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Nanoscale analysis reveals agonist-sensitive and heterogeneous pools of phosphatidylinositol 4-phosphate in the plasma membrane

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ABSTRACT

Phosphatidylinositol 4-phosphate [PtdIns(4)P] is the immediate precursor of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], which is localized to the cytoplasmic leaflet of the plasma membrane and has been reported to possess multiple cell biological functions. Direct evidence showing the distribution of PtdIns(4)P pools at a nanoscale when the plasma membrane PtdIns(4,5)P_2 is hydrolyzed by agonist stimulation is lacking. To analyze the distribution of PtdIns(4)P at a nanoscale, we employed an electron microscopy technique that specifically labels PtdIns(4)P on the freeze-fracture replica of the plasma membrane. This method minimizes the possibility of artificial perturbation, because molecules in the membrane are physically immobilized *in situ*. Using this technique, we observed no PtdIns(4)P in the caveolae of normal cultured human fibroblasts, although PtdIns(4,5)P_2 has been shown to be highly concentrated in them in our previous report. When cells were stimulated with angiotensin II, the level of PtdIns(4)P in the undifferentiated membrane transiently decreased to 64.3% at 10 s, began to increase at 30 s and largely increased to 341.9% at 40 s, and then returned to the initial level at 130 s after the stimulation. Interestingly, PtdIns(4)P localized at the caveolae at 70 and 130 s after the stimulation. These results suggest that the level of the PtdIns(4)P pool in the plasma membrane is sensitive and the distribution of PtdIns(4)P dramatically changes by agonist stimulation, and there are active sites of production or replenishment of PtdIns(4)P at undifferentiated membrane and caveolar areas.

1. Introduction

Phosphatidylinositol 4-phosphate [PtdIns(4)P] was originally thought to be the precursor of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], which plays critical roles in multiple cellular phenomena, such as ion channel regulation, endocytosis, exocytosis and cytoskeletal assembly. However, more recently, PtdIns(4)P itself has been found to serve as an essential signaling molecule in the Golgi complex, the endosomal system and plasma membrane in the control of membrane trafficking, cytoskeletal organization, lipid metabolism and signal transduction pathways, which are mediated by its direct interaction with PtdIns(4)P-binding proteins [1,2]. Additionally, several recent studies have found that a substantial pool of PtdIns(4)P resides in the plasma membrane [3–6]. It has been predicted that PtdIns(4)P is essential and plays critical roles in the plasma membrane as a key molecule in some cell phenomena. The functional roles of PtdIns(4)P need to be exerted in the right place and at the right time [1]. Further studies with sufficient spatial resolution for small scale detection [7,8] are needed to implicate the veritable role of PtdIns(4)P in the plasma membrane.

Imaging techniques using GFP-tagged domains, such as the pleckstrin homology (PH) domain of FAPP1 [9,10], OSH1 [11], OSBP [12] and OSH2 [11], and the P4M domain of SidM [6], that bind PtdIns(4)P in live cells at the light microscopy level may not have sufficient spatial resolution for small scale detection. Additionally, other limitations of the GFP-tagged domain method have been pointed out [13–16]: the GFP-tagged domain probe that is expressed in live cells may disturb intracellular signaling by sequestering lipids; dramatic changes in Golgi structure and function have been observed upon expression of a GFP-PtdIns(4)P-binding domain probe [10,17]; and the GFP-PtdIns(4)P-binding domain probe is not likely to detect PtdIns(4)P that is bound to endogenous proteins. However, methods using electron microscopy may detect local heterogeneities of lipids at a small scale [18,19], but the use of aldehyde-fixed cryosections is a potential problem, because lipids, including PtdIns(4)P, cannot be securely immobilized by chemical fixation and may be redistributed during the labeling procedure [20].

In the present study, we employed the quick-freezing and freeze-fracture replica labeling (QF-FRL) method, which enables nanoscale membrane lipid labeling by physically fixing membrane molecules [7,

Abbreviations: FAPP1, four-phosphate-adaptor protein 1; GFP, green fluorescent protein; OSBP, oxysterol-binding protein; OSH, oxysterol-binding protein homologs; P4M, PtdIns(4)P binding of SidM; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

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8,21–23]. In the QF-FRL method, live cells were quickly frozen without chemical fixation, and membrane halves split at the hydrophobic interface were cast by vacuum evaporation of carbon and platinum. Membrane lipids were thus physically stabilized, and the exposed hydrophilic surface could be probed without artificial perturbations. Using this method, we found that PtdIns(4)P was localized at the inner, but not outer, surface of the plasma membrane, and its relative cluster distribution in the undifferentiated flat plasma membrane was not susceptible to cholesterol depletion and actin-depolymerization. We also found that upon agonist stimulation, the concentration of PtdIns(4)P transiently increased in the undifferentiated flat plasma membrane, and was highly concentrated in caveolae, although it was not observed in unstimulated control cells. This result is the first demonstration of spatial–temporal heterogeneity of PtdIns(4)P in the plasma membrane. We also revealed that during agonist stimulation, caveolae, a membrane microdomain, implicated in diverse functions, also play a unique role in the PtdIns(4)P-related phenomena.

2. Materials and methods

2.1. Probes

For primary antibodies, anti-PtdIns(4)P mouse monoclonal (IgM) antibody was purchased from Echelon Biosciences (Salt lake city, UT, USA). For secondary gold-conjugated antibodies, 10 nm gold particle-conjugated anti-mouse IgM was purchased from BioCell (Cardiff, UK). Recombinant GST fusion protein containing the phospholipase C (PLC)- δ1 PH domain was expressed in *Escherichia coli* and purified as described previously [7]. The PH domain of PLC-δ1 was obtained from the GST-PLC-δ1 PH domain fusion protein by cleavage with PreScission protease (GE Healthcare Life Sciences, Pittsburgh, PA, USA) according to the manufacturer's protocol.

2.2. Liposome

The phospholipid liposome samples were made as described previously [7]. In brief, phosphatidylcholine (PtdC), phosphatidic acid (PA), phosphatidylethanolamine (PtdE), phosphatidylserine (PtdS), phosphatidylinositol (PtdIns) and phosphoinositides were purchased from Avanti (Alabaster, AL, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively. Ninety-five mol percent PtdC and 5 mol% of either PA, PtdE, PtdS, PtdIns or a phosphoinositide were dissolved in chloroform, dried under a dry nitrogen gas stream and rehydrated with an aqueous buffer to make liposomes. The liposome samples were left for 48 h to induce giant liposomes and were extruded through a polycarbonate membrane filter (pore size 0.1 or 0.2 μm) using a Miniextruder (Avanti) to obtain small unilamellar vesicles. The liposomes were pelleted, mixed with a buffer solution, and rapidly frozen by a high-pressure freezing machine (HPM010, Leica, Vienna, Austria).

2.3. Cell culture

Human fibroblasts and HeLa cells were obtained from the Japanese Collection of Research Bioresources Cell Bank, and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 0.05 mg/ml streptomycin, at 37 °C under 5% CO_2/95% air. For cholesterol depletion, human fibroblasts were treated with 5 mM methyl-β-cyclodextrin (MβCD; Sigma-Aldrich, St. Louis, MO, USA) in DMEM for 60 min. In some experiments, human fibroblasts were treated with 1 μM latrunculin A (Lat A; Wako Pure Chem., Tokyo, Japan) for 7 min, and with 1 μM angiotensin II (Ang II; Sigma-Aldrich) for the indicated times.

2.4. Quick-freezing and freeze-fracture

For quantitative lipid labeling on the plasma membrane of cultured human fibroblasts, a metal sandwich quick-freezing method was used as described previously [24]. Briefly, human fibroblasts grown on a small gold foil (~4 mm^2 in area; 20-μm-thick) were inverted onto prewarmed 10% gelatin on a copper foil (20-μm-thick) with the cell side down, and frozen by a quick press between two gold-plated copper blocks precooled in liquid nitrogen. For the analysis of the inside of cultured Hela cells, we used the high-pressure freezing method as described previously [25]. In brief, a shallow indentation (50-μm-deep) in an aluminum disk (3-mm diameter, 0.5-mm thick, Enginnering Office M. Wholwend) was filled with 10% gelatin, and a gold foil disk with cultured Hela cells was put on the aluminum disk with the cell culture side facing downward. The sample assembly was frozen using an HPM010 high-pressure freezing machine (Leica) according to the manufacturer's instructions. For freezing liposome, a gold EM grid (200 mesh) impregnated with liposome pellets was sandwiched between two flat aluminum disks, and processed similarly to Hela cells [23].

For freeze-fracture, the frozen liposome and cell sandwich was transferred to a cold stage of a Balzers BAF400 apparatus (Bal-Tec AG, Lichtenstein) with the gold side up and fractured at −95 °C (for analyzing the plasma membrane) or −130 °C (for analyzing the cell interior and liposome) and ~1×10^{-6} mbar. Replicas were made by electron-beam evaporation of carbon (C) (~5-nm-thick), followed by platinum (Pt; 2 nm), and then by C (20 nm) as described previously [8]. The replica thickness was controlled by a crystal thickness monitor (EM QSG100, Leica). Thawed specimens were treated with 2.5% sodium dodecyl sulfate in 0.1 M Tris–HCl (pH 8.0) at 60–70 °C overnight.

2.5. Labeling and electron microscopy observation

Labeling with probes was performed as described previously [8]. Briefly, after rinsing, freeze-fracture replicas were blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) at room temperature for 30 min. To block the unspecific binding of the anti-PtdIns(4)P antibody to PtdIns(4,5)P_2, replicas were pretreated with the PH domain (1 mg/ml) of PLC-δ1 in the blocking solution. Then, replicas were treated with primary antibodies at 4 °C overnight: anti-PtdIns(4)P mouse monoclonal antibody (1 μg/ml) diluted in PBS containing 1% BSA. After four washes with PBS containing 0.1% BSA, replicas were incubated with gold (10 nm)-conjugated secondary antibody in PBS containing 1% BSA at 37 °C for 30 min. Replicas were picked up on Formvar-coated grids and observed with a H7000KU electron microscope (HITACHI, Tokyo, Japan) operated at 75 kV.

2.6. Statistical analysis of immunogold labeling

The statistical analysis of the immunogold distribution on the plasma membrane has been described previously [7,8,21]. In brief, electron micrographs were digitized with an image scanner. The *x*-*y* coordinates of gold particles were obtained by Image Processing Tool Kit version 5 plug-in (Reindeer Graphics, Asheville, NC, USA) for Adobe Photoshop version 6 (Adobe system, Mountain view, CA, USA), and randomly chosen areas of 1 μm × 1 μm were analyzed by Ripley's K-function using a program provided by John Hancock [26]. For significance tests, 99% confidence envelopes for complete spatial randomness (CSR) were generated from Monte Carlo situations.

The caveolar area was defined by drawing a circle 150 nm in diameter around the dimples. Gold particles in and around deep and shallow dimples were counted and the average labeling density of 50 dimples was measured. The difference in the labeling density between control and 30-s, 40-s, 70-s or 130-s Ang II-stimulated samples was examined statistically with Student's *t*-test.