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Metabolic Labeling of Inositol Phosphates and Phosphatidylinositols in Yeast and Mammalian Cells

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Abstract

Inositol phosphate (IP) and phosphatidylinositol (PI) signaling are critical signal transduction pathways responsible for generating numerous receptor-mediated cellular responses. Biochemical and genetic studies have revealed diverse roles of IP and PI signaling in eukaryotic signaling, but detailed characterization of unique IP and PI signaling profiles in response to different agonists and among cell types remains largely unexplored. Here, we outline steady-state inositol metabolic-labeling techniques that can be leveraged to assess the IP and PI signaling state in eukaryotic cells. This flexible technique can be amended and optimized to your cell line of interest, perturbed with biochemical, genetic or pharmacological alteration, and used to provide comprehensive inositol profiling in various cellular systems.

Keywords

Inositol phosphates; phosphatidylinositol; metabolic labeling; signal transduction; HPLC; lipid signaling

Introduction

Phosphorylation is a ubiquitous signal used by cells to control biological processes. In addition to the widely studied phenomenon of protein phosphorylation, small molecules can also serve as cellular messengers. One such molecule is *myo*-inositol, a cyclohexanehexol obtained primarily from dietary sources [1–3]. In eukaryotic cells, soluble inositol phosphates (IPs) are derived from the lipid carrier phosphatidylinositol-4,5-bisphosphate (PIP₂). Stimulation of phospholipase C (PLC) cleaves PIP₂ to form diacylglycerol and inositol-1,4,5-trisphosphate (IP₃), a classic second messenger that stimulates Ca²⁺ release from the sarcoplasmic reticulum [1,2]. IP₃ can be phosphorylated at all six carbons and diphosphorylated at two, leading to over a dozen chemically unique molecules with distinct roles in regulating transcription, mRNA export, telomere maintenance, embryonic development, cell division, nutrient sensing, and acting as structural cofactors [4–10]. Inositol signaling is complex, with various kinases and phosphatases acting on IPs to create an array of small molecules amongst eukaryotic species (*Saccharomyces Cerevisiae*- Fig. 1; *Dictyostelium*- Fig. 2; Metazoans- Fig. 3). Therefore, equally diverse IP profiles are expected given the heterogeneity of cellular functions mediated through IP signaling.

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Similarly complex, PIPs can be phosphorylated or dephosphorylated and act as lipid signaling molecules capable of coordinating cell growth, division and acting as direct modifiers of nuclear and cell-surface receptors [11–17], in addition to a number of other cell signaling actions. Although PI comprises a minority of total cell-membrane lipid, its role in signaling is critical. Free PI generates PI(3)P, PI(4)P and PI(5)P through a number of kinases; PI(X)Ps can be further phosphorylated to both PIP₂ and PIP₃ species (Fig. 3). Genetic disruption and dysregulation of these numerous kinases and phosphatases have been implicated in a number of congenital and acquired diseases [18–23]. Similarly, PIPs can also be independently activated by PI3-K, which triggers Akt-induced activation of cellular growth, movement and proliferation [12,16], among other responses. Alteration of PI3-K signaling is observed in a number of diseases as well [17,20]. Thus, interrogation of the specific IP and/or PIP profiles in response to pharmacologic, chemical or genetic manipulation offers precise molecular signaling details in the biological system being tested.

Here, we detail experimental procedures to measure IPs and PIPs via steady-state metabolic labeling in yeast and mammalian cells. This technique can be modified to measure IPs and PIPs in numerous cell types, including post-mitotic cells. We anticipate that the techniques described herein enable investigators to discover new roles for IPs and PIPs in various eukaryotic cell systems.

Materials

Cell Culture

1. Cell line of interest (HEK293T, Cos7, Rat1, Hela, HT29, MEFs etc.). 6 or 12-well culture dishes (Corning, NY).
2. Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, St. Louis, MO) or Roswell Park Memorial Institute (RPMI) (Thermo Fisher, Cambridge, MA) media supplemented with 10% fetal bovine serum (Thermo Fisher, Cambridge, MA) and 1X penicillin/streptomycin (Sigma Aldrich, St. Louis, MO).
3. Inositol-free Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, St. Louis, MO) or inositol-free Roswell Park Memorial Institute (RPMI) (Thermo Fisher, Cambridge, MA) media supplemented with 10% dialyzed fetal bovine serum (Thermo Fisher, Cambridge, MA) and 1X penicillin/streptomycin (Sigma Aldrich, St. Louis, MO). We anticipate other media types can be used as long as they can be depleted of inositol.
4. 1X Phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO).
5. Hydrochloric acid (diluted to 0.5 M) (Sigma Aldrich, St. Louis, MO)
6. *Myo*-³H-inositol (Perkin-Elmer, Waltham MA) at 37.5 – 200 µCi/mL (depending on length of labeling experiment and cell type of interest). Alternatively, C¹⁴ and P³² can be used. However, the methods outlined here pertain specifically to ³H-inositol.

High Performance Liquid Chromatography (HPLC)

1. 1.7 M ammonium phosphate (Fisher Scientific, Fairlawn NJ) at a pH of 3.5. 10 mM ammonium phosphate is prepared by dilution with deionized water and passed through a 0.22 μ M cellulose acetate bottle top sterile filter (Corning Inc., Corning NY) prior to use in autoclave-sterilized buffer containers.
2. Buffer A: 10mM ammonium phosphate (pH= 3.5). Buffer B: 1.7M ammonium phosphate (pH= 3.5).
3. The HPLC system (Waters Bechmann or Agilent) equipped with an autosampler (desired) should contain a 4.6×125 mm Partisphere SAX strong anion exchange column (Whatman, Maidstone, England). Scintillation fluid (Perkin Elmer, Fairlawn NJ, Catalog number 50-905-0584) is collected at a ratio of 6 mL scintillation fluid per 1 mL HPLC fraction in 7 mL scintillation vials (Fisher Scientific, Fairlawn NJ) [24].
4. Scintillation counts are performed using a Tri-Carb 2100 TR (Packard, Fairlawn NJ).
5. It is also possible (desired) to have an in-line radioactivity detection system such as INUS beta-ram detector [24].

Soluble IP and PIP extraction

1. 372 μ L CHCl_3 and MeOH (Fisher Scientific, Fairlawn, NJ) at a ratio of 1:2 is added to 100 μ L of 0.5 M HCl extracted cells. In yeast, acid-washed bead beating (OPS Diagnostics, Lebanon NJ) is required to lyse the thick cell wall.
2. 100 mg/mL Bovine brain extract (Sigma Aldrich, St. Louis, MO) in CHCl_3 (Fisher Scientific, Fairlawn, NJ).
3. For deacylation and PIP extraction, a speed vacuum (Thermo Fisher, Cambridge, MA), propanol, 33% methylamine, butanol, petroleum ether, ethyl formate, and glass tubes (Fisher Scientific, Fairlawn, NJ) will be needed.

Methods

Interrogation of IP and PIPs in steady-state equilibrium enables robust analysis of genetic, biochemical or pharmacological manipulations of inositol signaling. Our group has published extensively in IP labeling in a variety of cell types and organisms [25–29,8,24,30]. Here, we offer trouble-shooting tips and guidelines for metabolic labeling in varying cell lines. The protocol described below details measurement of IPs and PIPs in yeast and mammalian cells by HPLC.

Cell culture and isolation of IPs and PIPs

1. Maintain cell line in regular growth media, then exchange inositol-complete to inositol-free medium for at least 24 hours prior to radiolabeling.
2. Plate your cell line of interest approximately 100,000–250,000 cells in 1 mL media (density of $\sim 10,000$ – $30,000$ cells/ cm^2 in a 6-well culture dish and allow

cells to equilibrate overnight. Cell lines that are both adherent to the dish and in suspension can be used.

3. Supplement inositol-free media with 37.5 – 200 $\mu\text{Ci/mL}$ of radiolabeled inositol and allow cells to double approximately 2–4 times. Depending on the cell line, this may range from 2–7 (or more) days.
4. Once cell line is near confluence, remove media, wash with 1X PBS and add 0.5 M HCl for 5 minutes prior to manual scraping of cells from surface of plate. Amount of HCl can be varied according to dish size and cell count. Alternatively, cells can be scraped via manual force in PBS or trypsinized, and HCl can be added directly to the cell pellet.
5. For yeast, grow overnight culture of yeast in complete synthetic medium. Inoculate 1 mL of fresh complete synthetic medium containing 20–200 $\mu\text{Ci/mL}$ ^3H -inositol with 10 μL of overnight culture and grown to log phase or saturation.
6. Harvest yeast by centrifugation (5 minutes at 14,000 rpm). Aspirate old medium and wash cells with 1 mL of PBS. Spin down cells, aspirate PBS and resuspend cell pellet in 100 μL of 0.5 M HCl.

Extraction of soluble IPs and PIPs

1. Per 100 μL of 0.5 M HCl, add 372 μL of $\text{CHCl}_3\text{:MeOH}$ (1 part CHCl_3 : 2 parts MeOH) and 100 μL acid-washed beads (for yeast) or vortex mammalian cells. Then, add 125 μL CHCl_3 and 125 μL 2M KCl (do not make master mix), followed by an additional 2 minutes of bead beating. For mammalian cells, vortex for approximately 15 seconds or until thoroughly homogeneous. Next, spin down for 5 minutes at 14,000 rpm.
2. Carefully separate the upper aqueous and lower organic layers. The upper aqueous layer contains soluble IPs, whereas the lower organic layer contains phosphatidylinositols.
3. For PIP extraction, add 10 μL of 100 mg/mL of bovine brain extract in CHCl_3 and dry speed vacuum at 35 $^\circ\text{C}$ for approximately 1 hour or until sample is dry.
4. Next, add 300 μL of deacylation reagent (10 parts 33% methylamine in ethanol, 3 parts water, and 1 part butanol; mix in glass tube; scale master mix accordingly) followed by 2 hours of shaking at 53 $^\circ\text{C}$. Perform all handling of methylamine in a chemical hood as this reagent is highly flammable. Once complete, spin down to pellet, add 150 μL of ice-cold propanol, and dry speed vacuum at 80 $^\circ\text{C}$. Drying time will vary depending on the volume in your sample.
5. Once the speed vacuum cycle is complete, dissolve in 200 μL water and 300 μL extraction solution (20 parts 1-butanol, 4 parts petroleum ether, 1 part ethyl formate; mix in glass tube and keep on ice); vortex to mix. Spin down for 5 minutes at 14,000 rpm and retain lower phase.

6. Add an additional 300 μ L of extraction solution, vortex, spin down for 5 minutes at 14,000 rpm and retain lower phase. Repeat this step 2 additional times, retaining the lower phase. Dilute this phase as necessary in 10 mM ammonium phosphate and sterile filter prior to HPLC analysis.

High Performance Liquid Chromatography

100 μ L of sample is injected onto a 1 mL Partisphere SAX column. Flow rate is maintained at 1 mL per minute and 1 mL fractions are manually collected into 7 mL scintillation vials. Once the run is complete, add 6 mL of scintillation fluid to each vial and count use a scintillation counter. Example HPLC traces for IP and PIP analysis can be found in Figure 4.

Notes

1. The total number of cell doublings, rather than the time cells are maintain in radiolabeled inositol determines the degree of saturation towards steady state and strength of scintillation counts detected. However, post-mitotic cells can be efficiently labeled by increasing the concentration of radiolabeled inositol and prolonged the labeling time *in vitro*.
2. Similarly, the total concentration of radiolabeled inositol used in culture varies from cell line to cell line, required optimization by the investigator. The concentration of radiolabeled inositol and labeling length of time should be empirically determined for each cell line of interest.
3. Typically, collection of 1 mL fractions every minute during the HPLC gives the highest resolution of IP and PI species.
4. Although automated beta-ran detectors have been employed to measure inositol species, we find that manual scintillation counts are often more sensitive to detection of IPs.
5. A comprehensive set of standards should be run for accurate determination of IP and PI species identification as elution times can vary depending on the column.
6. As described previously [28], higher-order IPs (IP₇, IP₈) may require transduction of inositol phosphate multikinase (IPMK) or robust activation of G protein-signaling via transfection of a constitutively active G protein-coupled receptor. In addition, to identify inositol pyrophosphates derived from IP₅ or IP₆, elution fractions can be treated with diphosphoinositol phosphate phosphatase (DIPP) as previously described [26].
7. Due to sample-to-sample variation, raw counts obtained by scintillation should be interpreted with caution as saturation of inositol labeling is not always 100%. Normalization to free inositol or IP₃ can be useful, but interpretation of these results should be done with rigor.
8. Metabolic labeling can be performed in 12-well plates as well, however, robust cell counts are more easily achieved when the cell line can undergo multiple doublings, which rapidly outpaces the space available in 12-well culture.

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*Saccharomyces Cerevisiae**Phosphatidylinositol* *Inositol Phosphate* **Figure 1:**

Diagram of the inositol phosphate and phosphatidylinositol signaling pathways in *Saccharomyces cerevisiae*, adapted from [29].

Dictyostelium

Figure 2:
Diagram of the inositol phosphate and phosphatidylinositol signaling pathways in *Dictyostelium*, adapted from [31].

Metazoan

Figure 3:

Diagram of the inositol phosphate and phosphatidylinositol signaling pathways in metazoans. To determine if a novel pyrophosphate is derived from IP₅ or IP₆, one can treat the fraction collected by HPLC with diphosphoinositol phosphate phosphatase (DIPP) as previously described [26]. Identification of the alternative pathway to generate IP₆ is detailed previously [32].

Figure 4:

Example IP HPLC trace from HT29 cells and PIPs from T lymphocytes. Elution times can vary depending on the column. Thus, we recommend using radiolabeled yeast standards when initiating a new experiment or changing columns.

Table 1

HPLC Gradient for IP analysis

Time (minutes)	% Buffer B	Approximate retention time (minutes)
0–5	0	
5–70	Linear gradient to 100%	IP ₃ (33)
		IP ₄ (45)
		IP ₅ (58)
70–100	100%	IP ₆ (75)

Table 2

HPLC Gradient for PIP analysis

Time (minutes)	% Buffer B	<i>Approximate retention time (minutes)</i>
0–5	0	
5–12	0–7	PI (5)
12–15	7	
15–30	7–14	
30–40	14–60	PI(3)P (15)
		PI(4)P (17)
		PI(3,5)P ₂ (36)
		PI(4,5)P ₂ (38)
40–45	60	PI(3,4,5)P ₃ (42)
45–55	60–100	
55–65	100	
65–80	0	