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Phosphoinositide Analysis using the HPLC System Equipped with a Fraction Collector and the TSKgel SAX Column

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Abstract

Phosphoinositides (PIs) are minor components of phospholipids involved in numerous cell functions, such as cytoskeletal reorganization, ion channel and transporter regulation, chromatin remodeling, membrane trafficking and the recruitment of signaling molecules. Due to their low abundance and rapid turnover rate in cells, it is difficult to quantify the cellular contents. Radiolabeling methods using [^{3}H]inositol and the HPLC system equipped with a flow scintillation analyzer and partisphere SAX column (Whatman) are usually employed for this purpose. In this technical review, we describe a simple and economical method for detecting cellular PIs using [^{3}H] inositol labeling and the HPLC system equipped with a fraction collector (DC-1500C, Waters) and the TSKgel SAX column (Tosoh). This method is useful for quantifying the cellular contents of the PIs and can be applied in screening for inhibitors of PI kinases and phosphatases.

Key words: Phosphoinositides, [^{3}H]inositol, HPLC, PI kinase

Introduction

Phosphoinositides (PIs) are minor components of cellular phospholipids. PIs are reversibly phosphorylated/dephosphorylated at positions D3, D4 and D5 of their inositol head group by PI kinases and phosphatases, and seven different PIs have been identified in mammals (Fig. 1).[1, 2] PIs are involved in crucial cellular processes, including cytoskeletal reorganization, membrane trafficking, endocytosis, exocytosis, ion channel and transporter regulation, chromatin remodeling and signaling molecule recruitment.[1-4] With respect to analyzing the cellular contents of PIs, radiolabeling methods using [^{3}H]inositol and the strong anion-exchange HPLC system have been employed for more than two decades.[5-10] However, since lipophilic PIs are simply not suitable for HPLC analysis, the lipids are generally changed to water-soluble deacylated products (glycerophospholnositols, groPIs) for treatment prior to chromatography. The strong anion-exchange HPLC column can be used to resolve [^{3}H]labeled-deacylated PIs ([^{3}H]groPIs) based on the structural differences in their inositol head groups.

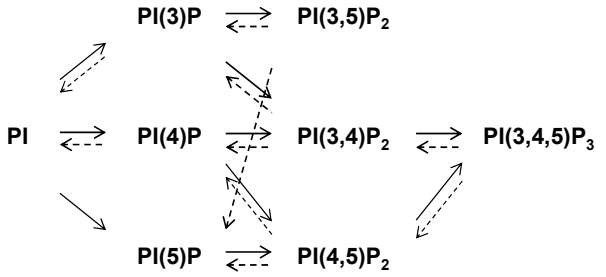

Figure 1. Synthetic pathways of PIs. PIs are phosphorylated (arrows) and dephosphorylated (dashed arrows) by PI kinases and phosphatases, respectively. Seven different PIs are formed by different combinations of phosphorylation at D3, D4 and D5 of the inositol ring.

Equilibratory labeling or pulse labeling of cells or tissues with radioisotopes has uncovered cellular PI contents and their metabolic pathways. For example, PI(4,5)P_2 was previously thought to be exclusively generated from PI(4) by PI(4)P 5-kinase; however, HPLC analysis demonstrated

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an alternative pathway that synthesizes PI(4,5)P_2 by PI(5)P 4-kinase.[6] In addition, PI(3,5)P_2 was first identified to be an osmotic stress-dependent PI based on a HPLC analysis.[7] In this technical review, we describe a simple and economical method for the quantitative analysis of cellular PI contents using the HPLC system equipped with a fraction collector (DC-1500C, Waters) and the TSKgel SAX column (Tosoh).

Set-up for the preliminary and subsequent experiments

The reagents and equipment used in this assay are listed in Table 1, and a summary of the methods used to analyze the [^{3}H]groPIs are shown in Table 2. It is necessary to determine the number of cells included in the assay prior to starting the experiments. We seeded three different amounts of cells into 6-cm culture dishes (for example, 3.0 x 10^5, 5.0 x 10^5 or 1.0 x 10^6 cells / dish) using culture medium. The day after incubation at 37°C, 5% CO_2, cells

Table 1. Reagents and equipment used in the assay

Reagents

Cell culture and [^{3}H] inositol labeling

Reagents	Supplier	Catalog No.
D-PBS(-)	Wako	045-29795
DMEM	Wako	044-29765
Medium 199	Gibco	11150-059
FBS	JRH Biosciences	12103-78P
[2-^{3}H] *myo*-inositol	Perkin Elmer	NET114A

Lipid extraction and deacylation

Reagents	Supplier	Catalog No.
Hydrochloric acid	Nacalai tesque	37314-15
Potassium chloride	Nacalai tesque	28514-75
Chloroform	Wako	038-02606
Methanol	Wako	131-01826
Brain total lipid extract	Avanti Polar Lipids	131101C
1-Butanol	Sigma	B-7906
40 % methylamine in H_2O	Sigma	426466
1-Propanol	Sigma	279544
Petroleum ether	Sigma	184519
Ethyl formate	Sigma	112682

HPLC analysis and detection

Reagents	Supplier	Catalog No.
Ammonium phosphate monobasic	Sigma	216003
Phosphoric Acid	Sigma	695017
Clear-sol I	Nacalai tesque	09135-93

Equipment

Equipment	Supplier	Model or Catalog No.
Dialysis membrane (MWCO : 3,500-5000)	Spectrum Lab.	133198
0.45 μm filter	Millipore	SLHVR04NL
HPLC	Waters	Alliance e2695
HPLC operation system	Waters	Empower™3
Fraction collecter	Waters	DC-1500C
Liquid scintillation Analyzer	Perkin Elmer	Tri-Carb 2900TR
TSKgel SAX column 6.0 x 150 mm	TOSOH	0007157
Partisphere SAX column 4.6 x 125 mm	Whatman	4621-0505

Table 2. Flow chart procedures to analyze the [^{3}H]groPIs

7.5×10^5 CCL39 cells
↓ seed into 6 cm culture dish containing 5 ml of 7.5 % FBS/DMEM
↓ incubate for 24h at 37 °C, 5 % CO_2
↓ wash with 5 ml of M-199 medium x 2 times
↓ add 2 ml of M-199 medium/7.5 % dialyzed FBS
↓ add 20 µl of 1 µCi/µl [2-^{3}H] *myo*-inositol (final 10 µCi/ml)
↓ incubate for 48h at 37 °C, 5 % CO_2
↓ (add stimulation or inhibitors if necessary)
↓ wash with 5 ml of ice-cold PBS (-) x 2 times
↓ add 1 ml of 1 N HCl
↓ scrape cells using cell lifter
↓ transfer to 1.5 ml screw-cap tube
↓ 14,000 rpm for 5 min at 4 °C
ppt
↓ resuspend to 100 µl of 1 N HCl
↓ add 372 µl of chloroform : methanol (1:2)
↓ vortex for 30 sec
↓ add 125 µl of chloroform
↓ add 125 µl of 2 M KCl
↓ vortex for 30 sec
↓ 14,000 rpm for 5 min at 4 °C
lower (lipid) phase
↓ add 40 µl of 25 µg/µl brain total lipid extract
↓ dry up using a speed vac concentrator at room temperature
↓ add 300 µl of deacylation reagent*
(methanol : 1-butanol : 40 % methylamine in H_2O : H_2O = 40 : 10 : 24 : 14)
↓ rotate for 2h in the 53 °C oven
↓ 14,000 rpm for 5 sec at 4 °C
↓ add 150 µl of ice-cold propanol
↓ dry up using a speed vac concentrator at 80 °C
↓ add 200 µl of H_2O
↓ add 300 µl of extraction solution*, vortex vigorously
(1-butanol : petrpleum ether : ethyl formate = 20 : 4 : 1)
↓ 14,000 rpm for 5 min at 4 °C
lower (soluble) phase
↓ transfer to a new screw-cap tube
↓ add 300 µl of extraction solution, vortex vigorously
↓ 14,000 rpm for 5 min at 4 °C
lower (soluble) phase
↓ repeat (total 3 times)
lower (soluble) phase = [^{3}H]groPIs
↓
2 µl
↓ add 1 ml of clear-sol I
↓ [^{3}H] cpm count using liquid scintillation analyzer
600,000 cpm of [^{3}H] groPIs (x µl)
↓ fill up to 1,350 µl using H_2O
↓ add 150 µl of 100 mM $NH_4H_2PO_4$ (pH3.5)
↓ filtration using 0.45 µm filter
1 ml (400,000 cpm of [^{3}H]groPIs)
↓ HPLC analysis using TSKgel SAX column and $NH_4H_2PO_4$ (pH3.5) gradient
Flow rate : 1.0 ml/min
Buffer A : 10 mM $NH_4H_2PO_4$ (pH3.5)
Buffer B : 1.7 M $NH_4H_2PO_4$ (pH3.5)
↓ collect fractions every 30 sec using fraction collector
↓ add 1 ml of clear-sol I
↓ [^{3}H] cpm count using liquid scintillation analyzer

* Prepare before use

were washed twice with Medium 199, a low-inositol medium, and Medium 199 containing dialyzed FBS was then added. Two day after the culture, we checked the cell condition under a microscope and selected cells at 90-100% confluency. In the following section, we briefly describe the procedures used in each step.

Cell labeling using [^{3}H]inositol

The day before labeling the cells, we seeded 7.5 x 10^5 CCL39 cells (ATCC) into 6-cm culture dishes (in the case of HeLa cells, we seeded 3 x 10^5 cells/dish). The day after incubation at 37°C, 5% CO_2, were washed twice using 5 ml of Medium 199, after which 2 ml of Medium 199 containing 10 μCi/ml of [2-^{3}H] *myo*-inositol was added. We used 7.5% dialyzed FBS against a massive amount of 0.9% NaCl. The use of low-inositol medium and dialyzed FBS enhances the [^{3}H]inositol-labeling efficiency. After 48 hours of cell labeling, we treated the cells with inhibitors if necessary and washed them twice using 5 ml of ice-cold PBS. The reaction was then stopped by adding 1 ml of 1N HCl. After scraping the cells using a cell lifter, the cells were transferred into 1.5-ml screw-capped tubes. Following centrifugation at 14,000 rpm for five minutes, the supernatants were used as soluble inositol polyphosphates fraction and the precipitants were subjected to lipid extraction.

Lipid extraction from the cells and deacylation of lipids

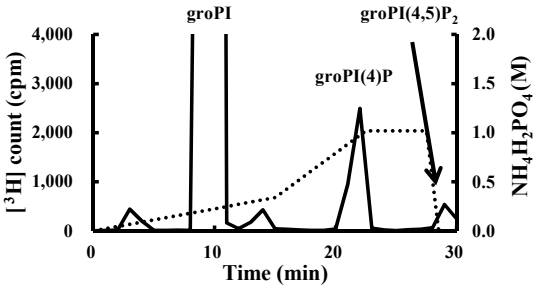
In order to analyze the PI contents, it is necessary to deacylate the lipid because lipophilic PIs are not suitable for HPLC analysis. For this purpose, we resuspended the cell precipitations into 100 μl of 1N HCl and then added 372 μl of chloroform : methanol (1:2). After vigorous vortexing, we added 125 μl of chloroform and 125 μl of 2 M KCl. Following additional vigorous vortexing, the contents were centrifuged at 14,000 rpm for five minutes, and the lower lipid phases were transferred into new 1.5-ml screw-capped tubes. After adding 40 μl of 25 μg/μl brain total lipid extract as a lipid carrier, the lipids were dried up using a vacuum concentrator at room temperature. The dried lipids were resuspended into 300 μl of deacylation reagent (methanol : 1-butanol : 40 % methylamine in H_2O : H_2O = 40 : 10 : 24 : 14) and then incubated for two hours at 53°C with gentle mixing. After two hours of incubation, 150 μl of ice-cold propanol was added and then dried up using a vacuum concentrator at 80°C. The contents were subsequently resuspended in 200 μl of H_2O, and then 300 μl of extraction solution (1-butanol : petroleum ether : ethyl formate = 80 : 16 : 4) was added. Following vigorous vortexing, the contents were centrifuged at 14,000 rpm for five minutes and the lower water phases were transferred into new 1.5-ml screw-capped tubes. The extraction step was repeated twice (for a total of three times), and the final products were subjected to the HPLC analysis. It is worth mentioning that the deacylation reagent and extraction solution were prepared just before use.

HPLC analysis of deacylated PIs

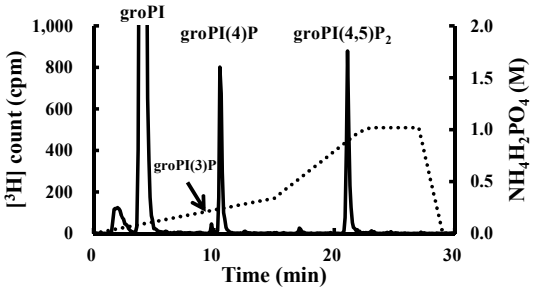
To date, the HPLC system equipped with a flow scintillation analyzer and partisphere SAX column (Whatman) has been frequently used to analyze [^{3}H] groPIs. However, a flow scintillation analyzer is an expensive apparatus and the supply of partisphere SAX column is temporarily unstable because the distribution agency recently changed from Whatman (UK) to Hichrom (UK). Therefore, we attempted to establish a simple and economical HPLC system equipped with a fraction collector (DC-1500C, Waters) and TSKgel SAX column (Tosoh, Japan). In this HPLC system, we injected 200,000 – 400,000 cpm of groPIs per each single run of the HPLC analysis, which was adequate to detect the groPI, groPI(4)P and groPI(4,5)P_2 signals (in the current HPLC analysis, we focused on these three major PIs due to the low abundance of other PIs in the cells). It is necessary, however, to apply approximately 3,000,000 cpm per run in order to detect minor PIs-, such as PI(3,4,5)P_3. Therefore, it is essential to optimize the experimental conditions, including the number of cells and amount of [^{3}H]inositol depending on the experiment. We usually prepared 1.0-1.5 ml of groPI samples following equilibration with 10 mM $NH_4H_2PO_4$ (pH3.5) (same as buffer A) and subsequently filtered the cells using 0.45-μm filters.

In order to compare the HPLC profiles of the two different columns (partisphere SAX column and TSKgel SAX column), we first separated the deacylated lipids using the following $NH_4H_2PO_4$ (pH3.5) gradients at a flow rate of 1 ml/min, HPLC program for groPIs separation : 0 – 15 min (Buffer A: 100%) → (Buffer A: 80%, Buffer B: 20%), 15 – 22.5 min (Buffer A: 80%, Buffer B: 20%) → (Buffer A: 40%, Buffer B: 60%), 22.5 – 27.5 min (Buffer A: 40%, Buffer B: 60%), 27.5 – 28.5 min (Buffer A: 40%, Buffer B: 60%) → (Buffer A: 100%), 28.5 – 30 min (Buffer A: 100%) (Fig. 2(C)). The fractions were collected using the fraction collector (DC-1500C) and the level of radioactivity was determined using the scintillation counter after mixing with the liquid scintillation cocktail. As shown in Figure

(A) **TSKgel SAX column (TOSOH)**
Fraction collector



(B) **Partisphere SAX column (Whatman)**
Flow scintillation analyzer



(C)

Time	buffer A 10 mM $NH_4H_2PO_4$ (pH3.5)	buffer B 1.7 M $NH_4H_2PO_4$ (pH3.5)
0 min	100%	0%
15 min	80%	20%
22.5 min	40%	60%
27.5 min	40%	60%
28.5 min	100%	0%
30 min	100%	0%

Figure 2. Comparison of the HPLC profiles between the TSKgel SAX column and the partisphere SAX column. (A) A total of 1.0 x 10^6 CCL39 cells were labeled with 10 μCi/ml of [^{3}H]inositol for 48 hours at 37°C, 5% CO_2. After deacylation of the lipids, 200,000 cpm of groPIs were analyzed using HPLC with the TSKgel SAX column and $NH_4H_2PO_4$ gradient. The fractions were collected every minute and the radioactivity was detected using the scintillation counter after mixing with the liquid scintillation cocktail. Under these condition, groPI, groPI(4)P and groPI(4,5)P_2 were eluted at 10, 22 and 29 minutes, respectively. The $NH_4H_2PO_4$ gradient is shown by the dashed line (right axis). (B) A total of 3 x 10^5 HeLa cells were labeled with 10 μCi/ml of [^{3}H]inositol for 48 hours at 37°C, 5% CO_2. After deacylation of the lipids, 400,000 cpm of groPIs were analyzed using HPLC with the partisphere SAX column and $NH_4H_2PO_4$ gradient. Radioactivity was detected every six seconds using an in-line flow scintillation analyzer (FLO-ONE β: Packard) after mixing with the liquid scintillation cocktail. Under these conditions, groPI, groPI(4)P and groPI(4,5)P_2 were eluted at 4, 10.5 and 21 minutes, respectively. Each elution position of the groPIs was previously identified using the radiolabeling PI standards.[15] The $NH_4H_2PO_4$ gradient is shown by the dashed line (right axis). (C) $NH_4H_2PO_4$ gradient used in this assay.

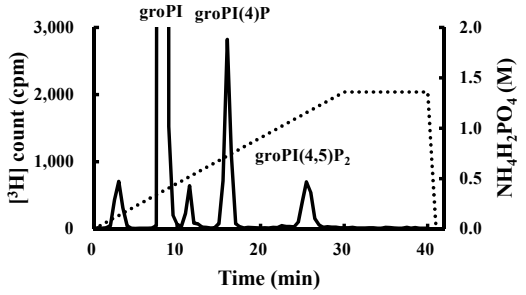
2 (A), groPI, groPI(4)P and groPI(4,5)P_2 were eluted at 10, 22 and 29 minutes, respectively, in the TSKgel SAX column. These retention times are longer than that for the partisphere SAX column (groPI, groPI(4)P and groPI(4,5)P_2 are eluted at 4, 10.5 and 21 minutes, respectively) in the same gradient (Fig. 2(B)). By obtaining the fractions every 30 seconds, it is possible to separate groPI, groPI(4)P and groPI(4,5)P_2; however, these conditions are inadequate to separate groPIPs (groPI(3)P and groPI(4)P) (as archived by in-line detection of [^{3}H] signaling using an in-line detector every six seconds (Fig. 2 (B)).

Because groPI(4,5)P_2 was eluted at the end of the gradient in the TSKgel SAX column, we modified the $NH_4H_2PO_4$ (pH3.5) gradients for following conditions, HPLC program for groPIs separation : 0 – 30 min (Buffer A: 100%) → (Buffer A: 20%, Buffer B: 80%) , 30 – 40 min (Buffer A: 20%, Buffer B: 80%), 40 – 41 min (Buffer A: 20%, Buffer B: 80%) → (Buffer A: 100%) , 41 – 50 min (Buffer A: 100%) (Fig. 3(C)). Based on this gradient, groPI, groPI(4)P and groPI(4,5)P_2 were eluted at 8.5, 16 and 25.5 minutes, respectively, in the TSKgel SAX column (Fig. 3(A)). We further determined the pharmacological responses of these major elution peaks. Wortmannin is a well-known PI 3-kinase inhibitor that inhibits PI(3,4,5)P_3 production[11] as well as PI(4)P production by inhibiting Type III PI 4-kinase.[12-14] We treated the [^{3}H]inositol-labeled CCL39 cells with 10 μM wortmannin for 30 minutes at 37°C, 5% CO_2. Following deacylation of the lipids, we separated the [^{3}H]groPIs using the TSKgel SAX column. As shown in Figure 3(B), the peak was eluted at 16 minutes, which corresponded to a decrease in groPI(4)P by approximately 74%, where as the peak eluted at 25.5 minutes, which corresponded to groPI(4,5)P_2 was not significantly decreased. These results are in agreement with those observed in previous reports.[13,14] Since treatment with wortmannin inhibits both PI 4-kinase and PI 3-kinase in CCL39 cells, the synthesis of PI(4)P from PI by PI 4-kinase (see Fig. 1) is thought to lower. On the other hand, the synthesis of PI(4,5)P_2 from PI(4)P by PI 5-kinase may remain unchanged, as wortmannin dose not inhibit PI 5-kinase. These pharmacological responses of the major elution peaks are consistent with the properties of groPI(4)P and groPI(4,5)P_2.

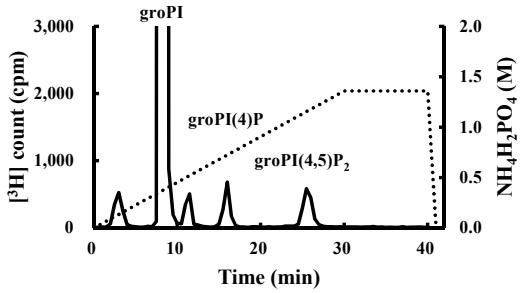
Concluding remarks

In this technical review, we showed a simple and economical method for detecting cellular PIs using [^{3}H]

(A) Control



(B) 10 μM wortmannin, 30 min



(C)

Time	buffer A 10 mM $NH_4H_2PO_4$ (pH3.5)	buffer B 1.7 M $NH_4H_2PO_4$ (pH3.5)
0 min	100%	0%
30 min	20%	80%
40 min	20%	80%
41 min	100%	0%
50 min	100%	0%

Figure 3. Wortmannin inhibits the PI(4)P production. (A, B) A total of 7.5 x 10^5 CCL39 cells were labeled with 10 μCi/ml of [^{3}H]inositol for 48 hours at 37°C, 5% CO_2 and then treated with (A) DMSO (control) or (B) 10 μM wortmannin for 30 minutes at 37°C, 5% CO_2. After deacylation of the lipids, 400,000 cpm of groPIs were analyzed using HPLC with the TSKgel SAX column and $NH_4H_2PO_4$ gradient. The fractions were collected every 30 seconds and the radioactivity was detected using the scintillation counter after mixing with the liquid scintillation cocktail. Under these conditions, groPI, groPI(4)P and groPI (4,5) P_2 were eluted at 8.5, 16 and 25.5 minutes, respectively. The $NH_4H_2PO_4$ gradient is shown by the dashed line (right axis). (C) $NH_4H_2PO_4$ gradient used in this assay.

inositol labeling and the HPLC system equipped with a fraction collector (DC-1500C, Waters) and the TSKgel SAX column (Tosoh). This HPLC system (modifying the $NH_4H_2PO_4$ gradient) can be used to separate groPI, groPI(4)P and groPI(4,5)P_2, with results almost comparable to those of the HPLC system equipped with a flow scintillation analyzer and partisphere SAX column (Whatman). This method is expected to be useful for quantifying the cellular contents of PIs and understanding their physiological functions. In addition, this tool can be applied in screening for inhibitors of PI kinases and phosphatases.

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