福岡大医紀（*Med. Bull. Fukuoka Univ.*）：40(3/4)，209–217，2013

Quantitative Analysis and Cell Distribution Imaging of Phosphoinositides

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Abstract：
Phosphoinositides are minor components of phospholipids in various cell types, but they have pivotal roles in cell functions. Due to their low abundance and rapid turnover rate in cells, it is difficult to detect the contents and cellular distribution of phosphoinositides. However, the development of phosphoinositide-labeling methods using radioisotopes and the identification of phosphoinositide-binding domains have helped to uncover the metabolic pathways of phosphoinositides and their physiological functions. In this review, we summarize the quantitative analysis of cellular phosphoinositides using [^{3}H] inositol labeling and a HPLC system. We also discuss the spatio-temporal imaging of the cellular localization of phosphoinositides using phosphoinositides-binding domains tagged with fluorescent proteins.

Key words：**Phosphoinositides, [^{3}H] inositol, HPLC, Phosphoinositide-binding domain**

Introduction

Phosphoinositides (PIs) comprise less than 10% of the total cellular phospholipids, but they have functions in a remarkable number of crucial cellular processes, such as cytoskeletal reorganization, membrane ruffling, endocytosis, exocytosis, regulation of ion channels and transporters, chromatin remodeling and the recruitment of signaling molecules.[1)-7)] PIs are reversibly phosphorylated/dephosphorylated at positions D3, D4 and D5 of their inositol head group by PI kinases and phosphatases in response to extracellular stimuli, such as hormones, neurotransmitters, cell growth factors and cell differentiation factors.[6),7)] At present, seven different PIs (PI (3) P, PI (4) P, PI (5) P, PI (3,4) P_2, PI (3,5) P_2, PI (4,5) P_2 and PI (3,4,5) P_3) have been identified in mammals (Fig. 1).[6),7)] To analyze the cellular contents of PIs, radiolabeling methods using [^{3}H] inositol and a HPLC system have been used for more than two decades.[8)-13)] By the equilibratory labeling or pulse labeling of cells or tissues using radioisotopes, the cellular PI contents and their metabolic flow have been uncovered.[8)-13)] Recently, numerous PI-binding domain-containing proteins have been identified, and it has been demonstrated that PIs exert their functions through their specific binding to PI-binding domains, which induce a conformational change of the protein structure or the assembly of PI-binding proteins in specific subcellular compartments.[7),14)-16)] Following the discovery of PI-binding domains, it was shown that they can be valuable tools to detect the subcellular distribution of PIs and their spatio-temporal dynamics in living cells.[7),17)] In this review, we summarize the quantitative analysis of the PI contents, and discuss the spatio-temporal imaging of the cellular localization of PIs and their physiological functions.

Application of the quantitative analysis of cellular PIs using HPLC systems

Mass measurements of PIs have been difficult due to their low abundance and rapid turnover. Therefore, radiolabeling methods using [^{3}H] inositol or [^{32}P] orthophosphates, combined with a HPLC system, have

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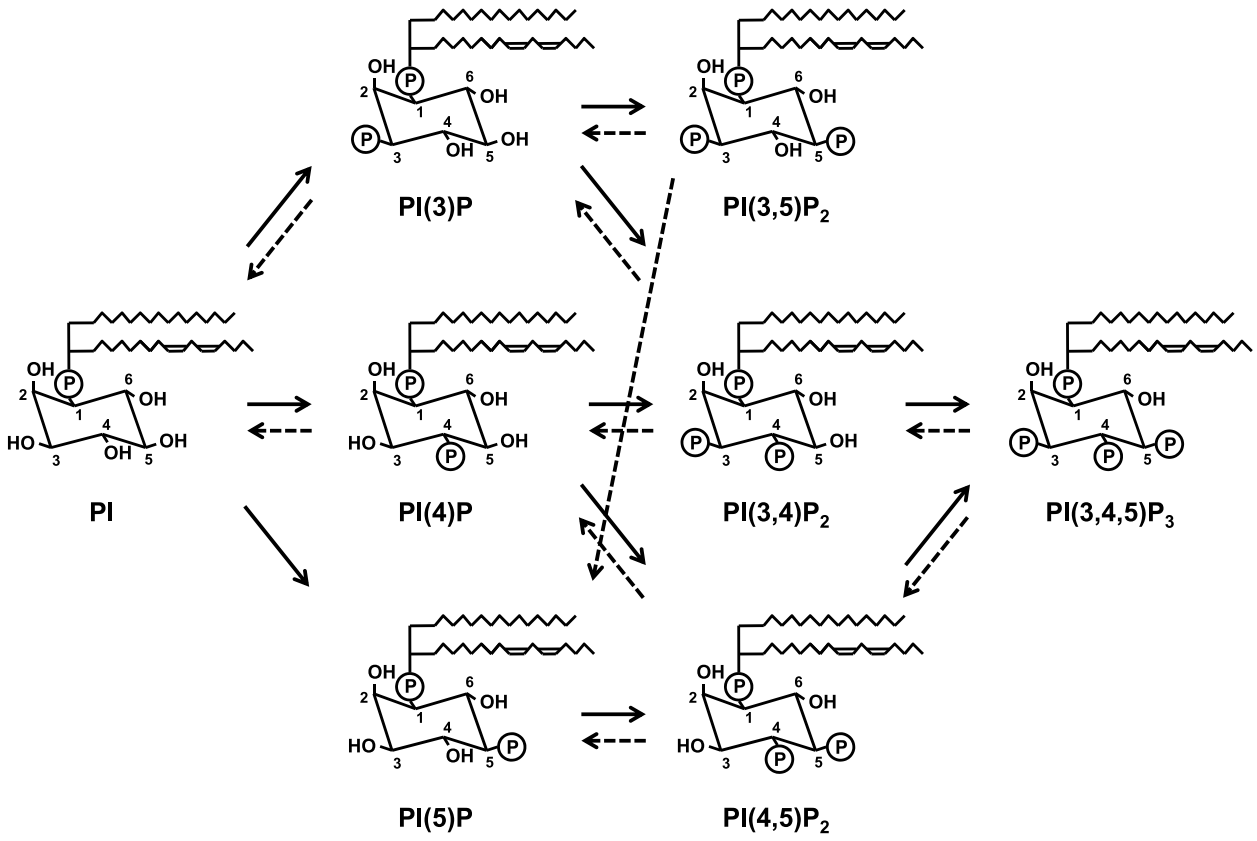

Figure 1 The metabolic pathways of the PIs. The PIs are phosphorylated (arrows)/dephosphorylated (dashed arrows) by PI kinases and phosphatases. There are seven different PIs that are generated by different combinations of the phosphate moiety on D3, D4 and D5 of the inositol ring.

been used as sensitive and quantitative ways to detect the cellular PI contents. These methods are also applied to analyze the substrate specificity of PI-metabolizing enzymes and the PI-metabolizing pathways.

It was previously thought that PI (4,5) P_2 was exclusively produced from PI (4) P by PI (4) P 5-kinase, however, the finding that type II PI (4) P 5-kinase (currently referred to as PI (5) P 4-kinase) catalyzes the phosphorylation of the D4 position of PI (5) P using a HPLC system demonstrated the existence of an alternative pathway to synthesize PI (4,5) P_2 (Fig. 1). [9] Additionally, the existence of PI (3,5) P_2 has been reported, and it has been suggested that hypo- or hyper-osmotic stress can respectively increase or decrease the PI (3,5) P_2 level in mammalian cells. [10] It has recently been shown that the mutation of FIG4, a PI (3,5) P_2 5-phosphatase, causes Charcot-Marie-Tooth disorder, a hereditary motor and sensory neuropathy. [18] It has also been demonstrated that the PI phosphatases, synaptojanin and their yeast homologues, INP52 and INP53, have dual PI 5-phosphatase activity and polyphosphoinositide phosphatase activity in distinct 5-phosphatase and SAC1 domains, respectively. [11] These enzymes regulate endocytosis, synaptic vesicle recycling, membrane trafficking and actin reorganization. [19) -21]

It has been difficult to quantify the PI (5) P levels because of its very low abundance in cells, and because it was difficult to achieve sufficient separation from the other PIPs, such as PI (4) P, by conventional HPLC system. Rameh and colleagues recently were able to successfully separate the PI (5) P from PI (4) P by developing a new HPLC method which uses tandem Partisphere Strong Anion exchange (SAX) columns and a higher pH elution buffer. [12] Using their new separation method, they showed that the PI (5) P levels are typically 1-2% of the PI (4) P levels in cells, such as epithelial cells, fibroblasts and myoblasts. [12]

One of the advantages of these methods is that they make it possible to separate and quantify the different phosphatidylinositol monophosphates and

phosphatidylinositol bisphosphates, which is impossible to do by mass-spectrometry. There is a limitation of the radioisotopic labeling efficiency of cells and tissues which enables the detection of the minor components of PIs using these methods. However, these methods are still useful to analyze the PI contents and PI metabolic flow in cells derived from mice with a knockout or transgenic expression of PI-metabolizing enzymes. It is also useful to screen for drugs that modify the PI metabolizing pathways.

Outline of the quantitative analysis of cellular PIs using a HPLC system

In this section, we will briefly summarize the methods used to quantitatively analyze the cellular PI contents using [^{3}H] inositol labeling and a HPLC system. To quantitatively analyze the cellular PI contents, it is necessary to efficiently label the PIs using a radioisotope. For this purpose, we usually label the cells in Medium 199, a low inositol containing medium, including *myo*- [2-^{3}H] inositol (10 μCi/ml), a head-group of PIs, for 48 – 72 hr. After equilibrium labeling of the cellular PIs, the total lipids are extracted from cells using $CHCl_3$/methanol.

To analyze the PI contents using a HPLC system, it is necessary to deacylate the lipids, because lipophilic PIs are not suitable for HPLC analysis. Therefore, the extracted total lipids are deacylated in methylamine-containing buffer, and the water-soluble deacylated products (glycerophosphoinositols, groPIs) are extracted. An appropriate amount of groPIs (usually 200,000 – 500,000 cpm of groPIs per 1 run) are subjected to a HPLC analysis using an anion exchange SAX column with a $NH_4H_2PO_4$ (pH 3.5) gradient.

Theoretically, the groPIs are separated by the $NH_4H_2PO_4$ (pH3.5) gradient depending on the number of phosphate groups on the inositol ring, and the elution position of each peak is assigned by the co-migration of standard products such as deacylated [^{3}H] PI (4) P or [^{3}H] PI (4,5) P_2 (Fig. 2). By counting the radioactivity of each fraction using an in-line detector or scintillation counter after mixing the samples with a liquid scintillation cocktail, it is possible to quantify the cellular PI contents. The details of these methods can be found in several excellent manuscripts.[8) -13)]

Spatio-temporal imaging of subcellular localization of PIs

The subcellular distribution of PIs was identified/

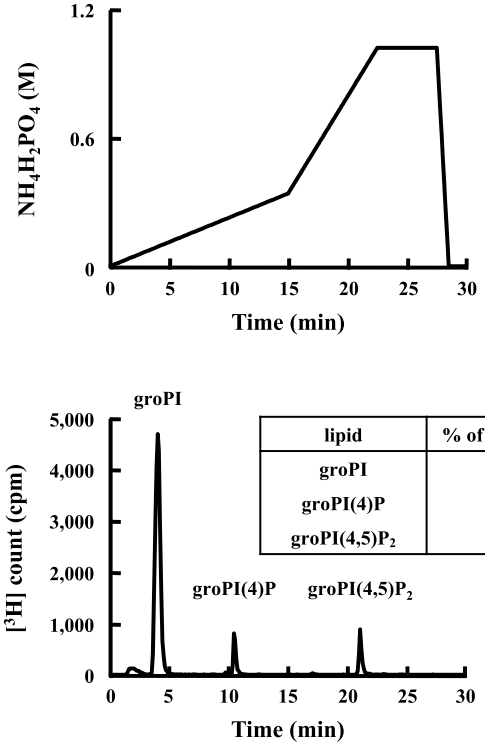

Figure 2 The results of the HPLC analysis of deacylated PIs (groPIs). A total of 3 x 10^5 HeLa cells were labeled with 10 μCi/ml [^{3}H] inositol for 48 hr at 37℃, 5%CO_2. After deacylation of the lipids, 400,000 cpm of groPIs were analyzed by HPLC using a SAX column and $NH_4H_2PO_4$ gradient. Using the $NH_4H_2PO_4$ gradient, the upper graph, groPI, groPI (4) P and groPI (4,5) P_2 were eluted at 4 min, 10.5 min and 21 min, respectively. The quantitative data for groPI, groPI (4) P and groPI (4,5) P_2 were depicted as the % of the total lipids in the inset. This figure was modified from reference 51 with permission.

speculated based on cell fractionation methods or immunofluorescent microscopy targeting PI-metabolizing enzymes. Therefore, it was very difficult to detect the subcellular distribution of PIs and the spatio-temporal dynamics in living cells until PI-binding domains were identified. In this section, we will briefly summarize the binding specificity of each PI-binding domain, the imaging of the subcellular distribution of PIs and their spatio-temporal dynamics in cells using PI-binding domains tagged with fluorescent proteins.

PI-binding domains

The pleckstrin homology (PH) domain, an approximately 120 amino acid structural domain, was first identified as a tandem repeat domain in pleckstrin, a major substrate of protein kinase C in the platelets, and was subsequently

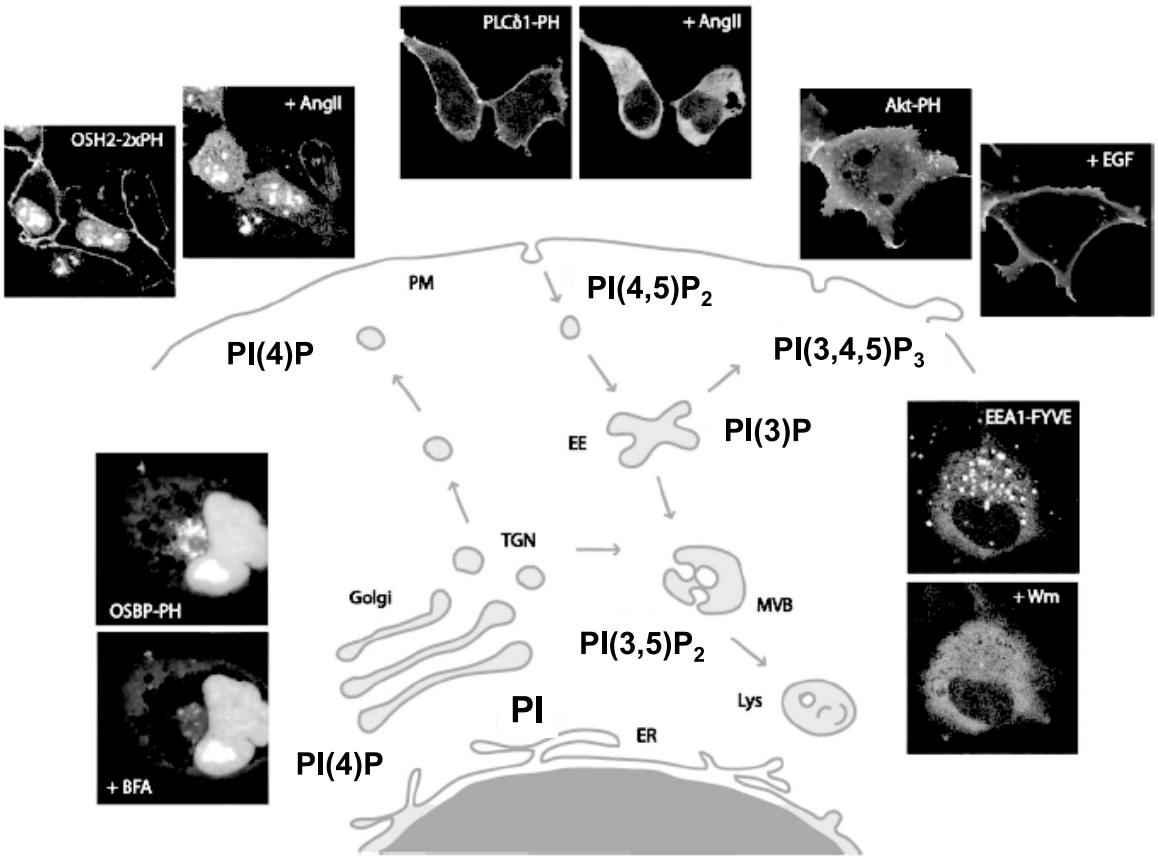

Figure 3 The subcellular localization of the PI-binding domains tagged with GFP. The PLC-δ1 PH domain, which specifically binds PI (4,5) P_2, localized to the plasma membrane. Following the hydrolysis of PI (4,5) P_2 after angiotensin II (+AngII) stimulation, GFP-PLC-δ1 PH translocates from the plasma membrane to the cytosol. The PH domain of the yeast OSH2 and the PH domain of human OSBP both bind PI (4) P, but are localized to the plasma membrane and Golgi, respectively. The Akt-PH domain, which recognizes PI (3,4) P_2 and PI (3,4,5) P_3, shows cytosolic localization in quiescent cells, however, this probe translocates to the plasma membrane after agonist stimulation, such as by EGF. The FYVE domain of EEA1 recognizes PI (3) P and is localized on the endocytotic membrane. This figure was modified from reference 17 with permission.

recognized as a PIs-binding module. [22)-24)] Following this discovery, PH domains have been identified in more than 250 proteins, including protein kinases, guanine nucleotide exchange factors and GTPase-activating proteins for small GTP-binding proteins, lipid transport proteins and phospholipases. Most of these proteins (but not all) bind PIs such as PI (4) P, PI (3,4) P_2, PI (4,5) P_2 and PI (3,4,5) P_3 with various degrees of specificity. [14), 15), 25), 26)]

One of the best-characterized PH domains is the PLC-δ1 PH domain, which specifically binds both PI (4,5) P_2 and the headgroup I (1,4,5) P_3, and these interactions are important for its activity and localization in the plasma membrane. [27)-29)] The three-dimensional structure of the isolated PLC-δ1 PH domain, which was co-crystallized with I (1,4,5) P_3, shows that the PH domain is composed of seven anti-parallel β-strands and one C-terminus α-helix. [30)] The positively charged amino acids on the loop between the β-strands (loop1/2 and loop3/4) have a crucial role for PI (4,5) P_2 and I (1,4,5) P_3 binding. The overall structure of PH domains is well conserved among proteins, and the positively charged amino acids on the loop region and additional contacts between the side chain of basic residues from elsewhere in the domain, as well as the phosphate group on inositol, define the binding specificity. [14)-16), 25)]

FYVE (Fab1, YOTB, Vac1, EEA1) domains, sequence motifs of about 70 residues, are involved in endocytosis

Table 1 Typical size, three-dimensional structure, binding specificity and examples of PI-binding domains

Domain	Typical size (amino acids)	Structure	Lipid	Examples of protein
ANTH	～280	α-helical solenoid	PI(4)P	EpsinR
			$PI(3,5)P_2$	Ent3
C2	～130	β-sandwich	$PI(4,5)P_2$	Synaptotagmin
ENTH	～150	α-helical solenoid	$PI(4,5)P_2$	AP180, CALM
FYVE	～70	Zn^{2+} finger	PI(3)P	EEA1, Hrs, PIKfyve
PH	～120	β-sandwich	PI(4)P	FAPP1/2, OSBP
			$PI(3,4)P_2$	Akt/PKB, TAPP1,2
			$PI(4,5)P_2$	PLC-δ1, dynamin
			$PI(3,4,5)P_3$	Akt/PKB, Btk, ARNO, GRP1
PX	～130	α + β structure	PI(3)P	SNX2,3,7,13
			$PI(3,4)P_2$	p47phox

Abbreviations are used as ANTH; AP180 N-termial homology, AP180; adaptor protein 180, ARNO; ADP-ribosylation factor nucleotide-binding-site opener, Btk; Bruton's tyrosine kinase, C2; conserved region-2, CALM; clathrin assembly lymphoid myeloid leukemia protein, EEA1; early endosome antigen-1, ENTH, epsin N-termial homology, FAPP; four-phosphate adaptor protein, FYVE; Fab1, YOTB, Vac1, EEA1, GRP1; general receptor for phosphoinositides-1, OSBP; oxysterol binding protein, PH; pleckstrin homology, PI(3)P; phosphatidylinositol 3-phosphates, PI(4)P; phosphatidylinositol 4-phosphates, $PI(3,4)P_2$; phosphatidylinositol 3,4-bisphosphates, $PI(3,5)P_2$; phosphatidylinositol 3,5-bisphosphates, $PI(4,5)P_2$; phosphatidylinositol 4,5-bisphosphates, $PI(3,4,5)P_3$; phosphatidylinositol 3,4,5-trisphosphates, PKB; protein kinase B, PLC; phospholipase-C, PX; phox homology, SNX; sorting nexin, TAPP; tandem PH-domain containing protein.

or vacuolar sorting through their specific binding to PI (3) P. [31)-34)] Based on their crystal structures, FYVE domains are cysteine-rich zinc finger structures comprising two short antiparallel β-sheets and a C-terminal α-helix. A sequence of conserved basic residues ((R/K) (R/K) HHCR) in the first β-strand contributes to a shallow, positively charged PI (3) P-binding site, and the structure is stabilized by two Zn^{2+}-binding clusters. [15), 16), 35)]

Phox-homology (PX) domains were initially identified in the $p40^{phox}$ and $p47^{phox}$ subunits of the neutrophil NADPH oxidase complex. [36)] These -130 amino acid domains were subsequently recognized as a 3-phosphorylated PIs binding domain, and they have been found in numerous proteins which participate in protein sorting, vesicular trafficking and PI metabolism. [37)] The PX domain contains three N-terminal β-sheets followed by four α-helices, and the PI binding pocket is constructed by the loop linking the β1-β2 strands and one of the helices. [38)]

In addition to above-mentioned structural domains, some other domains are also known to be PI-binding domains. In Table 1, the typical size (number of amino acids), three-dimensional structure, binding specificity for PIs and examples of proteins that contain each PI-binding domain are listed.

Subcellular localization of PIs

Following the discovery of PI-binding domains, it became possible to visualize the intracellular distribution of PIs and their spatio-temporal dynamics during numerous physiological processes such as signal transduction, cell motility, endocytosis and phagocytosis. [17)] Using PLC-δ1 PH tagged with the green fluorescent protein (GFP), it was shown that PI (4,5) P_2 localized to the plasma membrane, and the transient hydrolysis (translocation of a GFP-PLC-δ1 PH probe from the plasma membrane to the cytosol) was monitored upon exposure to extracellular stimuli, such as platelet

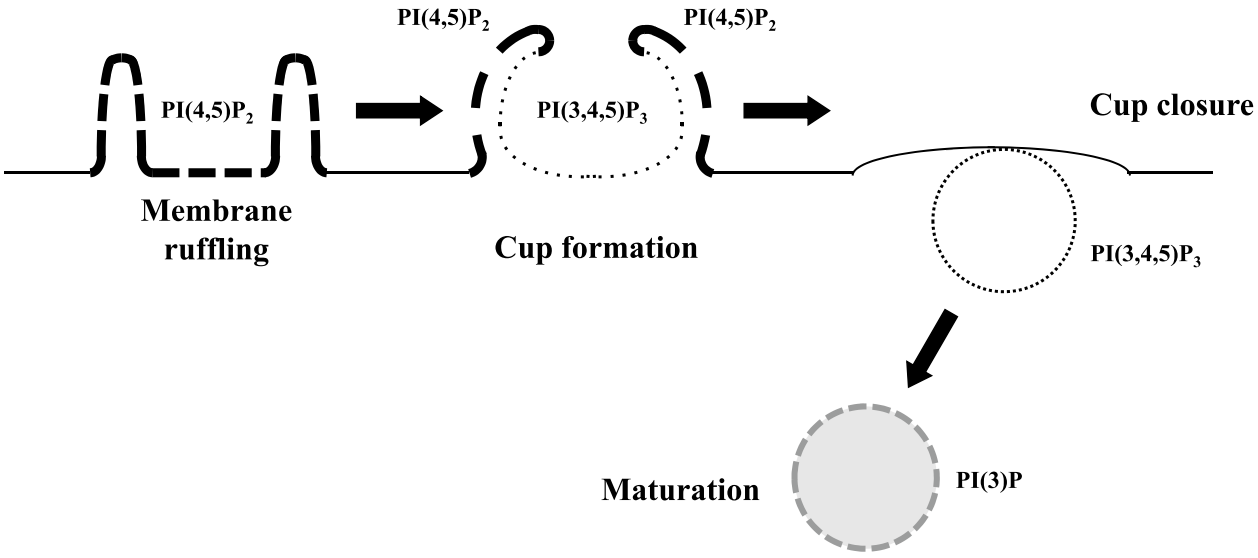

Figure 4 A schematic representation of the phagocytotic and macropinocytotic pathways and PI distribution of each of the processes. The PI (4,5) P_2 (bold dashed line) appeared at the location of membrane ruffling and then moved around the forming cup. At the point of cup closure, the PI (4,5) P_2 levels at the base of phagosomes/macropinosomes were dramatically decreased. Instead of a decrease in PI (4,5) P_2, the levels of PI (3,4,5) P_3 (dashed line) were increased at the base of the phagosome and they traveled throughout the entire phagosome cup. The PI (3,4,5) P_3 levels were maximized just before cup closure and then decreased. The internalized phagosomes acquire PI (3) P (grey dashed line), which is involved in vesicular fusion.

activation factor, angiotensin II and hypo-osmotic stress (Fig. 3).[29),39),40)] Using the Akt PH domain, which binds both PI (3,4) P_2 and PI (3,4,5) P_3, the transient production of these lipids was monitored after growth factor stimulation or chemoattractant stimulation (Fig. 3).[17),41)] PI (3) P was detected in the early endosome using GFP-tagged tandem FYVE (GFP-2x FYVE), and PI (4) P was detected in the Golgi using the OSBP-PH domain (Fig. 3).[34),42),43)] Therefore, the use of PI-binding domains tagged with GFP provides a powerful tool to detect the intracellular localization of specific PI species.

Redistribution of PIs during phagocytosis and macropinocytosis

Phagocytosis and macropinocytosis are important processes for the immune system to achieve the engulfment of an antigen from the extracellular fluid.[44)-46)] The actin-based membrane ruffling, cup formation, cup closure and maturation of phagosomes/macropinosomes are sequentially processed. These processes are tightly regulated by PIs, and the subcellular distribution of PIs during these processes can be visualized using PI-binding domains tagged with fluorescent proteins (Fig. 4).[45)-49)] Upon the cell surface binding of the antigen particle, the levels of PI (4,5) P_2 were increased at the base of the particle and then to move around the forming cup, which is important for actin polymerization.[45)-49)] At the point of cup closure, the PI (4,5) P_2 levels at the base of the phagosome were dramatically decreased, which induces actin depolymerization. Instead of a decrease in PI (4,5) P_2, PI (3,4,5) P_3 were increased at the base of the phagosome and traveled throughout the entire phagosome cup. The PI (3,4,5) P_3 levels were maximized just before cup closure, and then subsequently decreased.[45)-49)] The internalized phagosomes acquired PI (3) P, which is involved in vesicular fusion.[45)-49)] The transition of PI species recruits the different proteins involved in each step of the processes by binding through their PI-binding domains.

Conclusions and future issues

The development of methods that can be used to quantify the cellular PI contents and for the imaging of the subcellular localization of PIs has increased our understanding of the function of PIs in numerous cellular processes. However, the functions of minor

phosphoinositides are still unclear. To overcome this lack of information, it is necessary to identify additional PI-metabolizing pathways or to develop new inhibitors of PI-metabolizing enzymes. To efficiently screen for these compounds, it is necessary to develop a high throughput analytical system. It has recently been reported that a semi-automatic HPLC system using an auto-sampler and a HPLC system can be used for PI analysis [13] and a detection system for PIs using HPLC and a TOF-MASS analysis has also been described. [50] These methods will contribute to elucidating the full cellular functions of the PIs.

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（平成 25. 7. 3 受付，平成 25. 10. 10 受理）