3T3-L1 fibroblasts are seeded at 18×10^4 cells/well and 10×10^4 cells/well for 6-well and 12-well dishes, respectively, and fed with fresh media every 4 days. Differentiation is induced according to established protocols. Briefly, 3T3-L1 fibroblasts are allowed to grow at least 2 days past confluence (8 days after plating). Differentiation is induced (on day 0) with complete medium containing 0.25 μM dexamethasone, 4 μg/ml of insulin, and 500 μM 3-isobutyl-1-methylxanthine. After 4 days in differentiation media, the cells are

fed with complete medium containing 4 μg/ml of insulin (*see* Note 5). After 4 more days, the cells are refed every 2 days with complete medium. Differentiation is monitored by noting the accumulation of lipid droplets, which typically appear by day 4 of differentiation. Cells are considered fully differentiated between days 8 and 12.

16.3.2 In vivo *Phospholipase D Activity Assay*

Cellular PLD activity can be measured most readily using an *in vivo* transphosphatidylation reaction [16]. Measuring PLD activity through depletion of phosphatidylcholine substrate is not possible, as phosphatidylcholine makes up a significant proportion of total cellular phospholipid and the component metabolized by PLD is generally very small. In addition, there are, of course, numerous PLD-independent routes by which phosphatidylcholine can be metabolized. Similarly, phosphatidic acid, the product of PLD hydrolysis, can be rapidly synthesized and metabolized in an agonist-stimulated, PLD-independent manner and is also therefore unsuitable for use as a measurement of cellular PLD activity. PLD activity has been monitored using a single-cell microscope-based Forester Resonant Energy Transfer assay. However, this is unsuitable for routine activity screens and requires specialist equipment [18]. PLD retains the unique ability to transphosphatidylate primary alcohols such as ethanol or butan-1-ol and will, in the presence of primary alcohol, produce phosphatidlyalcohol in preference to phosphatidic acid. Accumulation of phosphatidylalcohol, which is generally metabolically stable, can be used as an indication of relative PLD activity when control (basal) and treated conditions are compared. However, we and others have found that alcohol substrate (such as butan-1-ol) decreases insulin receptor activation [10, 19, 20]. Therefore, insulin is added first for 5 minutes to preactivate insulin receptor before the addition of butan-1-ol to increase the sensitivity of the assay. PMA, a potent activator of cellular PLD, is used as a positive control.

16.3.2.1 MIN6 Phospholipase D Activity Assay

1. MIN6 pancreatic β-cells in 6-well plates are incubated in the presence of 4 μCi/ml [^{3}H] palmitic acid or myristic acid in supplemented low-glucose DMEM for a minimum of 7 h.
2. Media is aspirated and the cells briefly washed into modified KRB before being incubated in fresh modified KRB for 30 min.
3. For stimulated PLD activity, cells are then incubated with or without an appropriate stimulus, such as secretagogues including glucose (11.1 -> 25 mM), carbamoylcholine (cholinergic receptor agonist 100 μM), or hormones, in modified KRB containing 0.3% butan-1-ol for 30 min to 1 h. For

PMA-stimulated PLD activity, cells are incubated with 100 nM PMA and 0.3% butan-1-ol for 30 min.
4. At the end of incubation, aspirate the medium and add 0.5 ml of ice-cold methanol to each well of the 6-well plate.
5. Cells are scraped and collected into microcentrifuge tubes on ice.

16.3.2.2 L6 Myotube Phospholipase D Activity Assay

1. L6 myotubes in 6-well plates are incubated in the presence of 4 μCi/ml [^{3}H] palmitic acid or myristic acid in serum-free α-MEM for a minimum of 7 h.
2. For insulin-stimulated PLD activity, myotubes are incubated with 1, 50, or 100 nM insulin for 5 min prior to the addition of 0.3% butan-1-ol for 30 min. For PMA-stimulated PLD activity, myotubes are incubated with 100 nM PMA and 0.3% butan-1-ol for 30 min.
3. At the end of incubation, aspirate the medium and add 0.5 ml of ice-cold methanol to each well of the 6-well plate.
4. Myotubes are scraped and collected into microcentrifuge tubes on ice.

16.3.2.3 3T3-L1 Adipocyte Phospholipase D Activity Assay

1. 3T3-L1 adipocytes in 6-well or 12-well plates are serum-starved overnight (0.5% BSA in DMEM) in the presence of 4 μCi/ml [^{3}H] palmitic acid.
2. For insulin-stimulated PLD activity, adipocytes are incubated with 100 nM insulin for 5 min prior to the addition of 0.3% 1-butanol for 30 min. For PMA-stimulated PLD activity, cells are incubated with DMEM containing 100 nM PMA and 0.3% butan-1-ol for 30 min.
3. At the end of incubation, aspirate the medium and add 0.5 ml of ice-cold methanol to each well of the 6-well plate. It is important to note that special care must be taken not to damage the cell monolayer, because the monolayer is fragile at this time and can be damaged by suction or the careless addition of media.
4. Adipocytes are scraped and collected into microcentrifuge tubes on ice.

16.3.3 Phospholipid Extraction, Separation, and Quantification

1. To the methanol cell extracts, add 0.5 ml of chloroform, for a final chloroform:methanol ratio of 1:1. Mix well by vortex and incubate for 15 min at room temperature.
2. Add 0.45 ml of water or 0.88% KCl solution to each tube and mix well by vortex.
3. Centrifuge for 5 min at ~800 × *g* to promote phase separation.

4. Carefully remove the upper aqueous phase and take 20 µl of the lower organic phase for scintillation counting to determine total labeled phospholipids (*see* Note 6). The remainder of the organic phase is dried by vacuum centrifugation or under a nitrogen stream. At this point the samples can either be spotted onto pre-equilibrated TLC plates (as described below) or stored at −20°C for future processing.
5. LK5DF silica gel TLC plates are activated by soaking in 1% oxalic acid/2 mM EDTA in methanol:water (2:3), described in step 5 of Subheading 16.2.3, for 2 min before baking in an oven for at least 15 min at 110°C (*see* Note 7).
6. Resuspend the dried samples in 25 µl of chloroform/methanol (19:1;v/v). If desired, 50 µg of authentic PtdBut can be included in the chloroform/methanol resuspending solution as an internal standard, where iodine is to be used to visualize phospholipids.
7. Sample are spotted onto activated TLC plates at least 2 cm from the bottom of the plate (*see* Note 8).
8. When dry, the plate is developed in a TLC tank and pre-equilibrated (*see* Note 9) with the organic phase of ethylacetate:trimethylpentane:acetic acid:water (110:50:20:100; v/v/v/v). These solvents should be mixed in a separating funnel in a fume hood and allowed to stand until the upper organic and lower inorganic phases are separated. The upper phase is used to fill a TLC tank to a depth of no more than 2 cm.
9. Chromatographic separation in this solvent system takes approximately 1.5 h, by which time the solvent front is usually 2–3 cm from the top of the plate. The plate is removed from the TLC tank and briefly air-dried in the fume hood.

10a. The plate can be placed in a iodine atmosphere tank to stain for about 15–30 min. Use a soft pencil to mark the position of PtdBut, the retention factor (Rf) of which should be 0.3–0.4. Dry the plate in the hood until all the yellowish staining is gone.

10b. Alternatively, radiolabeled phospholipids can be visualized through a combination of autoradiography and fluorography. For this method, the TLC plates can be sprayed three times with a fluorescent intermediate autoradiography enhancer (EN^{3}Hance; *see* Note 10). The plates should be sprayed while still in the fume hood and allowed to dry for 10 min between each spray. The plates are exposed on film for about 5 days at –80°C. Once the film has been developed, the TLC plate can be oriented on the film over a lightbox to identify the position of PtdBut, the retention factor (Rf) of which should be 0.3–0.4. An example is shown in Fig. 16.1.

11. PtdBut spots are scraped from the plate into approximately 0.5 ml of methanol and scintillation fluid and used for counting in a scintillation counter.
12. An assessment of PLD activity is calculated as [^{3}H]-PtdBut and can be related to percentage of total phospholipids as required.

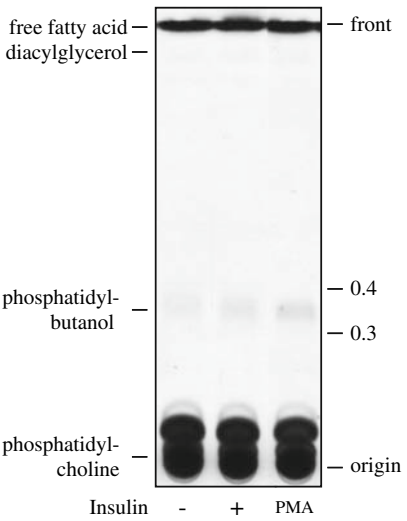

Fig. 16.1 An example of radiolabeled L6 myotube phospholipids separated by TLC. L6 cells were cultured as described and labeled with [^{3}H] myristic acid for 7 h before 10 minutes of treatment with or without 100 nM insulin or 10 nM PMA (as indicated) prior to the addition of 0.3% (v/v) butan-1-ol. Phospholipids were extracted and separated before visualization as described

Notes

1. MIN6 murine pancreatic β-cells are from Miyazaki et al. [21]; L6 rat skeletal myoblasts and 3T3-L1 murine fibroblasts are from ATCC.
2. Modified Krebs-Ringer bicarbonate buffer (KRB) is made up fresh from a 2x stock of 272 mM NaCl, 9.4 mM KCl, 10 mM $NaHCO_3$, 2.4 mM $MgSO_4$ (7 H_2O), 2.4 mM KH_2PO_4 with distilled water. To this are added $CaCl_2$ and HEPES to a final concentration of 1 mM and 10 mM, respectively, and glucose to a final concentration of either 2.8 or 25 mM. The solution is bubbled with ~5% CO_2 for 15 minutes and pH 7.4 confirmed. Albumin (bovine serum, BSA) is added to a final concentration of 5% (w/v) and the resultant solution is used within 1 h.
3. Add 400 μl of 500 mM EDTA to 60 ml of water and dissolve 1 g of oxalic acid in this solution. Once all the oxalic acid has dissolved, add 40 ml of methanol. Final concentrations are 1% oxalic acid:2 mM EDTA in methanol:water (2:3).
4. We have found that 3T3-L1 fibroblasts differentiate substantially better in an 8% CO_2 environment rather than 5% CO_2.
5. It is important to note that special care must be taken not to damage the cell monolayer, because the monolayer is extremely fragile at this time and can be damaged by suction or the careless addition of media.
6. Cell debris and protein form a white precipitate at the interface, which should also be carefully removed.
7. We use activated TLC plates. Once cooled, however, plates remain usable for several days/weeks.
8. Spots should be kept as small as possible to give the best resolution. To help achieve this, samples can be spotted in small increments (placed to be above the solvent level in the tank).
9. To help saturate the atmosphere of the tank, it should be lined with blotting paper prewetted with the organic solvent solution. Place the lid on the tank and allow it to saturate for at least 30 minutes before placing TLC plates inside.
10. As described in the manufacturer's instructions, it is important to avoid oversaturating the TLC plate when spraying with EN^{3}Hance.

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