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Role of flotillins in the endocytosis of GPCR in salivary gland epithelial cells

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ABSTRACT

Endocytosis has numerous functions in cellular homeostasis. Defects in the endocytic pathway of receptors may lead to dysfunction of salivary gland secretion. Therefore, elucidating the complex mechanisms of endocytosis may facilitate solutions for disease treatment and prevention.

The muscarinic type 3 receptor (M3R), a G-protein-coupled receptor (GPCR) located in the plasma membrane, is involved in numerous physiological activities such as smooth muscle contraction and saliva secretion. M3R enters cells through clathrin-mediated endocytosis (CME), while flotillins (flot-1 and -2), highly conserved proteins residing in lipid-raft microdomains, make use of clathrin-independent endocytosis (CIE) for their internalization. Since these two proteins use two discrete pathways for endocytic entry, the association of flotillins with CME is poorly understood.

We examined whether flotillins play a role in CME of M3R using immunoblotting, immunocytochemistry, confocal immunofluorescence microscopy, co-immunoprecipitation, and RNA interference techniques in secretory epithelial cells.

Upon stimulation with a cholinergic agonist, M3R, flot-1, and flot-2 each internalized from the plasma membrane into intracellular sites. The addition of chlorpromazine and cytochalasin D, well-known inhibitors of CME, inhibited internalization of M3R via CME. Filipin III and methyl-β-cyclodextrin (mβCD) acting as lipid raft inhibitors disrupted internalization of flot-1/2 via CIE. Interestingly, filipin III and mβCD slightly reduced expression level of M3R whereas chlorpromazine and cytochalasin D did not affect internalization of the flotillin isoforms. M3R and flot-1/2 colocalized and interacted with each other as they entered the cytosol during limited periods of incubation. Moreover, knockdown of flot-1 or -2 by flotillin-specific siRNA prevented internalization and reduced the endocytic efficiency of M3R. Our results suggest that flot-1 and -2 are partially involved in CME of M3R by facilitating its internalization.

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1. Introduction

Endocytosis has a wide variety of functions in cellular homeostasis, nutrient absorption, drug transportation, and receptor signaling regulation [1]. Malfunctions in endocytosis cause a number of fatal diseases such as cancer, brain diseases, and cardiovascular abnormalities [2–4]. From an exocrinological perspective, defects in the endocytic pathway of receptors lead to dysfunction of salivary gland secretion and may result in autoimmune diseases like Sjogren's syndrome [5,6]. Therefore, elucidating the complex mechanisms of endocytic pathways may facilitate solutions for disease treatment and prevention.

M3-muscarinic receptors (M3Rs) are a type of muscarinic acetylcholine receptor, which collectively include M1 to M5 pharmacological subtypes within G-protein coupled-receptor (GPCR) superfamilies [7]. M3Rs regulate physiological activities in the central and peripheral nervous systems, and are expressed not only in endocrine and exocrine glands, especially the salivary glands, but also in smooth muscles of the blood vessels, lungs, and brain in humans and rats [8–10]. Interaction of M3Rs as cholinergic receptors in parasympathetic nerves are associated with heart rate

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control, neurotransmission, smooth muscle contraction, and secretions from glandular tissues like the salivary glands [11,12]. In response to cholinergic stimulation, M3Rs are responsible for increasing intracellular calcium by binding to G protein to activate PLC (phospholipase C) and through further signaling cascades of second messengers [13].

Flotillins, also known as reggies, consist of two highly conserved isoforms, flotillin-1 (flot-1) and -2 (flot-2) in the flotillin subfamily of SPFH (stomatin, prohibitin, flotillin, HflK/C), which have similar structures and nucleic acid sequences [14]. Mainly found at the plasma membrane (PM), flotillins can also be expressed in lysosomes, endocytic compartments, and the nucleus [15]. In addition to the functions of flotillins in endocytosis and trafficking, they participate in signaling, actin cytoskeleton reorganization, and cellular adhesion [15]. Moreover, flot-1 and flot-2 act as scaffolding proteins for membrane lipid-rafts in lymphocytes, neurons, and other cell types [16]. One of the major characteristics of flotillins is that they are enriched in PM with detergent resistance, and intrinsically possess a tendency to associate with other molecules in lipid-raft microdomains [17].

Upon stimulation with acetylcholine, M3R in the PM in mammalian cells internalizes through clathrin-mediated endocytosis (CME) [18]. The receptor is delivered from the PM to early and late endosomal compartments and finally down-regulated for further signaling, moved to lysosomes for degradation, or recycled back to the membrane [19]. Activation of flotillins leads to creation of membrane curvature and subsequent cell entrance through clathrin-independent endocytosis (CIE) [20]. A number of former studies have suggested that proteins using CIE are unlikely to affect clathrin-mediated endocytic pathways of other proteins since lipid raft proteins are less likely to colocalize with clathrins [15]. On the other hand, there is evidence that components for GPCR signaling are organized in membrane microdomains, especially the lipid rafts in which enriched cholesterol and sphingolipids are located [21].

It has been frequently reported that M3R and flot-1 and -2 undergo endocytosis by two distinct pathways for internalization. However, whether flotillins that conventionally access flotillin-dependent endocytic pathways are implicated in clathrin-mediated endocytosis of GPCR remains unclear. Our study introduces novel evidence for the substantial activity of flot-1/2 during clathrin-mediated endocytosis of M3R in human submandibular gland (HSG) epithelial cells.

2. Material and methods

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Welgene (Gyeongsan, Korea), and penicillin streptomycin was purchased from Gibco (Carlsbad, CA, USA). Carbamylcholine chloride, chloropromazine, filipin, and methyl-β-cyclodextrin were purchased from Sigma Aldrich (St. Louis, MO, USA), and cytochalasin D was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). M3R rabbit and mouse polyclonal antibodies were purchased from Abcam (Cambridge, England). Flot-1 and -2 rabbit polyclonal and mouse monoclonal primary antibodies, Gαq rabbit polyclonal antibody, secondary normal mouse IgG, and HRP-conjugated donkey anti-rabbit IgG were purchased from Santa Cruz Biotechnology Inc. Normal donkey serum and normal goat serum were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA), and goat anti-mouse IgG was obtained from Koma Biotech, Inc., Seoul, Korea. For immunostaining, VECTASHIELD® Mounting Medium with DAPI was purchased from Vector Laboratories (Burlingame, CA, USA). Alexa Fluor 594 (Red)-conjugated donkey anti-mouse IgG secondary antibody and Alexa Fluor 488 (Green)-conjugated goat anti-rabbit IgG antibody were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Transfection and RNAi

HSG cells were either planted in a 60 mm dish for knockdown of clathrin and flot-1/2. Flot-1, -2, or clathrin (Origene, Rockville, MD, USA) siRNAs diluted in Opti-MEM (Gibco) and Lipofectamine™ 2000 reagent with the same medium were mixed and applied to each dish, according to the manufacturer's instructions. Five hours post-transfection, medium in each dish was removed and replenished with complete growth medium to prevent toxicity. After 60 h of transfection, the cells were harvested for further experiments.

2.3. Membrane fractions and Western blotting

The cells were washed with PBS and lysed by sonication with membrane fractionation buffer containing 1 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride ($3 \times 30s$, Branson Sonifer) at pH 7.4. The samples were centrifuged at $600 \times g$ at 4 °C, and the supernatants were again centrifuged at $20{,}000 \times g$ at 4 °C. Membrane and cytosolic protein concentrations were measured by Thermo Scientific Pierce's BCA protein assay kit (Rockford, IL, USA). Both the membrane proteins and corresponding supernatants were resolved by 10% SDS/PAGE. The protein samples were transferred to nitrocellulose membranes (0.45 μm, GE Healthcare) containing bound proteins, blocked with 10% skim milk overnight at 4 °C, and detected with polyclonal antibody specific for M3R at a dilution of 1:1000, polyclonal antibody specific for flot-1/2 at a dilution of 1:8,000, polyclonal antibody specific for Gαq at a dilution of 1:1,000, or polyclonal antibody specific for clathrin HC at a dilution of 1:1,000, followed by HRP-conjugated anti-rabbit or anti-mouse IgG as secondary antibodies. Development of the corresponding protein bands was performed with the ECL substrate kit (Thermo Scientific Pierce, USA).

2.4. Co-immunoprecipitation

Co-immunoprecipitation of flot-1 and flot-2 was performed in EBC buffer solution containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 100 mM NaF, 50 μg/mL PMSF, 10 μg/mL aprotinin, 5 μg/mL leupeptin, 0.2 mM Na_3VO_4, and 100 mM NaF. After lysis by sonication in EBC buffer and centrifugation to separate the membrane and cytosol, protein concentrations were measured with BCA protein assay kit. Cytosolic proteins were pre-cleared with a mixture of normal rabbit IgG and protein A/G PLUS-agarose (Santa Cruz Biotechnology Inc.), according to the manufacturer's instructions. The sample mixture was rotated for 30 min at 4 °C. The isolated supernatants were incubated overnight at 4 °C with rabbit anti-flot-1 or -2 polyclonal antibodies and protein beads. The next day, supernatants were discarded after centrifugation, and the remaining bead-antibody-protein complex was washed using the same lysis buffer. After elution of the complex with 2X sample buffer, the same procedures for Western blotting were carried out.

2.5. Immunostaining

Cells seeded on coverslips were washed with PBS with 0.1% tween-20, and fixed with 4% paraformaldehyde for 10 min at RT. After a blocking step using a PBS-based-blocking solution containing 10% normal goat serum, 10% normal donkey serum, 5% FBS, 2% BSA, and 0.1% Triton X-100, the cells were incubated overnight at 4 °C with M3R or flot-1/2 primary antibodies. After the cells were washed in PBS, fluorescently-labeled secondary antibodies were

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