이학석사 학위논문

타액선 상피세포의 GPCR 내포작용 과정에 관여하는 Flotillin 의 역할

Role of Flotillins in the Endocytosis of GPCR in Salivary Gland Epithelial Cells

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ABSTRACT

Role of Flotillins in the Endocytosis of GPCR in Salivary Gland Epithelial Cells

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The muscarinic type 3 receptor (M3R), a GPCR protein 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 (flotillin-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, flotillin-1, and flotillin-2 each internalized from the plasma membrane into intracellular sites. The addition of chlorpromazine, filipin III, cytochalasin D, and methyl-β-cyclodextrin (mβCD) affected internalization of M3R and flotillin-1/2 via CME and CIE, respectively. Filipin III and mβCD, lipid raft inhibitors, reduced internalization of M3R slightly, whereas chlorpromazine and cytochalasin D, inhibitors of CME, did not affect endocytosis of the flotillin isoforms. M3R and flotillin-1/2 colocalized and interacted with each other as they entered the cytosol during limited periods of incubation. Interestingly, knockdown of flotillin-1 or -2 by flotillin-specific siRNA prevented internalization and reduced the endocytic efficiency of M3R. Our results suggest that flotillin-1 and -2 are partially involved in CME of M3R by facilitating its internalization.

Keywords: endocytosis, GPCR, muscarinic type 3 receptor, flotillin, internalization
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INTRODUCTION

Endocytosis has a wide variety of functions in cellular homeostasis, nutrient absorption, adhesion and migration, drug transportation, receptor signaling regulation, and neurotransmission (Conner and Schmid 2003). Malfunctions in endocytosis cause a number of fatal diseases such as cancer, brain diseases, and cardiovascular abnormalities (Cataldo et al. 2000; Polo et al. 2004; Robertson 2009). 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 (Hayashi 2011; Jin et al. 2012). 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 (Wess 1993). M3Rs regulate physiological activities in the central and peripheral nervous systems (Bonner et al. 1987; Bonner et al. 1988), and are expressed not only in endocrine and exocrine glands, especially the salivary glands, but also in smooth muscles of the blood vessels, lungs, brain, and pancreas in humans and rats (Garssen et al. 1993; Lin et al. ; Preiksaitis et al. 2000; Renuka et al. 2006; Vilaró et al. 1994). Interaction of M3Rs as cholinergic receptors in parasympathetic nerves are associated with heart rate control, neurotransmission, smooth muscle contraction, and secretions from glandular tissues like the salivary glands (Gautam et al. 2004; Kruse et al. 2012). 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 (Wang et al. 2004). More specifically, odd-numbered types of muscarinic receptors (M_1, M_3, M_5) are linked to $G_{q/11}$ for the activation of PLC inducing phosphatidylinositol (PI) turnover, while even-numbered types of muscarinic receptors (M_2, M_4) are coupled to G_i proteins to inhibit adenylyl cyclase (AC) which activates cAMP and PKA phosphorylation (Wang et al. 2004). Current research suggests that muscarinic agonists may resolve a number of clinical diseases such as Alzheimer's disease, schizophrenia, and autoimmune diseases. Muscarinic inhibitors, in contrast, may help treat Parkinson's disease and gastric ulcer (Eglen et al. 1999).

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 (Otto and Nichols 2011). Mainly found at the plasma membrane, flotillins can also be expressed in lysosomes, endocytic compartments, and the nucleus (Glebov et al. 2006). In addition to the functions of flotillins in endocytosis and trafficking, they participate in signaling, actin cytoskeleton reorganization, and cellular adhesion

(Glebov et al. 2006). Moreover, flot-1 and flot-2 act as scaffolding proteins for membrane lipid-rafts in lymphocytes, neurons, and other cell types (Head et al. 2014). One of the major characteristics of flotillins is that they are enriched in plasma membranes with detergent resistance, and intrinsically possess a tendency to associate with other molecules in lipid-raft microdomains (Solomon et al. 2002). For that reason, they can be utilized as protein markers for diseases such as Alzheimer's disease (AD), Parkinson's disease, and Niemann-Pick type C disease (NPC) (Browman et al. 2007; Zhang et al. 2011).

In general, upon stimulation with acetylcholine, M3R in the plasma membrane (PM) in mammalian cells internalizes through clathrin-mediated endocytosis (CME) (Wolfe and Trejo 2007). 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 (Popova et al. 2013). At the same time, activation of flotillins leads to creation of membrane curvature and subsequent cell entrance through clathrin-independent endocytosis (CIE) (Frick et al. 2007). 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 (Glebov et al. 2006). 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 (Chini and Parenti 2004).

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 flotillin-1/2 during clathrin-mediated endocytosis of M3R in human submandibular gland (HSG) epithelial cells. We investigated colocalization and interactions of M3R and flotillin-1/2 for a limited period of time in the course of internalization upon cholinergic stimulation. Both flotillin-1 and -2 affected internalization of M3R, since a lack of flotillins reduced the endocytic efficiency of M3R. However, the absence of M3R is unlikely to influence any steps of the clathrin-independent endocytic pathway of flotillins.

MATERIALS AND METHODS

Reagents, Chemicals, 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 all purchased from Sigma Aldrich (USA), and cytochalasin D was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). M3R rabbit polyclonal antibody and M3R mouse polyclonal antibody were purchased from Abcam (Cambridge, England). Flotillin-1 and -2 rabbit polyclonal and mouse monoclonal primary antibodies, Gαq rabbit polyclonal antibody and secondary normal mouse IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Normal donkey serum and normal goat serum for immunostaining were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). HRP-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology Inc.) and goat anti-mouse IgG (Koma Biotech, Inc., Seoul, Korea) were used as secondary antibodies. For immunostaining, nuclei were stained with VECTASHIELD® Mounting Medium with DAPI (4',6-diamidino-2-phenylindole) obtained from Vector Laboratories (Burlingame, CA, USA). M3Rs were labeled with Alexa Fluor 594 (Red)-conjugated donkey anti-mouse IgG secondary antibody from Invitrogen (Carlsbad, CA, USA), and flotillin-1 and -2 were labeled with Alexa Fluor 488 (Green)-conjugated goat anti-rabbit IgG antibody from Invitrogen (USA).

Cell Culture

The HSG cell line was cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin streptomycin at 37°C in a humidified incubator containing 5% CO_2. For chemical treatments and confocal microscopy experiments, HSG cells were grown on 100- or 60-mm dishes and glass coverslips in 35-mm dishes. General subcultures at about 90% confluence were routinely split 1:3. Before chemicals were applied to cells, culture medium in each dish was discarded and cells were washed twice with PBS.

Transfection and RNAi

For knockdown of clathrin and flotillin-1/2, HSG cells were either planted in a 60-mm dish for cell scraping or on glass coverslips for immunostaining, and were grown to 70% confluence before transfection. Flotillin-1, -2 (Santa Cruz Biotechnology Inc., USA), or clathrin (Origene, Rockville, MD, USA) siRNAs

diluted in Opti-MEM obtained from Gibco (USA) and LipofectamineTM 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 hours of transfection, the cells were harvested for further experiments.

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 0.3mM phenylmethylsulfonyl fluoride (3×30s, Branson Sonifer) at pH 7.4. The samples were centrifuged at 600 xg at 4°C, and the supernatants were again centrifuged at 20,000 xg 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 and western blotting. The protein samples were transferred to nitrocellulose membranes (0.45 μm, GE Healthcare) containing bound proteins, blocked with 10% skim milk for three hours at RT or overnight at 4°C, and detected with polyclonal antibody specific for M3R at a dilution of 1:1000, polyclonal antibody specific for flotillin-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 (enhanced chemiluminescence) substrate kit (Thermo Scientific Pierce, USA).

Co-immunoprecipitation

Co-immunoprecipitation of flotillin-1 and flotillin-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 scraping of the samples, lysis by sonication in EBC buffer, and centrifugation to separate the membrane and cytosol, protein concentrations were measured with the BCA protein assay kit. Cytosolic proteins were pre-cleared with a mixture of normal rabbit IgG and protein A/G PLUS-agarose obtained from Santa Cruz Biotechnology Inc. (USA), according to the manufacturer's instructions. The sample mixture was rotated for 30 minutes at 4°C. After centrifugation, the isolated supernatants were incubated overnight at 4°C with rabbit anti-flotillin-1 or -2 polyclonal antibodies and the protein beads. The next day, supernatants were discarded after centrifugation, and the remaining bead-antibody-protein complex was washed seven times using the same lysis buffer. After elution of the complex with 2X sample buffer, the same procedures for Western blotting were carried out and of the bands were detected using the ECL substrate kit.

Immunostaining and Confocal Microscopy Assays

Cells seeded on coverslips coated with poly-L-ornithine were washed with PBS with 0.1% tween-20, and fixed with 4% paraformaldehyde for 10 minutes at room temperature. 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 flotillin-1/2 primary antibodies. After the cells were washed three times in PBS, fluorescently-labeled secondary antibodies were attached and incubated for one hour in the dark. M3Rs were labeled with Alexa Fluor 594 (Red)-conjugated donkey anti-mouse IgG at a dilution of 1:100 followed by labeling of flotillin-1 or -2 with Alexa Fluor 488 (Green)-conjugated goat anti-rabbit IgG at a dilution of 1:200. Sample coverslips on slides were mounted with DAPI and visualized using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Jena, Germany).

Statistical Analysis

Densities of the immunoblotted bands were quantified using ImageJ software, and statistical data for diagrams and graphs were analyzed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). All data are presented as the mean ± SEM from experiments performed in triplicate. P-values less than 0.05 were considered significant and are marked with an asterisk.

RESULTS

Dose-dependent internalization of M3R

First, I compared the effect of carbamylcholine chloride (CCh) concentrations ranging from 10 μM to 1 mM on M3R endocytosis in human submandibular gland (HSG) cell line to figure out the ideal concentration of the CCh acting as an agonist for internalization for the following experiments. 1 mM of carbachol group represents a greater reduction in expression level of the M3R membrane internalization than any other groups (Fig. 1A & B). Therefore, I have chosen 1 mM of carbachol as the standard concentration for stimulating HSG cells.

Internalization of M3R and flotillin-1/2

M3R, flotillin-1, and flotillin-2 are proteins primarily expressed on the plasma membrane (PM) in secretory epithelial cells. To biochemically observe temporal patterns of internalization of M3R and flotillin isoforms at the membrane and in the cytosol after stimulation with a cholinergic agonist, HSG cells were incubated with 1 mM of carbamylcholine chloride (CCh) for the specified time periods, and expression of M3R and flotillin-1/2 in the membrane and cytosolic fractions was analyzed by immunoblotting.

In order to eliminate probable alternative stimulants or other possibilities that could cause internalization of M3R and flotillin-1/2, cells were also incubated in serum-free media without application of agonists in a time-dependent manner. Expression of M3R at the PM did not show any remarkable changes for two hours (Fig. 2A upper). Similarly, neither flotillin-1 nor flotillin-2 exhibited noticeable declines in expression at the PM (Fig. 2A center & bottom). Collectively, M3R and flotillin-1 and -2 were not internalized without agonist stimulation.

Aspects of the internalization for M3R, flotillin-1, and -2 were investigated by incubating the cells with CCh as a stimulant for indicated time periods. There was no significant change in the expression of M3R for the first 15 minutes of CCh stimulation. However, 30 minutes after agonist stimulation, expression of M3R at the PM began to gradually decrease over time (Fig. 2B upper). Stimulation of HSG cells resulted in significant loss of cell surface receptors of M3R up to an hour. In contrast, two hours after CCh addition, expression of M3R that had translocated to the cytosol seemed to return to the PM. Expression of M3R in the cytosol was almost inversely proportional to that of M3R at the PM (Fig. 2C & D). In concert, flotillins also internalized from the PM into the cytosol in response to agonist stimulation, in the same time-dependent manner shown in Fig. 2A (Fig. 2B middle & bottom). 15 minutes after CCh stimulation, the distribution of flotillin-1 and -2

between the membrane and cytosol began to change. Like M3R, flotillin-1 and -2 showed a lack of translocation from the membrane to the cytosol until 15 minutes after addition of CCh. In contrast to the trend of M3R internalization, however, expression of flotillin-1 and-2 at the cell surface continued to decrease for up to two hours after CCh stimulation (Fig. 2B & C). Collectively, these results indicate that M3R and flotillin-1 and -2 distinctively internalize upon agonist stimulation, yet flotillin-1 and -2 tend to exhibit characteristics similar to endocytosis. Furthermore, the degree of reduction in M3R expression at the PM was more dramatic than that of the flotillins. Over time, M3R seemed to recycle back to the plasma membrane after two hours of agonist stimulation while flotillin-1 and flotillin-2 continued to internalize. Gαq was used as the negative control, since it does not internalize from the PM to intracellular sites (McConalogue et al. 1998).

The endocytic pathways of internalization for M3R and flotillin-1/2

Biochemical approaches were utilized to identify the nature of discrete endocytic pathways of M3R and flotillin isoforms. In order to re-confirm that the M3R internalizes via clathrin-mediated endocytosis (CME) and that flotillin-1/2 internalize through clathrin-independent endocytic pathways (CIE) in secretory epithelial cells, several inhibitors for two different endocytic pathways were applied.

Stimulation with agonist 1 mM CCh for 60 minutes diminished the expression of M3R and flotillin-1 and -2 at the PM (Fig. 3A-D). Application of 1 mM of CCh with 10 μM of chlorpromazine (CPZ), an inhibitor of CME by deactivating recruitment of clathrin and AP2 adaptor (Vercauteren et al. 2010), inhibited the internalization of M3R, but exerted no significant effects on flotillins, which continued internalization (Fig. 3A & E). Endocytosis of M3R was also inhibited by cytochalasin D (cyto D), a drug that destabilizes actin filaments and eventually prevents CME (Itoh et al. 2008). However, this inhibitory effect, which drastically reduced M3R internalization, showed no significant influence on flotillin-1/2 internalization (Fig. 3B & E). Meanwhile, treatment with 15 μM filipin III (FLPIII), a lipid-raft inhibitor that interacts with cholesterol and modifies properties of cholesterol-enriched regions (Boucrot et al. 2015), and simultaneous addition of CCh to HSG cells resulted in almost complete inhibition of endocytosis of the flotillin isoforms. Interestingly, decreased M3R internalization was observed after addition of FLPIII and CCh at the same time compared to the loss of M3R at the PM with CCh treatment alone for 60 minutes (Fig. 3C). These results deviated from the prediction that the internalization of the M3R would be unaffected by lipid-raft inhibitors. In addition, application of 10 mM methyl-β-cyclodextrin (mβCD), another lipid-raft inhibitor (Colin et al. 2011), induced inhibition of M3R internalization much like filipin III (Fig. 3D), while endocytosis of flotillins was disrupted by mβCD, as expected.

Taken together, these findings indicate that internalization of flotillin-1 and -2 was only affected by inhibition of typical lipid-raft inhibitors FLPIII and mβCD, whereas inhibition of lipid-raft microdomains against GPCRs could possibly have slight inhibitory effects on internalization of M3R in secretory epithelial cells (Fig. 3E).

Interaction of M3R with flotillin-1 or flotillin-2

In previous experiments, we observed that M3R in HSG cells internalized through CME upon the addition of an agonist. Next, we tested whether flotillins could also take part in the endocytic pathway of M3R. Confocal microscopy was used to verify relevant regional clues between M3R and flotillin-1 or -2, both of which do not appear to correlate to one another as they are conventionally known to internalize via different endocytic pathways. Intracellular localization and association of M3R and flotillins were studied by immunostaining and co-immunoprecipitation during the course of CCh stimulation in HSG cells (Fig. 4).

In untreated cells, M3R slightly colocalized with flotillin-1 at the PM (Fig. 4A). However, significant colocalization of M3R with flotillin-1 was observed in cytosolic regions of the cells 30 minutes after agonist administration. An hour after CCh application, areas of M3R and flotillin-1 overlapped at the intracellular site and represented a similar intensity of colocalization compared to those following 30-minute agonist stimulation (Fig. 4A). Similar colocalization was seen for M3R and flotillin-2 as well (Fig. 4B). M3R barely colocalized with flotillin-2 in unstimulated cells. Fifteen minutes post-carbachol addition, M3R sparsely colocalized with flotillin-2. However, as M3R entered the intracellular site 30 minutes after CCh addition, the two proteins began to colocalize to one another. After one hour of CCh exposure, substantial colocalization of the M3R and flotillin-2 was also observed (Fig. 4B). This observation was concordant with previous data demonstrating M3R colocalization with flotillin-1. These findings confirm a correlation of the internalization of M3R with flotillins.

Next, we evaluated possible interactions between M3R and flotillin-1 or -2 by co-immunoprecipitation (co-ip) to examine whether M3R was internalized together with flotillins. Co-ip of M3R with flotillins from the cytosol in HSG cells was tested in a time-dependent manner after CCh stimulation (Fig. 4C & D), and 10% input was used as the control group. Flotillin-1 did not co-immunoprecipitate with M3R from the cytosol in unstimulated cells, since its expression was not clearly observed. From 15 minutes to 60 minutes after agonist addition, a prominent expression in co-ip of M3R with flotillin-1 and -2 from the cytosol was observed (Fig. 4C & D). M3R and flotillin-2 did not actively interact until after 15 minutes of stimulation with CCh. The association level of the two proteins peaked around 30 minutes after CCh addition (Fig. 4D). These experiments support the view that the carbachol-evoked involvement of flotillins in clathrin-mediated endocytosis is followed by an

association with M3R internalization and those interactions somewhere between 30 to 60 minutes post-stimulation.

Knockdown of flotillin-1 or -2 by siRNA reduces the clathrin-mediated endocytic activity of the M3R

Since M3R colocalized with flotillin-1/2 and they interacted in HSG cells after specific periods of time, we further investigated whether the internalization of M3R in the absence of flotillin families could result in different aspects of internalization activity. Expression of flotillin-1 or -2 was knocked down in HSG cells by RNA interference using siRNA (Fig. 5A & B) The siRNA control group, CCh-treated group, and CCh with siFlotillin-1/2 groups were compared to determine whether flotillin-1/2 could be cargo proteins that affect efficiency of M3R endocytosis in secretory epithelial cells.

As seen in Fig. 5A, stimulation with CCh for only 60 minutes resulted in decreased M3R expression, much like that seen in Figures 2 and 3. On the other hand, knockdown of flotillin-1 in the presence of CCh attenuated the diminishing expression of M3R, resulting in a reduction of the agonist-evoked internalization of M3R (Fig. 5A). In the same way, 1 mM of CCh was added to HSG cells after knockdown of flotillin-2. Untreated cells and those treated with CCh or siRNA-control exhibited no significant changes as compared to Fig. 5A. However, knockdown of flotillin-2 induced a substantial increase in the membrane level of M3R, compared to the level of M3R expression in the CCh-added group (Fig. 5B). In conclusion, CCh stimulation with siFlotillin-1/2 RNA interference-treated cells yielded a significant decrease in the endocytic efficiency of M3R (Fig. 5D). Flotillin-2 knockdown with the agonist caused similar recovery-like effects on reduced M3R expression in the PM due to diminished efficiency of M3R internalization (Fig. 5A, B, & D).

In the final analysis, internalization of flotillin-1/2 in the absence of clathrin was tested using siRNA transfection and Western blotting, in order to examine whether GPCR affects the internalization of proteins that utilize flotillin-dependent endocytosis. Clathrin heavy chain (HC) was knocked down by three types of siRNA: duplexes A, B, and C (Origene, USA), where duplex A had the highest efficiency in the knockdown of clathrin HC in M3R (Fig. 5C upper). As expected, there was no remarkable change in untreated cells, scrambled siRNA controls, and in the CCh-treated group. Moreover, knockdown of clathrin HC did not affect the internalization of flotillin-1 or -2 (Fig. 5C lower). These results support speculation that the internalization of agonist-activated flotillin families may partly involve CME of M3R as an intermediate state during the course of M3R internalization in secretory cells, whereas the absence of clathrin did not influence endocytosis of flotillins.

Figure 1

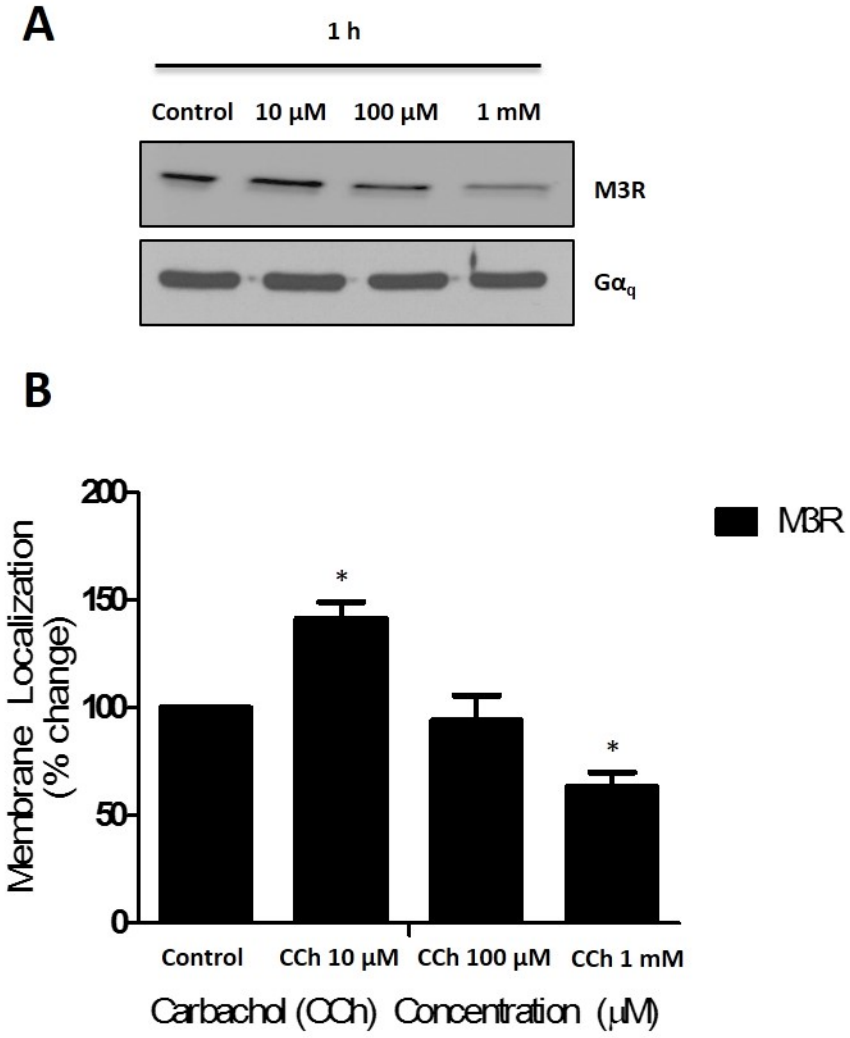


Fig 1. Dose-dependent internalization of M3R. (A) The effect of varying concentration of CCh on internalization of M3R. The HSG cells were incubated for 1 hour with indicated carbachol concentrations. 30 µg of M3R membrane protein for each lane was subjected to SDS-PAGE and immunoblotting with anti-M3R. The different intensities of the bands show that the endocytosis of M3R membrane protein is dose-dependent. Gαq was used as control groups. (B) The densitometry

analysis shows the different expression levels of M3R membrane protein depending on the various carbachol concentrations. 1 mM of carbachol concentration group represents the greatest reduction in expression level of the M3R membrane internalization in the group, and this concentration value was applied as a standard concentration for stimulating cells in further experiments. Three independent experiments were performed with similar results.

Figure 2

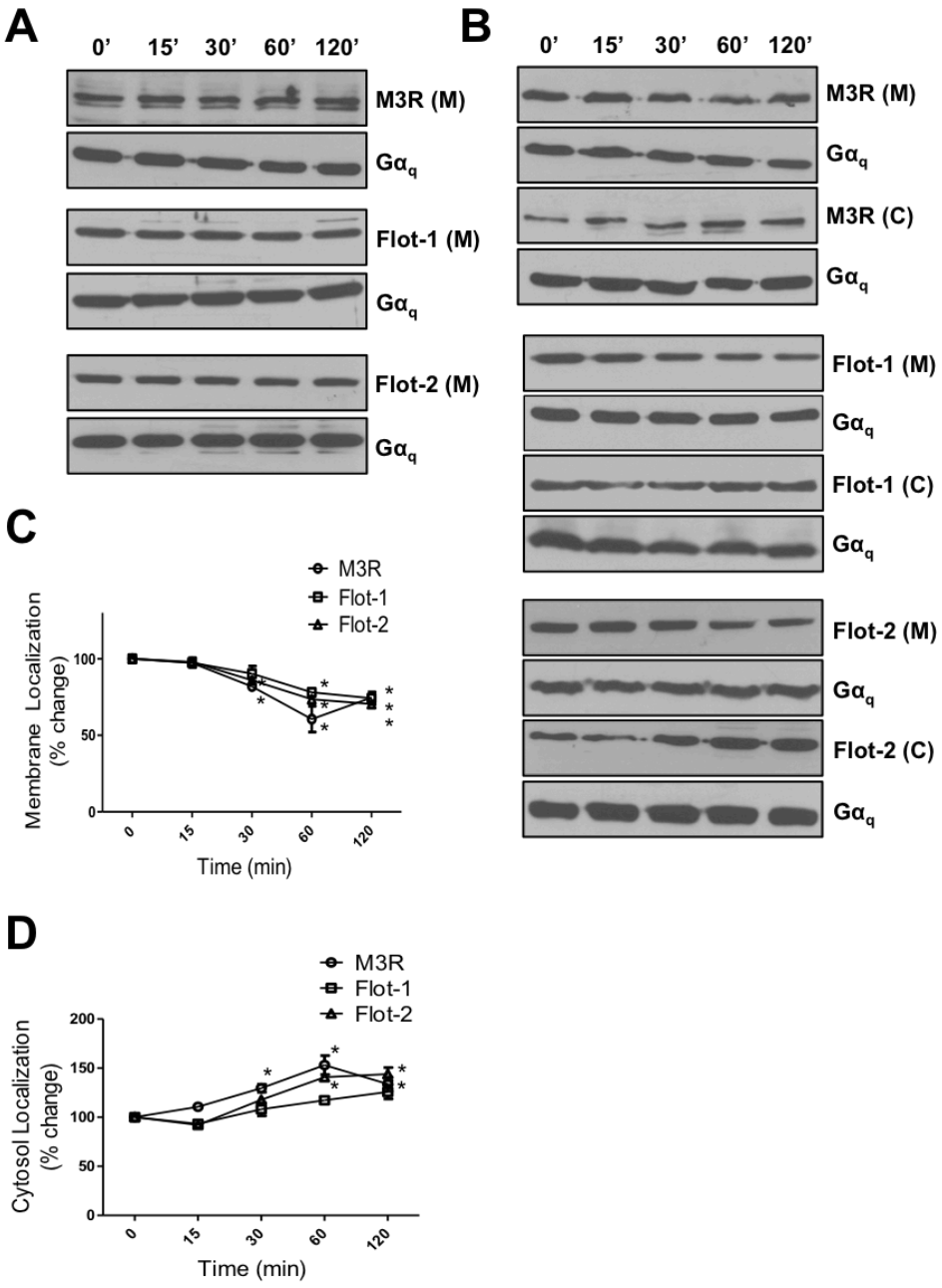

A
0’ 15’ 30’ 60’ 120’
M3R (M)
Gαq
Flot-1 (M)
Gαq
Flot-2 (M)
Gαq
B
0’ 15’ 30’ 60’ 120’
M3R (M)
Gαq
M3R (C)
Gαq
Flot-1 (M)
Gαq
Flot-1 (C)
Gαq
Flot-2 (M)
Gαq
Flot-2 (C)
Gαq
C
M3R
Flot-1
Flot-2
Membrane Localization (% change)
100
50
0
0 15 30 60 120
Time (min)
D
M3R
Flot-1
Flot-2
Cytosol Localization (% change)
200
150
100
50
0
0 15 30 60 120
Time (min)

Fig 2. Upon agonist stimulation, M3R and flotillin-1/2 are internalized from plasma membrane into intracellular sites in proportion to the indicated incubation times. (A) Endocytosis of the M3R and flotillin-1/2 in the serum-free medium without CCh stimulation. All types of protein in each group did not internalize in the absence of agonist stimulation. Membrane and cytosol fractions were subjected to SDS/PAGE (50 μg of membrane protein in each lane) and western blotting with anti-M3R, -flotillin-1, -flotillin-2, and Gαq, followed by the experimental procedures. Gαq was used as control groups since it remained unchanged both at the membrane and in the cytosol. (M) and (C) stand for membrane and cytosol, respectively. **(B)** HSG cells were incubated with 1 mM CCh for the indicated periods of time, as described. Expression of the internalization of M3R, flotillin-1, and -2 at the plasma membrane in proportional to the incubation time, and at the cytosol in inversely proportional to the incubation time, measured by immunoblottting. **(C & D)** Change of membrane and cytosolic localization for M3R and flotillin-1/2 at the plasma membrane, and in the cytosol, respectively. Three independent experiments were performed with similar results. Two-way ANOVA was used for statistical analysis, representing * $P < 0.05$.

Figure 3

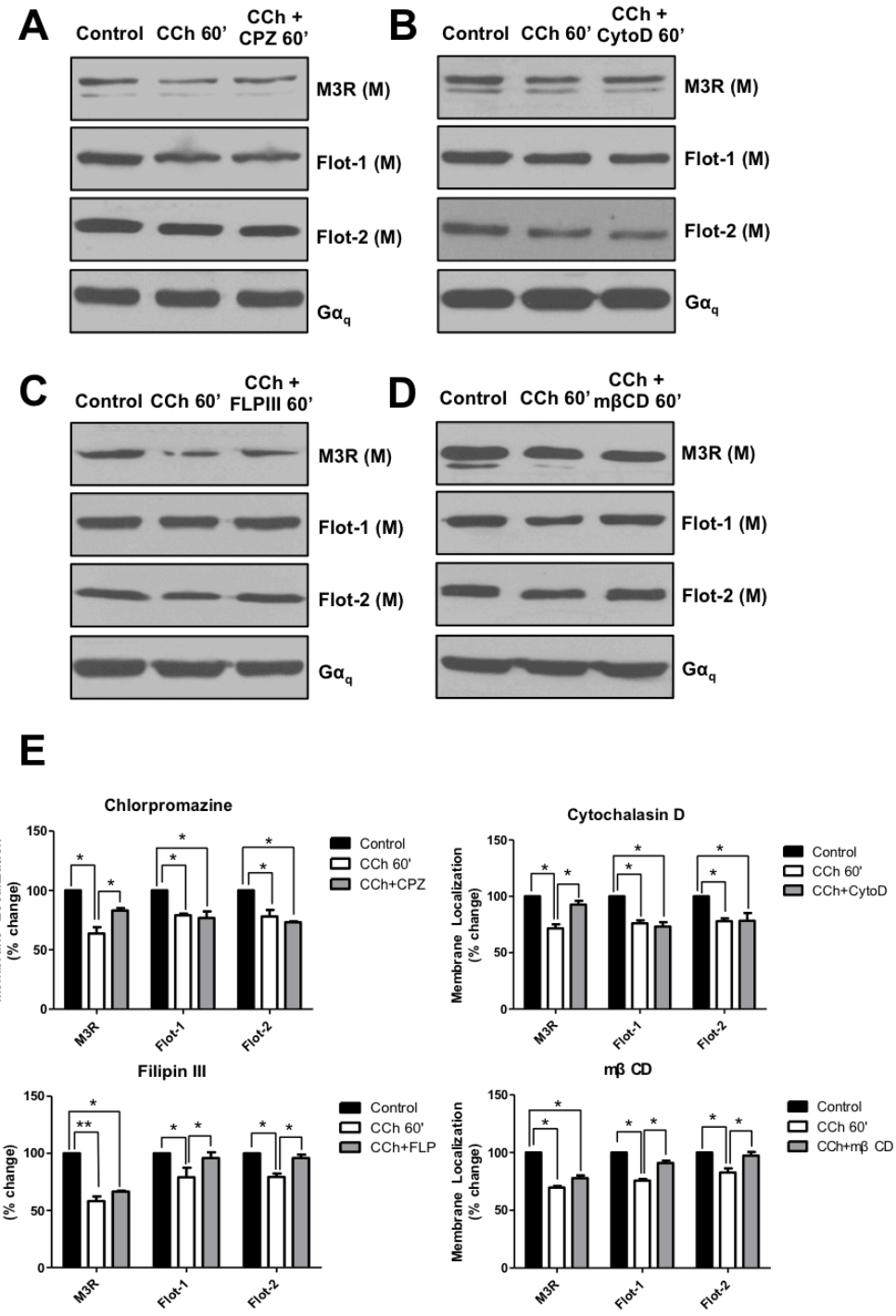
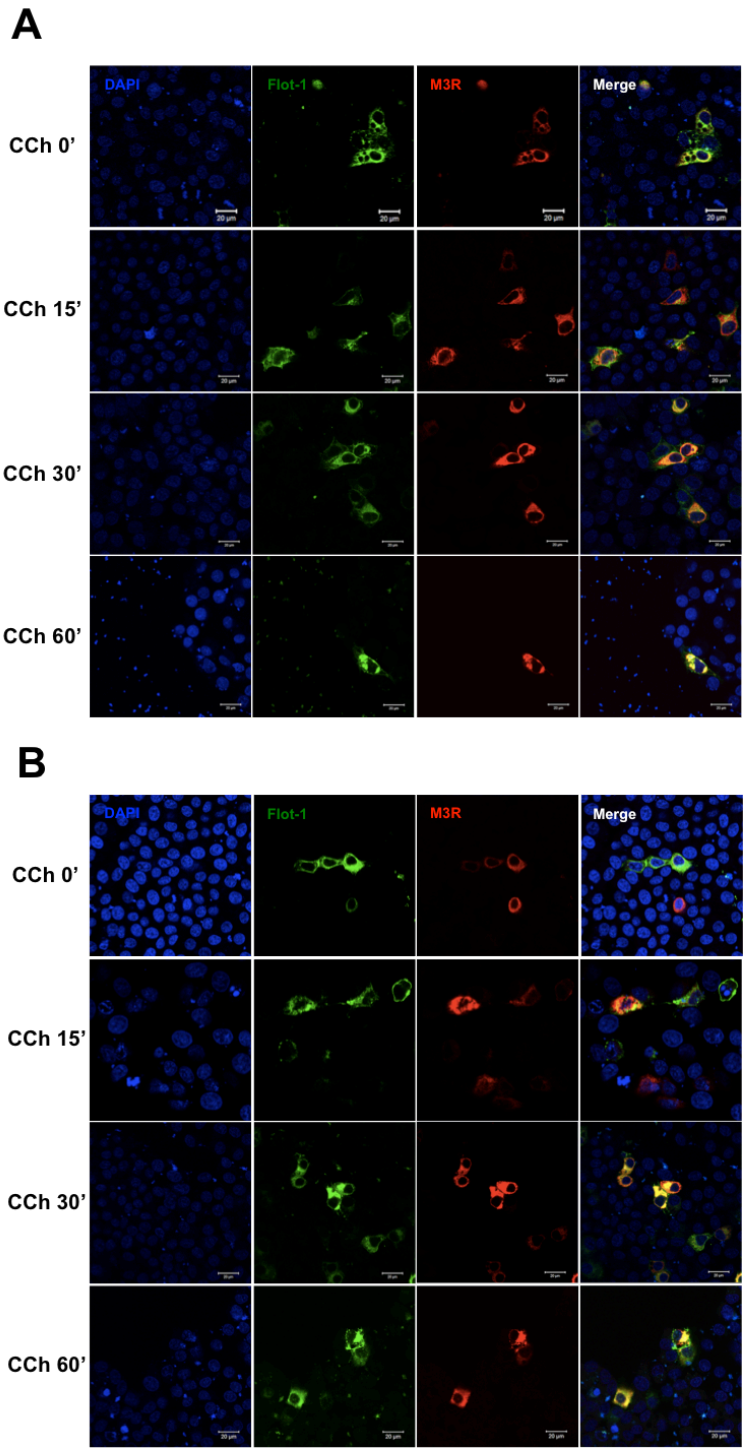

A
Control
CCh 60'
CCh + CPZ 60'
M3R (M)
Flot-1 (M)
Flot-2 (M)
Gαq
B
Control
CCh 60'
CCh + CytoD 60'
M3R (M)
Flot-1 (M)
Flot-2 (M)
Gαq
C
Control
CCh 60'
CCh + FLPIII 60'
M3R (M)
Flot-1 (M)
Flot-2 (M)
Gαq
D
Control
CCh 60'
CCh + mβCD 60'
M3R (M)
Flot-1 (M)
Flot-2 (M)
Gαq
E
Chlorpromazine
Membrane Localization (% change)
Control
CCh 60'
CCh+CPZ
M3R
Flot-1
Flot-2
Cytochalasin D
Control
CCh 60'
CCh+CytoD
Filipin III
Control
CCh 60'
CCh+FLP
mβ CD
Control
CCh 60'
CCh+mβ CD

Fig. 3. Chemical treatments of chlorpromazine, filipin, cytochalasin D, and methyl-β-cyclodextrin represent internalization of the M3R and flotillin-1/2 via CME and CIE, respectively. Expression of the M3R, flotillin-1, and flotilin-2 internalization upon stimulation of 1 mM CCh without or with various antagonists. Cells in control group were unstimulated. CCh 60' stands for the incubation of the cells in the presence of CCh for one hour. After membrane fractions, 50 μg of membrane protein for each lane were subjected to SDS-PAGE and immunoblotting with anti-M3R, antil-flotillin-1, and anti-flotillin-2. **(A)** CCh + CPZ 60' indicates the incubation of cells for one hour using 1 mM CCh and chloropromazine, an inhibitor of the CME **(B, C, & D)** CCh + Cyto D (inhibitor of CME), CCh + FLP III (inhibitor of lipid-raft), and CCh + mβCD (inhibitor of lipid-raft) incubated in HSG cells for one hour. **(E)** Statistical graph by densitometry for membrane expressions of the M3R and flotillins. The results are the means ± S.E.M of three independent experiments. Immunoreactive bands were quantified by Image J and statistically analyzed by two-way ANOVA through GraphPad Prism 5. Asterisks above the bar * $P < 0.05$ compared to the control group.

Figure 4


A
DAPI
Flot-1
M3R
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CCh 0'
CCh 15'
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CCh 30'
CCh 60'

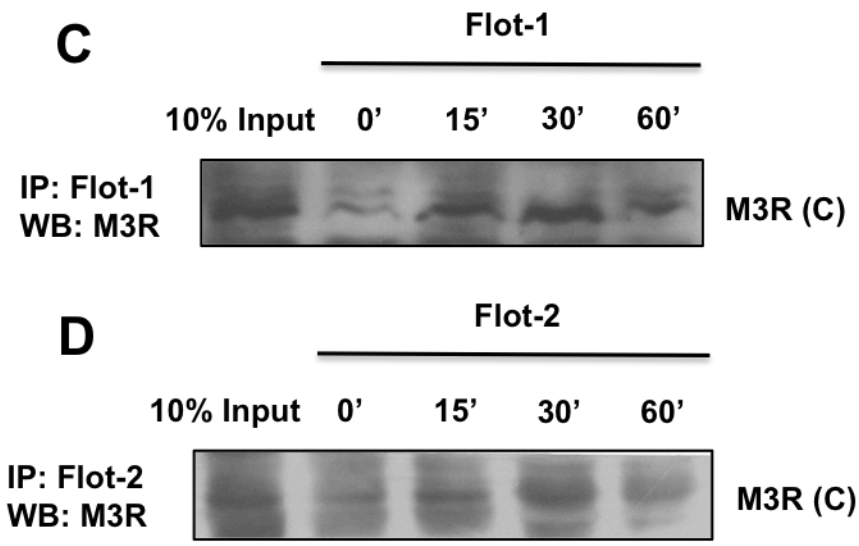

Fig. 4. M3R co-localized and interacted with flotillin-1 and -2 for specific periods of time after internalization by stimulation of carbachol in HSG cells. Cells were treated with 1 mM CCh for the indicated periods before fixation and immunostaining. Confocal micrographs of untreated (0 min) and CCh-treated (15, 30, and 60 min) cells are shown. Three individual experiments with similar results were performed. **(A)** Co-localization of M3R and flotillin-1. Flotillin-1 appears in green and M3R in red. Areas of M3R/flotillin-1 co-localization appear in light orange/yellow. **(B)** Co-localization of M3R and flotillin-2. Flotillin-2 appears in green and M3R in red. Areas of the M3R-flotillin-2 co-localization are seen in yellow. In unstimulated cells, M3R and flotillin-1 are barely overlapped to each other at the plasma membrane. Note their intracellular co-localization increasing at 30~60 minutes after CCh addition. **(C)** Co-immunoprecipitation (co-IP) for M3R and flotillin-1 or -2. HSG cells were subjected to co-IP with 10% input (control), anti-flotillin-1 or anti-flotillin-2, and anti-M3R antibodies. IPs were subjected to immunoblotting.

Figure 5

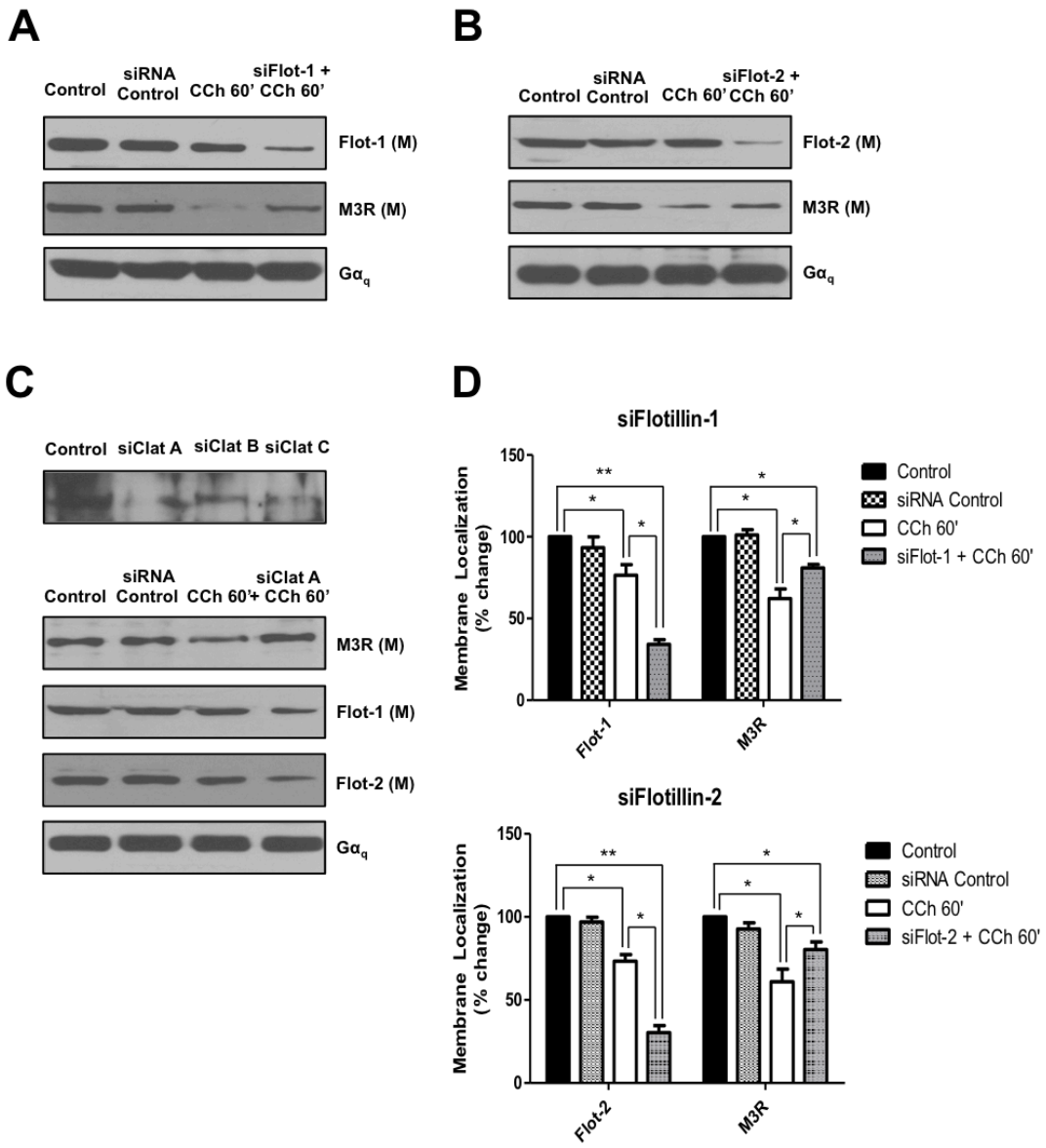

Fig. 5. Knockdown of flotillin-1 or -2 by siRNA reduces the clathrin-mediated endocytic activity of the M3R. HSG cells were transfected with siRNA duplexes (Origene) targeting flotillin-1 or -2 for 60 h post-transfection with final

concentration of 10 nM. Control siRNA denotes the universal scrambled negative control siRNA duplexes. **(A)** Internalization of M3R in absence of flotillin-1. Immunoreactive bands represent membrane protein of the M3R after CCh stimulation for one hour, and flotillin-1 and -2 with successful knockdown. **(B)** Internalization of M3R in absence of flotillin-2. **(C)** A representative immunoblotting bands of the M3R and flotillin-1/2 internalization under the condition in which the clathrin heavy chain (clathrin HC) functions have been lost in the cell. **(D)** Densitometries for the statistical analyses of the M3R internalization in the absence of flotillin isoforms. The results are the means ± S.E.M of three individual experiments with similar results. * $P < 0.05$ compared to the control group.

DISCUSSION

Receptors like GPCRs in the plasma membrane take part in diversified pathways and in a great deal of coordinated interactions between other kinds of proteins and lipids. Moreover, several kinds of transmembrane proteins have been implicated in lipid rafts and are involved in the regulation of myriad cellular functions (Barnett-Norris et al. 2005). Recent papers suggest that flotillins may act as adaptor proteins for endocytosis of cargo proteins like NPC1L1 and DAT receptors (Cremona et al. 2011; Ge et al. 2011). Such transmembrane proteins may interact with flotillins during their trafficking and internalization from the plasma membrane to the cytosol. However, it has been unclear whether other types of GPCRs such as M3R may also be affected by adaptor proteins mediated by different endocytic pathways other than CME.

Through this current research, we found that flotillin isoforms residing in lipid-raft microdomains possess the possibility to associate with M3R internalization in response to agonist stimulation, and influence the endocytic efficiency of the M3R in secretory epithelial cells. The observed effects of lipid-raft inhibitors on M3R internalization, co-localization and interaction between M3R and flotillins, and aggregation of the endocytic efficiency of M3R in the absence of flotillins all support our observations.

To observe the internalization pattern of M3R and flotillin-1/2, HSG cells were treated with CCh in a time-dependent manner. Expression of M3R and flotillins at the membrane and in the cytosol were measured in order to investigate the movement of each protein depending on incubation time. According to experiments in which the cells were treated in serum-free medium without an agonist to eliminate any other possible variables that could cause internalization of the two proteins, PM expression of M3R and flotillin-1/2 was not quantitatively different for two hours of incubation, demonstrating that internalization of M3R and flotillin-1/2 were only induced by cholinergic agonists in a dose- and time-dependent fashion (Fig. 2A).

Expression of M3R in the cytosol inversely increased against M3R expression in the plasma membrane. Flotillin-1 and -2 internalization behaved similarly (Fig. 2A). The alteration of expressional changes of the two proteins between the PM and cytosol represents the tendency of their movement at the cellular level. The explicit differences between M3R and flotillin-1/2 internalization from the plasma membrane to the cytosol was that the expression level of M3R increased back at the plasma membrane two hours after CCh treatment, while the flotillins continued to move into the cytosol, even after two hours. This distinction may be due to different recycling systems or differences among cell types, since flotillins do not recycle back to the PM until at least two hours of agonist incubation.

Some types of GPCR exist as preassembled complexes in lipid-raft microdomains or in cholesterol-rich regions, where they can be recruited and are ready for internalization without agonists (Ostrom and Insel 2004). In contrast, some other GPCRs cannot be assembled in lipid-rafts without agonist stimulation. If cholesterol-rich regions are not working or are inhibited by antagonists, proteins such as flotillins, originally enriched in lipid-raft microdomains, would not function appropriately and assist CME in the lipid-raft regions, leading to malfunction of CME of the GPCR. For that reason, we chose inhibitors (filipin III and mβCD) for flotillin isoforms to observe whether GPCRs such as M3R could fully internalize even if the lipid-raft regions or flotillin-enriched regions were functionally inhibited. Conversely, we confirmed that the internalization of flotillins would not be disrupted by CME inhibitors (chlorpromazine and cytochalasin D) in secretory epithelial cells.

Collectively, internalization of flotillin-1 and -2 was only affected by inhibition of the typical lipid-raft antagonists, whereas inhibition of the lipid-raft microdomains toward GPCR remain possible influences with slight inhibitory effects on internalization of M3R (Fig. 3E). Moreover, the newly discovered aspects of the relationship between M3R internalization and lipid-raft inhibitors led us to further experiments to elucidate the complexity.

Flotillins appeared to partially participate in CME of M3R, perhaps beginning through their mutual interaction beneath the PM regions and continuing to colocalize in the cytosol for specific moments. M3R colocalization with flotillins in HSG cells was studied by confocal microscopy, where variations in the intensity of merged colors between M3R with flotillin-1 or -2 revealed how their colocalization visually differed over four subsequent CCh incubation periods. We discovered transient colocalization of M3R with flotillin-1 or -2 in the cytosol at around 30~60 minutes post-agonist stimulation (Fig. 4A & B). Data from immunoblotting after co-immunoprecipitation also demonstrated that M3R interacted with flotillin-1 for up to an hour after CCh stimulation (Fig. 4C). Flotillin-2 also co-immunoprecipitated with cytosolic M3R, especially 30 and 60 minutes after carbachol stimulation, although it did not co-immunoprecipitate with M3R in untreated cells (Fig. 4D). The evidence indicates that M3R and flotillin-1/2 somewhat colocalized and interacted with each other for approximately 30 minutes, and inconsistently so. Such results support the endocytic aspects of M3R and flotillins shown in Fig. 2, such that the expression levels of these two proteins in the PM were gradually reduced together up to an hour, providing additional clues that they might have different fates after up to two hours.

Flotillins might mediate M3R internalization through their interactions for limited periods of time. Although flotillin-1/2 did not apparently participate in the mechanism of CME, both actually colocalized and interacted with M3R, as seen in Fig. 4. Based on the data, we suggest that flotillins might act as assistant proteins or

adaptor proteins such as AP2, epsins, and β-arrestins to aid clathrin-coated pit creation of M3R (Meister et al. 2014; Wendland 2002). Further M3R internalization may depend on flotillins, which can be especially essential in endocytic mechanisms within lipid-raft regions. According to our flotillin knockdown experiments, the endocytic efficiency of M3R internalization in the absence of flotillin-1 or flotillin-2 was not as dynamic as that of M3R internalization with flotillins. The paradox between the fact that flotillins lack colocalization with clathrin, yet still act as a component affecting the endocytic efficiency of M3R could be due to multiple alternate endocytic pathways of M3R (Doherty and McMahon 2009). Flotillins in lipid-raft microdomains may be detected by M3R that directly binds to cholesterol, and M3R may attempt to recruit flotillins to clathrin-coated pits to form M3R-flotillin complexes and lead to internalization. However, it is well known that the possibilities of interaction or coexistence between clathrin, GPCRs, and flotillins are insufficient to predict strong relationships between the two discrete proteins during internalization. Furthermore, the technique of RNA interference was inadequate to conclusively evaluate our hypotheses due to imperfect loss-of-function of flotillins to CME of M3R. Accordingly, we cannot rule out other scenarios where M3R and flotillins may not interact with each other. Additional data are needed to show that flotillins are recruited to the clathrin-coated pits of M3R at specific locations and times before they form a complex and are ready to internalize. For example, we need further experiments to support additional significant interactions of flotillins with adaptor proteins like AP2 rather than just clathrins. Moreover, observing flotillin internalization in the absence of M3R would also help identify more specific relationships between the two protein types in secretory epithelial cells.

Scrutinizing such novel endocytic mechanisms that represent potential interventions and the effect of adaptor proteins such as flotillins to clathrin-mediated endocytosis of M3R may help further unravel mechanisms of CME associated with additional proteins beyond flotillins. Such findings can contribute to a better understanding of receptor internalization from a physiological and pharmaceutical perspective.

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국문초록

타액선 상피세포의 GPCR 내포작용 과정에 관여하는 Flotillin 의 역할

Muscarinic type 3 receptor (M3R) 는 세포막에 존재하는 GPCR 단백질 종류의 하나이다. M3R 은 평활근의 수축이나 타액 분비와 같은 여러가지의 생리학적 현상에 관여하는 물질로 알려져 있다. 이 수용체는 clathrin 조절에 의한 내포작용 (CME, clathrin-mediated endocytosis) 에 의해 세포의 내부로 진입하는 과정을 거친다. 한편 flotillin-1 과 flotillin-2 는 lipid-raft microdomain 에 위치하는 단백질로서, scaffolding 단백질로도 잘 알려져 있는 물질이다. 이들은 clathrin 조절에 의존하지 않는 별도의 내포작용 (CIE, clathrin-independent endocytosis) 을 통해 세포 내부로 유입된다. 이처럼 M3R 과 flotillin 은 서로 전혀 다른 내포작용 기전들에 의해 세포 안으로 들어가기 때문에, M3R 내포 작용 과정에서의 flotillin 역할에 대해서는 명확히 알려져 있지 않다. 따라서 clathrin 에 의해 조절되는 M3R 의 내포 작용에서 flotillin 이 관여를 하는지, 또 어느 정도 규모의 영향을 주는지를 밝히는 과정이 본 연구의 목적이다.

M3R 의 내포 작용 안에서 flotillin 이 어떠한 역할을 하는지 알기 위해 여러가지 실험 기법이 동원되었다. 내포작용에 의해 세포 내부로 진입하는 M3R 과 flotillin 의 양상을 관찰하기 위해 membrane fraction 을 통해 두 단백질들의 membrane 과 cytosol 을 분리한 후, 단백질 양의 차이를 비교하기 위해 immunoblotting 기법이 사용되었다. 자극에 의한 내포작용을 관찰하기 위해 carbachol 약물 처리 후, 시간에 따른 두 단백질의 위치 이동을 관찰하기 위해 immunocytochemistry 기법과 confocal immunofluorescence microscopy 촬영을 이용하였고, 두 물질의 상호 작용 여부에 대해 알기 위해 co-immunoprecipitation 기법을 활용하였다. 마지막으로 flotillin 또는 clathrin 의 발현을 억제시킨 상태에서 M3R 과 flotillin 의 내포작용을 관찰하기 위해 siRNA 간섭 기술을 사용하였다.

Acetylcholine 자극에 의해 M3R, flotillin-1, 그리고 flotillin-2 는 각각 세포막에서 세포기질로 이동함을 확인하였다. M3R 은 자극 후 60 분에 가장 많은 양이 세포기질에서 나타난 반면, flotillin 동형 단백질들은 자극 후 120 분까지 지속적으로 세포기질로 이동함을 관찰하였다. Chlorpromazine, filipin III, cytochalasin D, methyl-β-cyclodextrin (mβCD) 을 이용해 이루어진 약물 처리 기법을 통해 실질적으로 타액 상피세포에서 M3R 은 CME 를, 그리고 flotillin 은 CIE 를 거쳐 내포작용을 일으키는지 재확인하고자 하였다. Chlorpromazine 과 cytochalasin D 약물처리를 통해 M3R 의 CME 가 예상대로

억제됨을 확인하였고, 반대로 filipin III 와 mβCD 약물처리를 통해 flotillin 의 CIE 가 억제됨을 확인하였다. 그러나 filipin III 와 mβCD 을 이용한 약물 억제 적용 시 M3R 의 internalization 양의 감소 양상을 통해 약간의 억제가 이루어졌음을 관찰하였다. 추가 실험을 통해 M3R 과 flotillin-1/2 가 세포의 자극 후 특정 시간동안 같은 위치에 존재하고 서로 관여함을 관찰하였다. 흥미롭게도, siRNA 간섭 기술을 통해 flotillin-1 또는 -2 의 발현을 억제한 후 세포를 자극했을 때, 내포작용에 의해 세포기질로 진입한 M3R 의 양이 다소 감소하였음을 확인하였다.

연구결과를 통해 M3R 내포 작용에서 flotillin 의 존재 유무에 따라 M3R 내포 작용의 효율성이 변화할 수 있음을 발견하였고, 반대로 clathrin 을 억제시켜 M3R 의 내포작용을 억제한 상황이 flotillin 의 내포작용 과정에는 영향을 주지 않음을 깨달았다. 따라서 flotillin-1 또는 flotillin-2 는 M3R 내포작용 과정에서 일방적으로 관여하는 단백질일 수 있는 가능성을 확인하였다.

주요어: endocytosis, GPCR, muscarinic type 3 receptor, flotillin, internalization
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