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Fixation-induced cell blebbing on spread cells inversely correlates with phosphatidylinositol 4,5-bisphosphate level in the plasma membrane

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ABSTRACT

While most attention has been focused on physiologically generated blebs, the molecular mechanisms for fixation-induced cell blebbing are less investigated. We show that protein-fixing (e.g. aldehydes and picric acid) but not lipid-stabilizing (e.g. OsO_4 and $KMnO_4$) fixatives induce blebbing on spread cells. We also show that aldehyde fixation may induce the loss or delocalization of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane and that the asymmetric distribution of fixation-induced blebs on spread/migrating cells coincides with that of PIP_2 on the cells prefixed by lipid-stabilizing fixatives (e.g., OsO_4). Moreover, fixation induces blebbing less readily on PIP_2-elevated spread cells but more readily on PIP_2-lowered or lipid raft-disrupted spread cells. Our data suggest that fixation-induced lowering of PIP_2 level at cytoskeleton-attaching membrane sites causes bleb formation via local breakdown of the membrane–cytoskeleton coupling.

1. Introduction

Plasma membrane blebs are spherical, dynamic, functional cell protrusions. Cell blebbing is a common phenomenon during many cell physiological behaviors including cell spreading [1,2], migration [3,4], cytokinesis [5,6], apoptosis [7,8], and viral infection [9]. Physiologically produced blebs are commonly regarded to be directly driven by the hydrostatic pressure that is exerted on the cytoplasm by the contractile actomyosin cortex [10,11]. Expansion of a physiologically produced bleb lasts ~30 s whereas its retraction lasts ~2 min and many proteins (particularly myosin motor proteins) dynamically participate in these processes [12,13].

Cell fixation with aldehyde fixatives at certain concentrations is also able to induce cell blebbing [14,15,16,17], based on which a technique mainly using formaldehyde as the fixative for isolating plasma membranes or giant plasma membrane vesicles has been developed and applied widely [18,19,20]. Unlike physiologically produced blebs, fixation-induced blebs expand for 10–30 min and retract quickly (within seconds) or detach from the plasma membrane [16,21]. Aldehydes are mainly protein-stabilizing fixatives that, especially at relatively high concentrations, can completely fix most proteins in/on cells rapidly including those that may participate in cell blebbing under physiological conditions. It suggests that the molecular mechanisms for fixation-induced cell blebbing may be unique although we believe that fixation-induced blebs are also the mechanical consequence of hydrostatic pressure. Until now, however, while most attention in this research field has been paid to physiologically generated blebs the molecular mechanisms for fixation-induced cell blebbing remain unclear.

In this study, we focus on fixation-induced blebbing of spread adherent cells. We reveal that fixation-induced bleb formation inversely correlates with the level of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$ or $PI(4,5)P_2$ or PIP_2) in the plasma membrane. The inability of protein-stabilizing fixatives (e.g., aldehydes) to stabilize PIP_2 may cause local loss or delocalization of PIP_2 from cytoskeleton-attaching sites of the plasma membrane

Abbreviations: DIC, differential interference contrast; HUVECs, human umbilical vein endothelial cells; PIP_2, phosphatidylinositol 4,5-bisphosphate; TBS, Tris-buffered saline; DAG, 1,2-diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; MβCD, methyl-β-cyclodextrin; PI3K, phosphoinositide-3 kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C

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(i.e., lower PIP_2 level at these sites) where local detachments of the plasma membrane from the cell cortex occur, followed by hydrostatic pressure-powered blebbing.

2. Materials and methods

2.1. Reagents, cell lines and cell culture

Paraformaldehyde (Solarbio, Beijing, China), Picric acid (AiKeda Chemical Technology, Chengdu, China), osmium tetroxide (OsO_4; Ted Pella, CA, USA), potassium permanganate ($KMnO_4$; Shanghai Chemical Company, Shanghai, China), wortmannin (Sigma) and LY294002 (inhibitors of phosphoinositide-3 kinase (PI3K); Calbiochem), U73122 and m-3M3FBS (inhibitor and activator of phospholipase C, respectively; Calbiochem), methyl-β-cyclodextrin (MβCD; Invitrogen), and phorbol-12-myristate-13-acetate (PMA; Sigma) were all purchased commercially. The stock or working solutions of all reagents were prepared, stored, and used according to the manufacturer's instructions. All working solutions were freshly prepared for each experiment.

Human umbilical vein endothelial cells (HUVECs) were purchased from Xiangya Central Experiment Laboratory (Hunan, China) and were routinely cultured in DMEM (Gibco) supplemented with 10% (w/v) fetal calf serum (Hyclone, South Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. For all experiments, cell cultures had been passaged ~5 times.

Human THP-1 monocytic leukemia cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). THP-1 cells were routinely cultured in RPMI 1640 media (Hyclone) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Before stimulation, approximately 1.5×10^5/ml THP-1 cells were seeded in 12-well plates and cultured at 37 °C in a 5% CO_2 incubator for 2 days. Then, the cells were differentiated for 2 days in growth medium with addition of PMA (100 ng/ml), washed twice with PBS, and cultured in growth medium without PMA.

2.2. Cell fixation by different types of fixatives

Approximately 2×10^4/ml HUVECs were plated in a petri dish and cultured at 37 °C in a 5% CO_2 incubator for 24 h. After washing with PBS, cells were fixed at room temperature (except the OsO_4 experiments) with the following fixation strategies: (a) 4% paraformaldehyde (pH ~7.0) for 20 min; (b) 1.5% or 1% picric acid solution (pH ~7.1) for 1 h; (c) 2%, 1%, or 0.5% $KMnO_4$ solution (pH ~7.1) for 1 h; (d) different concentrations of $KMnO_4$ for 1 h and then 4% paraformaldehyde for 20 min; (e) 1% or 0.5% OsO_4 solution (pH ~7.1) at 4 °C for 2 h; (f) 1% or 0.5% OsO_4 at 4 °C for 2 h and then 4% paraformaldehyde for 30 min. All fixative solutions were prepared or diluted using PBS and freshly prepared for each experiment. After fixation, the cells were immediately imaged by confocal microscope. In order to make sure whether a slight change in ionic strength of fixative solutions influences the genesis of blebs, we also prepared 3% paraformaldehyde (pH ~7.2) and 3% paraformaldehyde (pH ~7.3) by diluting 4% paraformaldehyde (already in PBS when purchased) with PBS and double distilled water, respectively.

2.3. Measurement of fixation-induced blebs on migrating cells

Approximately 5×10^4/ml HUVECs in a petri dish were cultured at 37 °C in the incubator for 24 h to create a confluent cell monolayer. A p100 pipet tip was used to scrape in a straight line to make a "scratch" in the cell monolayer. After cell debris was removed by two washes with PBS and the medium was refreshed, an image of a section of the scratch was taken as a reference image by confocal microscope. Then, the cells were cultured for an additional 6 h in the incubator. After a 20-min fixation with 4% paraformaldehyde, the section of the scratch was imaged again by confocal microscope. The number and radius of blebs on migrating cells in the scratch in each image was counted or measured.

2.4. Inhibitors/activator or MβCD treatments

Approximately 2×10^4/ml HUVECs were plated in a Petri dish and cultured at 37 °C in the incubator for 24 h. After washing with PBS, cells were treated at 37 °C in the incubator with various working reagent solutions at different concentrations: (a) 100, 75, 50, or 25 nM wortmannin for 1 h; (b) 50, 40, 30, or 20 μM LY294002 for 30 min; (c) 10, 8, 6, or 4 μM U-73122 for 30 min; (d) 100 μM m-3M3FBS for 2 min; (e) 10 or 5 mM MβCD for 30 min; (f) 50 μM LY294002 for 30 min, washing with PBS twice, and then 10 mM MβCD for 30 min. After washing with PBS, cells were fixed with 4% paraformaldehyde for 30 min and immediately imaged by confocal microscope. For THP-1-derived macrophages, 100 nM wortmannin, 50 μM LY294002, 10 μM U-73122, and 100 μM m-3M3FBS were used.

2.5. Fluorescence staining of PIP_2 and cholesterol

PIP_2 staining was performed according to the manufactory provided protocol. Briefly, the cells pre-treated with or without reagents (OsO_4, wortmannin, m-3M3FBS, and others as indicated in the corresponding figure legends) were fixed with 4% paraformaldehyde for 20 min at room temperature and rinsed with Tris-buffered saline (TBS) three times. The cells were permeabilized with 0.5% saponin for 15 min at room temperature and washed three times with TBS. After the block with 1% BSA (Solarbio) in TBS overnight at 4 °C, the cells were incubated with biotinylated mouse anti-PIP_2 IgM (Echelon Biosciences, UT, USA) at a final concentration of 10 μg/ml in TBS for 60 min at 37 °C followed by TBS washes and subsequent incubation with streptavidin-conjugated Alexa Fluor 555 (Life Technologies, USA) in TBS (1:1000) for 30 min at 37 °C. After rinsing thoroughly with distilled water, the cells were imaged with confocal microscopy.

Cholesterol cell-based detection assay kit (Cayman, USA) was used to fluorescently detect cellular cholesterol. Briefly, the cells pretreated with or without MβCD were fixed with cell-based assay fixative solution for 10 min. After washing with cholesterol detection wash buffer three times, the cells were incubated with filipin solution in the dark for 60 min. After washing with wash buffer two times, the cells were immediately imaged with confocal microscopy.

2.6. Flow cytometry

The treatments and PIP_2 staining of cell samples for flow cytometry were similar to the procedures described above. Flow cytometric acquisition and analysis were performed in a FASCScalibur flow cytometer (BD Biosciences, USA).

2.7. Confocal microscopy

An LSM710 confocal microscope (Carl Zeiss, Germany) was used. All images for observation and measurement of fixation-induced blebs were differential interference contrast (DIC) images and obtained with a Zeiss Plan-Neofluar objective (10×/0.30 or 20×/0.50 or 40×/0.75). The fluorescence images for PIP_2/cholesterol detection were obtained with a 63× oil immersion objective. Occasionally, a FV1000 confocal microscope (Olympus, Japan) with an UPLAPO objective (40×/0.95) was used for PIP_2 imaging.