

The internalization of the M₂ and M₄ muscarinic acetylcholine receptors involves distinct subsets of small G-proteins

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Abstract

Multiple mechanisms exist for the endocytosis of receptors from the cell surface. While the M₁, M₃, and M₄ subtypes of muscarinic acetylcholine receptors internalize through the well-characterized mechanism of clathrin coated vesicles, the mechanism of M₂ endocytosis is not well defined. Because the M₂ and M₄ receptors transduce their signals through the same second messengers but internalize through different pathways, we tested the ability of several small G-proteins to regulate the agonist-induced endocytosis of M₂ and M₄ in JEG-3 human choriocarcinoma cells. Dominant-negative Rab5 as well as both wild-type and dominant-negative Rab11 inhibited M₄ but not M₂ endocytosis. In contrast, a dominant-negative Arf6 as well as wild-type Rab22 increased M₂ but not M₄ endocytosis. We used immunocytochemistry to show that in unstimulated cells, the M₂ and M₄ receptors co-localize on the cell surface, whereas after stimulation M₂ and M₄ are in distinct vesicular compartments. In this study, we demonstrate that agonist-induced internalization of the M₂ receptor utilizes an Arf6, Rab22 dependent pathway, while the M₄ receptor undergoes agonist-induced internalization through a Rab5, Rab11 dependent pathway. Additionally, we show that Rab15 and RhoA are not involved in either pathway in JEG-3 cells.

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Introduction

Muscarinic acetylcholine receptors (mAChRs) are members of the G-protein coupled receptor (GPCR) superfamily whose members couple with heterotrimeric G-proteins to transduce extracellular signals into intracellular signal transduction cascades (Lanzafame et al., 2003; van Koppen and Kaiser, 2003). The mAChR family consists of five subtypes (M₁–M₅) which can be divided into two groups based on the signal transduction pathway to which they couple most efficiently; M₁, M₃, and M₅ couple to the G_q family of G-proteins, while M₂ and M₄ couple to the G_i family (van Koppen and Kaiser, 2003).

Several mechanisms are responsible for regulating cell signal transduction following stimulation of a GPCR. In

seconds to minutes after agonist exposure, desensitization, involving G-protein coupled receptor kinase (GRK) and β -arrestin, uncouples the receptor from its G-protein (Ferguson, 2001). Agonist-induced receptor endocytosis also occurs rapidly to remove receptors from the cell surface. Receptor endocytosis has several functions that depend on the specific receptor and cell type, including coupling receptors to different signal transduction cascades (Pierce et al., 2001), dephosphorylation and resensitization of receptor function (Claing et al., 2002), and initiating downregulation (von Zastrow, 2003) of the receptor. Additionally, internalization of one receptor can influence the signaling of an unrelated receptor, as endocytosis of the epidermal growth factor receptor contributes to the signal transduction of the adrenergic receptors (Pierce et al., 2000). Several mechanisms of receptor endocytosis have been characterized. The most well understood mechanisms are mediated by clathrin coated pits (Le Roy and Wrana, 2005), where a clathrin matrix assembles causing an invagination which is then pinched from the membrane in a dynamin-dependent fashion, and caveolae,

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which utilize caveolin and are sometimes associated with lipid rafts (Nichols, 2003; Parton and Richards, 2003). Perplexingly, several receptors have been shown to not internalize through either of these pathways, though the pathways that they utilize are not yet well-characterized. M_1 , M_3 , and M_4 mAChRs undergo agonist-induced endocytosis in a dynamin-dependent manner through clathrin coated pits, while M_2 displays a unique sensitivity to dynamin in HEK cells and does not utilize clathrin coated pits (Tolbert and Lameh, 1996; Schlador and Nathanson, 1997; Shockley et al., 1999; van Koppen, 2001; Roseberry and Hosey, 2001; Roseberry et al., 2001; Delaney et al., 2002). Some studies have shown an interaction between M_2 and caveolin in cardiomyocytes (Feron et al., 1997), although the internalization of the M_2 receptor in HEK293 cells has been reported to be independent of caveolae (Roseberry and Hosey, 2001).

We have previously shown that JEG-3 choriocarcinoma cells are an attractive system for the analysis of agonist-induced internalization of the M_2 receptor. Initial work showed that the M_2 but not the M_1 receptor undergoes agonist-induced internalization in these cells (Goldman et al., 1996). These observations provided the first evidence that multiple pathways exist for agonist-induced internalization of the mAChRs. In addition, while M_2 internalization can be increased by overexpression of GRK2 and β -arrestin-1 (Schlador and Nathanson, 1997), this is not the preferred endocytic pathway for M_2 as demonstrated by the observation that $M_2/M_1(6)$, an internalization deficient mutant M_2 , is able to undergo endocytosis only when co-expressed with these proteins (Schlador et al., 2000). The differential internalization of M_1 and M_2 has allowed identification of five specific amino acid residues residing in the third intracellular loop as well as the sixth and seventh transmembrane domains required for this M_2 -specific internalization pathway (Schlador et al., 2000). Like M_1 , the M_3 receptor is also relatively resistant to agonist-induced internalization in JEG-3 cells (Goin and Nathanson, unpublished data), while the M_4 receptor exhibits internalization that is comparable in magnitude to M_2 (Schlador, 2000).

The Rab and Arf families of small G-proteins were originally discovered as GTPases involved in transport within the trans-Golgi network, but it has recently become clear that they are also involved in internalization of cell surface receptors. In fact, some members of these families are found on the plasma membrane and endosomes where their main function is in regulating vesicular transport (Takai et al., 2001). Arf6, Rab5, Rab11, and Rab4 have all been implicated in the endocytosis or recycling of the beta-adrenergic receptor following ligand stimulation (Premont et al., 1998; Seachrist et al., 2000; Moore et al., 2004). Additionally, Arf6 has been implicated in M_2 endocytosis (Delaney et al., 2002; Houndolo et al., 2005) while Rab5 and Rab11 have been implicated in M_4 endocytosis and recycling, respectively (Volpicelli et al., 2001; Volpicelli et al., 2002). To date, there has not been a systematic comparison of the regulation of different muscarinic receptor subtypes by small G-proteins. Therefore, we sought to determine whether the endocytic pathways of M_2 and M_4 , which are highly homologous receptors with similar signal transduction properties, are completely distinct or if their pathways involve some

shared modulators. We demonstrate here that the M_2 endocytic pathway utilizes Arf6 and Rab22 while M_4 uses Rab5 and Rab11 for its endocytic pathway. We further demonstrate that M_2 and M_4 do not co-localize in vesicles during their initial stages of endocytosis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin (P/S) were obtained from Life Technologies. Lipofectamine2000 was from Invitrogen. N-[3 H]methylscopolamine ([3 H]NMS, 80–82 Ci/mmol) was purchased from Amersham. The chamber slides were from Nalge Nunc International. The anti-HA rabbit polyclonal antibody was from Pierce. The Alexa 568 conjugated anti-mouse antibody and the Alexa 488 conjugated anti-rabbit antibody were from Molecular Probes. The anti-Flag mouse monoclonal M2 antibody, carbamylcholine chloride (carbachol), atropine and all other reagents were purchased from Sigma.

Plasmids

The Flag- M_2 pcDNA3.1 construct was generated by digesting pCDPS-Flag- M_2 (Schlador and Nathanson, 1997) with KpnI and EcoRI to remove the Flag- M_2 coding region, which was then ligated into the pcDNA3.1 vector (Invitrogen). The HA- M_4 , HA-RhoA, HA-RhoAS19N, and HA-RhoAG14V constructs were purchased from the Guthrie (now the UMR) cDNA Resource Center. Rab5-GFP and Rab5S34N-GFP were the generous gift of Dr. Nigel Bunnett (UCSF) (Schmidlin et al., 2001). Dr. Aimee Powelka kindly provided the Arf6, Arf6T27N, and Arf6Q67L constructs (Powelka and Buckley, 2001). Dr. Ann Richmond (Vanderbilt University School of Medicine, Nashville, TN) kindly provided the Rab11 and Rab11S25N constructs (Fan et al., 2003). Rab15, Rab15N121I, Rab15T22N, and Rab5Q67L constructs were the generous gift of Dr. Lisa Elferink (University of Texas Medical Branch, Galveston, TX) (Zuk and Elferink, 1999). The Rab22, Rab22S19N, and Rab22Q64L constructs were the generous gift of Dr. Luis Mayorga (Universidad Nacional de Cuyo, Argentina) (Mesa et al., 2001).

Cell culture and transfection

JEG-3 choriocarcinoma cells (American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% FBS and 1% P/S in a 10% CO₂ environment at 37 °C. For assays, a 10 cm plate was transected using Lipofectamine2000 with 6–10 μ g DNA each of both receptor and G-protein expression vectors.

Binding assays

Cell surface expression of mAChRs was measured using the binding of the membrane-impermeable radioligand N-[3 H]

methylscopolamine to intact cells using a previously described method (Schlador et al., 2000). Briefly, 24 h post-transfection each 10 cm plate was split into two 6-well plates. 48 h post-transfection, triplicate wells were stimulated with 1 mM carbachol for 15 or 30 min. All cells were placed on ice and washed three times with ice-cold phosphate-buffered saline (PBS: 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4). All wells were then incubated for 4 h at 4 °C with 1 nM [^3H]NMS in 2 ml PBS. Three control wells also received 1 μM atropine to measure non-specific binding. Following the incubation, all wells were washed three times with ice-cold PBS, solubilized with 0.5 ml 1% Triton X-100 and transferred to scintillation vials containing 3.5 ml scintillation fluid for counting. The percent of receptor remaining on the cell surface was determined by normalizing the 15 and 30 min time points to the 0 min time point for each transfection type.

Recycling assays

24 h post-transfection each 10 cm plate was split into two 6-well plates. 48 h post-transfection, triplicate wells were stimulated with 1 mM carbachol for 10 min. Following stimulation, one set of cells was washed 3 times and placed in the 37 °C incubator for 30 min. Cells were then placed on ice and washed three times with ice-cold PBS. All wells were then incubated for 4 h at 4 °C with 1 nM [^3H]NMS in 2 ml PBS. Three control wells also received 1 μM atropine to measure non-specific binding. Following the incubation, all wells were washed three times with ice-cold PBS, solubilized with 0.5 ml 1% Triton X-100, and transferred to scintillation vials containing 3.5 ml scintillation fluid for counting. The percent of receptor remaining on the cell surface was determined by normalizing the carbachol treated cells to unstimulated cells for each transfection type.

Statistical analysis

ANOVA was performed comparing all data from each individual experiment using Stat View (SAS, Cary, NC) statistical analysis software. For ANOVA with significant p values ($p < 0.05$), the Fisher's PLSD post-hoc test was performed to

obtain p values between each mock transfected and G-protein transfected culture as well as p values between wildtype and mutant G-protein transfected cultures for each set of conditions at each time point. Figures show conditions in which the $p \leq 0.05$.

Immunocytochemistry and confocal microscopy

Receptor cellular localization was visualized using a modified version of a previously described protocol (Beattie et al., 2000). 24 h following cotransfection of N-terminal tagged receptors, cells from each 10 cm plate were split onto chamber slides at a density of 3.5×10^5 cells/chamber. 48 h following transfection, live cells were labeled with antibodies directed at either the Flag or HA epitope of the receptor (4 $\mu\text{g}/\text{ml}$ or 1:150, respectively, in 0.5 ml PBS) for 30 min at room temperature. After excess antibody was removed (2 washes in DMEM), cells were incubated for up to 30 min in 1 mM carbachol at 37 °C in 2 ml DMEM. Endocytosis was terminated by placing the cells on ice and washing twice with ice-cold PBS. An acid wash (0.5 M NaCl, 0.2 N acetic acid) was then applied to the cells for 3 min to remove any remaining surface antibodies; control cells did not receive this treatment. Cells were fixed (4% paraformaldehyde) then permeabilized (0.25% Triton X-100 in PBS). After several washes in PBST (0.1% Tween-20 in PBS), cells were incubated with secondary antibodies in blocking buffer (3% bovine serum albumin, 0.25% Triton, 0.1% Tween-20 in PBS) for 45–60 min in the dark. Receptors were labeled with Alexa 568 goat anti-mouse antibody (1:500, to label Flag-tagged receptors) or FITC goat anti-rabbit antibody (1:300, to label HA-tagged receptors). Images were collected using a Leica TCS SP1/NT confocal microscope (Leica Microsystems, Inc., Exton, PA.) with a 100 \times , 1.4 N.A. oil immersion lens.

Results

Previous work in our laboratory and others has shown that the M_2 and M_4 mAChRs internalize to similar extents with similar kinetics. We have focused on the endocytosis of these receptors in JEG-3 cells because our lab has previously characterized the mechanism of both M_2 and M_4 internalization in them, showing that while both M_2 and M_4 receptor internalization can be

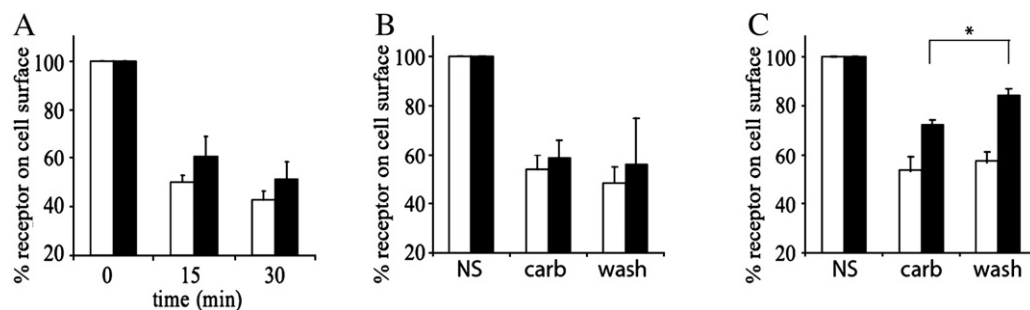


Fig. 1. Time course of M_2 and M_4 internalization in JEG-3 cells (A) JEG-3 cells were transfected with Flag- M_2 (white bars) or HA- M_4 (black bars) and stimulated for 0, 15 or 30 min with 1 mM carbachol. (B) JEG-3 cells were transfected with Flag- M_2 (white bars) or HA- M_4 (black bars) and either not stimulated (NS), stimulated for 10 min with 1 mM carbachol (carb) or stimulated then washed and allowed to recover for 30 min (wash). (C) HEK293 cells were treated as in (B). Cell surface receptors were measured using the binding of [^3H]NMS to intact cells as described in 'Materials and methods.' Data represent the means \pm S.E. of two to six independent experiments, each performed in triplicate.

regulated by overexpression of GRK and β -arrestin, M_2 preferentially internalizes through a mechanism independent of these proteins (Schlador et al., 2000; Schlador, 2000). In JEG-3 cells transiently transfected with either M_2 or M_4 , we observe a 40–50% loss of cell surface binding sites for the membrane-impermeable radioligand [3 H]NMS following 15 min of stimulation with 1 mM carbachol and further loss of cell surface binding with 30 min stimulation (Fig. 1A). These results indicate that M_4 internalizes to a similar extent as M_2 following agonist activation. The level of receptor expression usually ranged from 4–25 fmol per well over the course of the experiments reported here. We have previously shown that the extent of agonist-induced mAChR internalization in transiently transfected JEG-3 cells is independent of receptor density over a greater than 13-fold range of expression levels (Goldman et al., 1996).

The interpretation of results from internalization experiments can be dependent on whether or not the receptor is recycled following internalization. Previous reports have shown that M_4 recycles back to the cell surface in CHO, HEK293 and PC12 cells with the time required for recycling dependent on cell type (Bogatkewitsch et al., 1996; Krudewig et al., 2000; Volpicelli et al., 2002) whereas in HEK293 and JEG-3 cells M_2 does not recycle (Vogler et al., 1998; Schlador et al., 2000). In order to determine if the M_2 or M_4 receptors could exhibit significant recycling in JEG-3 cells during the 30 min agonist incubation period used in most of the experiments in this study, we examined the recycling of both M_2 and M_4 in JEG-3 cells following 10 min of carbachol stimulation and 30 min of recovery after the agonist was washed from the cells. We found that neither receptor recycled (Fig. 1B). As a control, we also examined the recycling of the receptors in HEK293 cells to ensure our experimental conditions are sufficient to detect recycling (Fig. 1C). Our results show that M_4 , but not M_2 , undergoes partial recycling to the surface of HEK293 cells following agonist stimulation. This is in agreement with previous studies in HEK293 cells (Vogler et al., 1998; Krudewig et al., 2000).

M_4 has previously been shown by immunocytochemistry to internalize through a clathrin coated pit mechanism dependent on dynamin, Rab5 and Rab11 in PC12 cells (Volpicelli et al., 2001; Volpicelli et al., 2002). To determine whether M_4

internalizes through a similar mechanism in our cell culture system, we first cotransfected JEG-3 cells with M_4 and either wild-type Rab5 (Rab5WT) or a dominant-negative Rab5 (Rab5S34N) which cannot exchange GDP for GTP. The cells were then stimulated with 1 mM carbachol for 15 or 30 min and the loss of M_4 from the cells' surface was measured by [3 H]NMS binding (Fig. 2A). At both the 15 and 30 min time points there was a significant decrease in the extent of M_4 internalization in cells cotransfected with Rab5S34N compared to cells cotransfected with Rab5WT, confirming the role of Rab5 in the endocytosis of M_4 in JEG-3 cells. Endocytosis of M_4 in cells transfected with Rab5WT was not statistically different from cells transfected with M_4 alone.

We next cotransfected M_4 and either wild-type Rab11a (Rab11aWT) or a dominant-negative Rab11a (Rab11aS35N), which cannot exchange GDP for GTP (Fig. 2B). In the cells cotransfected with either Rab11aWT or Rab11aS35N, there was a significant inhibition in the endocytosis of M_4 compared to M_4 alone at both the 15 and 30 min time points. These results show that in JEG-3 cells, M_4 internalizes through a mechanism involving Rab5 and Rab11.

We next tested the role of Rab15, another small G-protein that is associated with Rab5 and Rab11a mediated endocytosis (Zuk and Elferink, 1999), on M_4 internalization. JEG-3 cells were cotransfected with M_4 and either wild-type Rab15 (Rab15WT), a dominant-negative Rab15 (Rab15T22N), which cannot exchange GDP for GTP, a constitutively active Rab15 (Rab15Q67L), which lacks GTPase activity, or another dominant-negative Rab15 (Rab15N121I), which is unable to bind guanine nucleotides, following carbachol stimulation (Fig. 2C). ANOVA analysis shows no significant differences in M_4 internalization between these conditions, indicating that Rab15 is not involved in M_4 endocytosis. There is also no significant difference between cells transfected with M_4 and Rab15WT and those transfected with M_4 alone.

The M_2 mAChR is thought to internalize through a clathrin-independent mechanism (van Koppen, 2001; Roseberry and Hosey, 2001; Roseberry et al., 2001). Thus, we hypothesized that Rab5, Rab11a and Rab15 would not have an effect on M_2 endocytosis. To determine whether Rab5 is involved in M_2

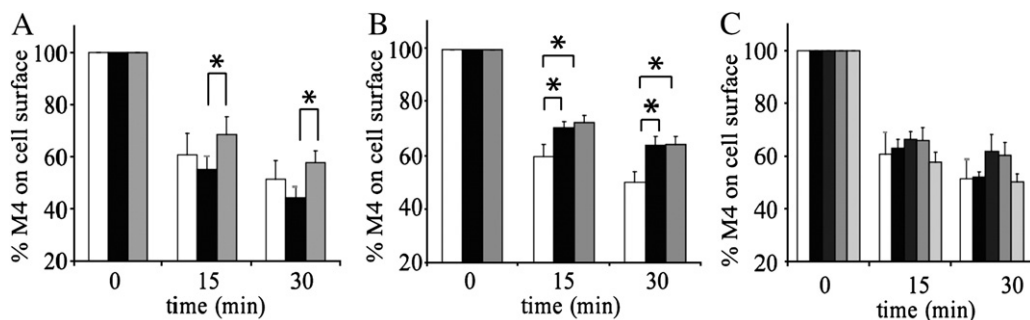


Fig. 2. The effects of Rab5, Rab11 and Rab15 on M_4 internalization in JEG-3 cells. (A) JEG-3 cells were transfected with Flag- M_4 alone (white bars) or with either Rab5WT (black bars) or Rab5S34N (dominant-negative; grey bars). (B) JEG-3 cells were transfected with either Flag- M_4 alone (white bars) or with either Rab11aWT (black bars) or Rab11aS35N (dominant-negative; grey bars). (C) JEG-3 cells were transfected with Flag- M_4 alone (white bars) and either Rab15WT (black bars), Rab15N121I (dominant-negative; dark grey bars), Rab15T22N (dominant-negative; medium grey bars), or Rab15Q67L (constitutively active; light grey bars). Following transfection, cells were stimulated for 0, 15, or 30 min with 1 mM carbachol. Internalization of M_4 receptors was measured using the binding of [3 H]NMS to intact cells as described in 'Materials and methods.' Data represent the means \pm S.E. of four to ten independent experiments, each performed in triplicate.

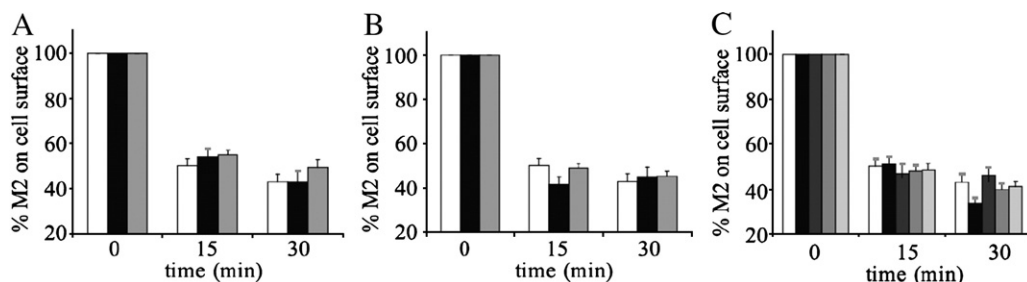


Fig. 3. The effects of Rab5, Rab11 and Rab15 on M₂ internalization in JEG-3 cells. (A) JEG-3 cells were transfected with Flag-M₂ alone (white bars) or with either Rab5WT (black bars) or Rab5S34N (dominant-negative; grey bars). (B) JEG-3 cells were transfected with Flag-M₂ alone (white bars) and either Rab11aWT (black bars) or Rab11aS25N (dominant-negative; grey bars). (C) JEG-3 cells were transfected with Flag-M₂ alone (white bars) or with either Rab15WT (black bars), Rab15N121I (dominant-negative; dark grey bars), Rab15T22N (dominant-negative; medium grey bars), or Rab15Q67L (constitutively active; light grey bars). Following transfection, cells were stimulated for 0, 15 or 30 min with 1 mM carbachol. Internalization of M₂ receptors was measured using the binding of [³H]NMS to intact cells as described in 'Materials and methods.' Data represent the means ± S.E. of three to five independent experiments, each performed in triplicate.

internalization, JEG-3 cells were cotransfected with M₂ and either Rab5WT or Rab5S34N and stimulated with 1 mM carbachol for 15 or 30 min. Cell surface receptors were measured by [³H]NMS binding (Fig. 3A). The results of this experiment show no difference between conditions. In addition, cells transfected with M₂ alone showed no significant difference compared with cells transfected with M₂ and Rab5WT. In JEG-3 cells cotransfected with M₂ and either Rab11aWT or Rab11aS35N, ~55% of the M₂ receptors internalized in an agonist dependent manner (Fig. 3B), indicating that Rab11a does not affect M₂ endocytosis. There was also no difference between cells cotransfected with Rab11aWT and M₂ and those transfected with M₂ alone. To determine whether Rab15 is involved in agonist-induced M₂ internalization, internalization assays were performed following stimulation of cells cotransfected with M₂ and either Rab15WT, Rab15T22N, Rab15Q67L or Rab15N121I (Fig. 3C). ANOVA analysis of the data shows no difference in internalization between the conditions. There are also no differences between cotransfected cells and those transfected with M₂ alone.

Several small G-proteins have been implicated in clathrin-independent endocytosis. Arf6 is associated with early endosome formation as well as the recycling of internalized proteins back to the cell surface. Previous studies using NMS binding and immunocytochemistry have shown Arf6 to regulate the initial steps of M₂ endocytosis in HeLa cells (Delaney et al., 2002). To test the role of Arf6 in M₂ agonist-induced endocytosis in JEG-3 cells, cells were cotransfected with M₂ and either wild-type Arf6 (Arf6WT), a dominant-negative Arf6 (Arf6T22N), which cannot exchange GDP for GTP, or a constitutively active Arf6 (Arf6Q67L), which lacks GTPase activity. We found that Arf6T22N caused a dramatic increase in internalization of the receptor, while there was no difference between M₂ internalization in the Arf6Q67L transfected cells when compared to cells transfected with Arf6WT nor between M₂ internalization in cells transfected with receptor alone when compared to cells transfected with Arf6WT (Fig. 4A). Recently, Rab22 has been shown to regulate the recycling phase of the Arf6 endocytic pathway (Weigert et al., 2004); therefore, we tested its ability to influence M₂ endocytosis in our system. 15 min following stimulation, we observed a significant increase

in internalization of the receptor from the cell surface when M₂ was co-expressed with Rab22WT compared to cells expressing M₂ alone or M₂ and a constitutively active Rab22 (Rab22Q69L), which lacks GTPase activity (Fig. 4B). This increase in internalization was not seen at the later time point. Co-expression of a

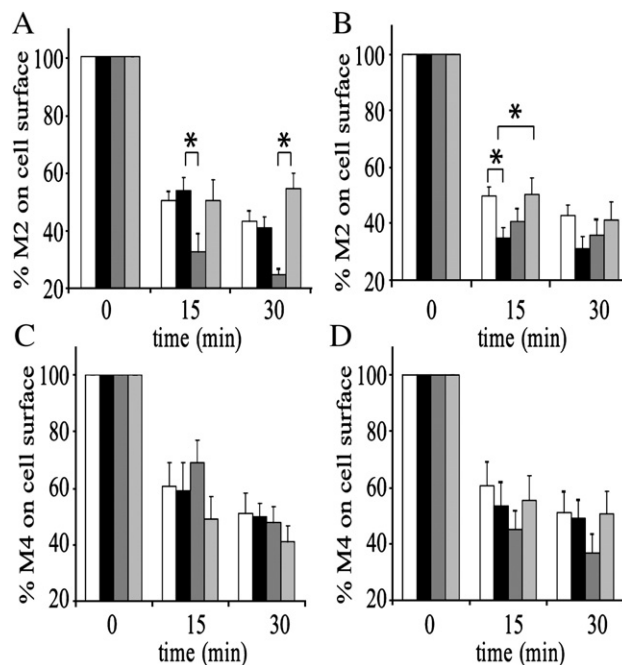


Fig. 4. The effects of Arf6 and Rab22 on M₂ and M₄ internalization in JEG-3 cells. (A) JEG-3 cells were transfected with Flag-M₂ alone (white bars) or with either Arf6WT (black bars), Arf6T22N (dominant-negative; dark grey bars) or Arf6Q67L (constitutively active; light grey bars). (B) JEG-3 cells were transfected with Flag-M₂ alone (white bars) or with either Rab22WT (black bars), Rab22S19N (dominant-negative; dark grey bars), or Rab22Q69L (constitutively active; light grey bars). (C) JEG-3 cells were transfected with Flag-M₄ alone (white bars) or with either Arf6WT (black bars), Arf6T22N (dominant-negative; dark grey bars), or Arf6Q67L (constitutively active; light grey bars). (D) JEG-3 cells were transfected with Flag-M₄ alone (white bars) or with either Rab22WT (black bars), Rab22S19N (dominant-negative; dark grey bars), or Rab22Q69L (constitutively active; light grey bars). Following transfection, cells were stimulated for 0, 15, or 30 min with 1 mM carbachol. Internalization of M₂ and M₄ receptors was measured using the binding of [³H]NMS to intact cells as described in 'Materials and methods.' Data represent the means ± S.E. of three to eight independent experiments, each performed in triplicate.

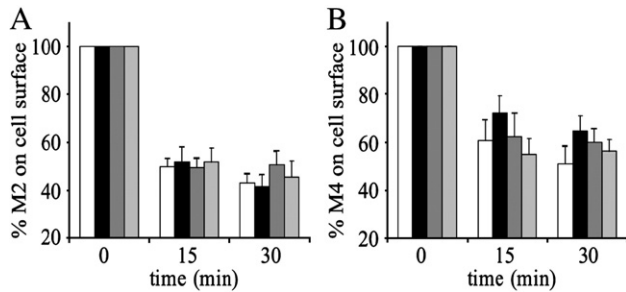


Fig. 5. The effects RhoA on M_2 and M_4 internalization in JEG-3 cells. (A) JEG-3 cells were transfected with Flag- M_2 alone (white bars) or with either RhoAWT (black bars), RhoAG14V (constitutively active; dark grey bars), or RhoAS19N (dominant-negative; light grey bars). (B) JEG-3 cells were transfected with Flag- M_4 alone (white bars) or with either RhoAWT (black bars), RhoAG14V (constitutively active; dark grey bars), or RhoAS19N (dominant-negative; light grey bars). Following transfection, cells were stimulated for 0, 15, or 30 min with 1 mM carbachol. Internalization of M_2 and M_4 receptors was measured using the binding of [3 H]NMS to intact cells as described in 'Materials and methods.' Data represent the means \pm S.E. of five to six independent experiments, each performed in triplicate.

dominant-negative Rab22 (Rab22S19N), which cannot exchange GDP for GTP, showed no significant difference in M_2 endocytosis when compared to all other conditions. We carried out similar experiments to determine whether Arf6 and Rab22 have a role in M_4 endocytosis by cotransfecting M_4 with the various small G-protein constructs (Fig. 4C and D). None of these constructs influenced the agonist-induced endocytosis of M_4 when compared to their wild-type counterparts. Taken together, these results indicate that M_2 endocytosis is dependent on both Arf6 and Rab22 and M_4 endocytosis is independent of these small G-proteins.

Sequestration of M_1 and M_2 mAChRs has been demonstrated to be inhibited by overexpression of RhoA in HEK293 cells (Vogler et al., 1999a,b). To determine the role of RhoA in M_2 and M_4 endocytosis in our system, we cotransfected JEG-3 cells with M_2 or M_4 and either wild-type RhoA (RhoAWT), a dominant-negative RhoA (RhoAS19N) which is unable to exchange GDP for GTP, or a constitutively active RhoA (RhoAG14V), then stimulated with 1 mM carbachol for 15 or 30 min. Cell surface receptors were measured by [3 H]NMS binding (Fig. 5A and B). No significant changes in M_2 or M_4 endocytosis occurred with co-expression of any RhoA constructs, indicating RhoA does not influence the endocytosis of either receptor subtype in our system.

Our results thus far have placed M_2 and M_4 in separate endocytic pathways, which do not share any small G-protein regulators. To further confirm the differences between M_2 and M_4 endocytic pathways, we used immunocytochemistry to determine if the receptors would co-localize following stimulation. We tested whether the receptors are in distinct areas of the cell at all time points following stimulation by cotransfecting JEG-3 cells with Flag-tagged M_2 and HA-tagged M_4 . In order to decrease background staining and to ensure that all receptor observed following stimulation were receptors internalized from the cell surface; we labeled live JEG-3 cells with a primary antibody directed at either a Flag- or HA-tagged receptor. Cells were stimulated with 1 mM carbachol for 5, 15 or 30 min, then

placed on ice and any antibody remaining on the cell surface was removed using an acid wash. After being fixed and permeabilized, the cells were labeled with fluorescent secondary antibodies and visualized by confocal microscopy. In non-stimulated, non-acid washed cells, some, but not all of the M_2 and M_4 co-localized on the membrane (Fig. 6). The acid wash successfully stripped the antibody off of the cell surface of non-stimulated cells, assuring that all labeling in the stimulated, acid washed cells consisted of internalized receptors. At the 5 and 15 min time points, the M_2 and M_4 receptors were localized to distinct vesicles, as evidenced by the complete lack of yellow in the merged confocal images. At 30 min approximately half of the cells showed significant co-localization, as seen by the

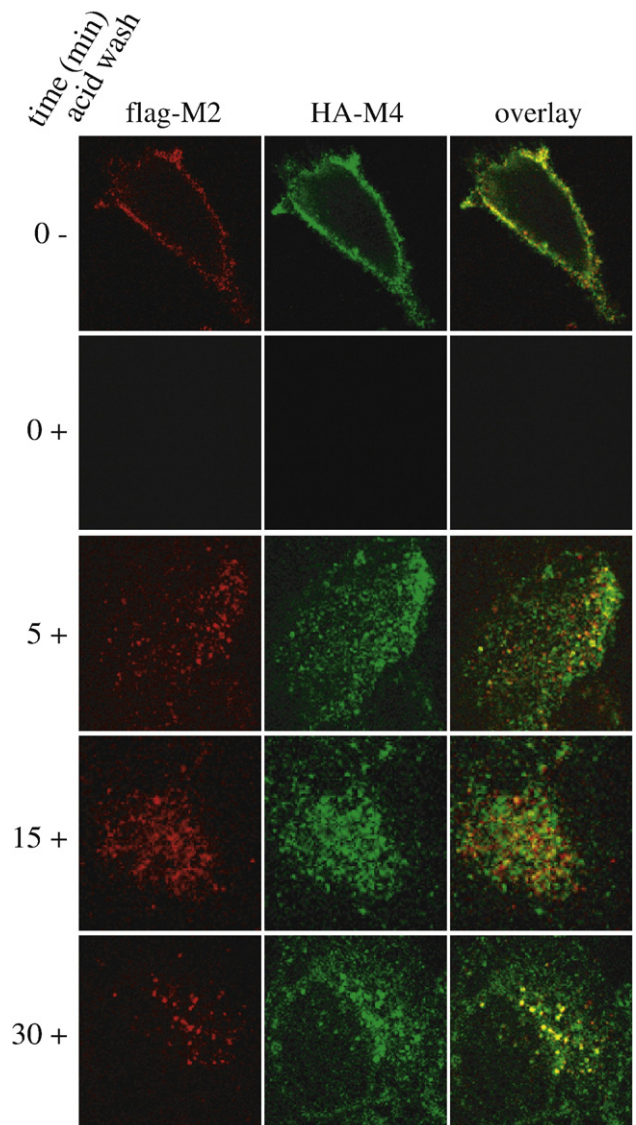


Fig. 6. Co-localization of M_4 with M_2 . JEG-3 cells were transfected with HA- M_4 and Flag- M_2 . The cells were stimulated for various times as indicated in the first column, acid washed as indicated in the second column and stained and analyzed by confocal microscopy as described in 'Materials and methods.' The first column of images represents staining for HA- M_4 only. The second column of images represents staining for Flag- M_2 only. The third column of images is an overlay of the first two columns. Images were taken at 100 \times magnification and are representative examples of images taken from five independent experiments.

presence of yellow signal due to the overlap of the M₂ and M₄ signals in the merged confocal images (Fig. 6), and the other half showed the receptors to be in distinct compartments as at the earlier time points (data not shown). Thus the M₂ and M₄ receptors initially undergo agonist-induced internalization using distinct vesicular pathways, consistent with the biochemical studies shown here, while the pathways appear to begin to merge at 30 min after agonist stimulation.

Discussion

In this study we used overexpression of wildtype and mutant G-proteins to show that M₂ and M₄ internalize through distinct pathways using different subsets of small G-proteins. It has been shown previously that M₁, M₃, and M₄ require β -arrestin and dynamin for endocytosis (Vogler et al., 1998; Vogler et al., 1999a,b). Although M₂ internalization is able to utilize this pathway, it preferentially uses a pathway independent of these proteins in JEG-3 cells (Schlador and Nathanson, 1997; van Koppen, 2001). Here we have compared the M₂ and M₄ endocytic pathways for their reliance on small G-proteins.

We first investigated the reliance of M₂ and M₄ endocytosis on a variety of small G-proteins that have previously been shown to associate with endosomes. We hypothesized that M₄ internalization would be affected by small G-proteins known to associate with clathrin coated pits, Rab5, Rab11, and Rab15, while M₂ internalization would be affected by those known to be involved with clathrin-independent endocytosis, Arf6, Rab22, and RhoA.

Recent studies have shown that Rab5, Rab11, and Rab15 are involved in clathrin coated pit mediated endocytosis (Stenmark et al., 1994; Moore et al., 1995; Zuk and Elferink, 1999; Wang et al., 2000). Additionally, Rab5 and Rab11 have been implicated in M₄ internalization and recycling, respectively, in PC12 cells (Volpicelli et al., 2001; Volpicelli et al., 2002). We first tested the role of these G-proteins on agonist-induced internalization of M₄ in JEG-3 cells (Fig. 2A, B). Our studies indicate that Rab5 plays a role in M₄ internalization in JEG-3 cells. This finding correlates with earlier studies in PC12 cells though our results differ slightly. Volpicelli et al. used immunocytochemistry to demonstrate that the dominant-negative Rab5S34N caused a decrease in M₄ endocytosis at up to 5 min following agonist stimulation, but not at later time points. They suggest that this may be because the receptor is quickly transferred out of the early endosomes and into recycling endosomes which allows for more receptor to internalize. Our studies found a decrease in M₄ endocytosis up to 30 min following agonist stimulation. The difference in the time points of our results could be due to increased sensitivity of the binding assay versus immunocytochemistry or differences in cell types: PC12 cells recycle M₄ (Volpicelli et al., 2002) whereas JEG-3 cells do not (Fig. 1B).

We tested if the inability to detect recycling of the M₄ receptor in JEG-3 cells reflected a lack of sensitivity of the assay or an intrinsic property of the JEG-3 cells. As shown in Fig. 1C, the M₄ receptor (but not the M₂ receptor) underwent partial recycling in HEK293 cells after agonist removal. This difference in regulatory properties is not surprising. Previous studies have shown that both the specificity of functional coupling and the

regulatory properties and trafficking of a given receptor can be dependent on the celltype in which the receptor is expressed (Tietje and Nathanson, 1991; Goldman et al., 1996; Schlador et al., 2000). Indeed, even different stocks of the same cell line obtained from different laboratories in the same institution can show significant differences in the regulation of a specific receptor (Lefkowitz et al., 2002). Thus, the use of more than a single cell line can detect important aspects of receptor biology that would be missed by the use of a single celltype.

We found that co-expression of M₄ with either wild-type Rab11 or dominant-negative Rab11S35N resulted in an inhibition of endocytosis when compared to cells expressing only M₄ (Fig. 2B). It is somewhat surprising that both the wild-type and dominant-negative Rab11 had similar effects on M₄ internalization. There are several possible explanations for this observation. Volpicelli et al. (2002) showed that Rab11a was involved in endosomal recycling of M₄ in PC12 cells. It is possible that the overexpression of wild-type Rab11 increases the rate of recycling, thus leading to more receptors on the cell surface. Because the M₄ receptor does not recycle in JEG-3 cells, it is more likely that the wild-type Rab11 is increasing delivery of newly synthesized M₄ to the plasma membrane (Urbe et al., 1993). The effects of the dominant-negative Rab11S35N suggest that Rab11 may be involved in the early stages of M₄ endocytosis. It would not be unusual for a single small G-protein to have multiple roles in endocytosis, as Arf6 was initially implicated in the recycling phase of endocytosis (D'Souza-Schorey et al., 1995) and it has recently been found to also be involved in the early stages of endocytosis of the M₂ receptor (Delaney et al., 2002; Houndolo et al., 2005). In fact, Rab11 has recently been shown to regulate both the endocytosis and insulin-induced translocation to the plasma membrane of GLUT4 (Uhlig et al., 2005). Finally, it is possible that Rab11 can act as a protein scaffold independently of its activity as a GTPase. It would be interesting to determine if both mutant and wildtype Rab11 interact with one or more other proteins which regulate the trafficking of the M₄ receptor but not the M₂ receptor. Since Rab15 is a regulator of early endocytosis that co-localizes with Rab5 on early endosomes and with Rab11 on recycling endosomes (Zuk and Elferink, 1999), we wanted to know if it was also involved in M₄ endocytosis. In JEG-3 cells cotransfected with M₄ and either wild-type Rab15WT, dominant-negatives Rab15T22N, Rab15N121I, or constitutive active Rab15Q67L, there were no significant differences in either rate or extent of internalization between any of the conditions (Fig. 2C) indicating that Rab15 does not play a role in M₄ agonist-stimulated endocytosis.

We found that neither Rab5, Rab11 nor Rab15 are involved in M₂ endocytosis. Because these proteins have been implicated in clathrin-mediated internalization, these results are consistent with the idea that M₂ does not internalize through clathrin coated pits.

Several small G-proteins have also been shown to associate with a clathrin-independent endocytic pathway. Arf6 is distinct amongst the small G-proteins in that it is membrane associated in unstimulated cells (Cavenagh et al., 1996; Yang et al., 1998). It is also the only small G-protein previously reported to be involved in M₂ endocytosis (Delaney et al., 2002; Houndolo et al., 2005). The first implication of Arf6 in the endocytosis of M₂ showed that the constitutively active Arf6Q67L inhibited endocytosis while

the dominant-negative and wild-type Arf6 constructs had no effect (Delaney et al., 2002). We showed that Arf6 is involved in M₂ agonist-induced endocytosis in JEG-3 cells; we found that the dominant-negative Arf6T22N will stimulate internalization while Delaney et al. (2002) found that the constitutively active Arf6Q67L inhibits internalization. The lack of effect of the dominant-negative Arf6 reported by Delaney et al. (2002) and the lack of effect of the constitutively active Arf6 found in the current study suggest that endogenous Arf6 plays a more important role in regulating M₂ internalization in JEG-3 cells than in the HeLa cells used by Delaney et al. The decrease in M₂ at the cell surface when co-expressed with dominant-negative Arf6 is not due to a blockade of receptor recycling following internalization, because we are unable to observe M₂ recycling in these cells. The decreased internalization by constitutively active Arf6 found by Delaney et al. (2002) and the increased internalization by dominant-negative Arf6 found here suggest an inhibitory role for Arf6 in M₂ endocytosis.

Rab22, has high sequence homology to Rab5 and associates with endosomes (Olkonen et al., 1993), but has not yet been implicated in the internalization of GPCRs. More recently, Rab22 has been implicated in the recycling phase of the clathrin-independent endocytosis of MHCI (Weigert et al., 2004). Our results show that co-expression with Rab22WT increases M₂ internalization 15 min following stimulation with carbachol. Studies to date have shown Rab22 to be associated with early endosomes and to interact specifically with EEA1 (Mesa et al., 2001; Kauppi et al., 2002), suggesting a role for Rab22 in vesicle fusion in a manner similar to Rab5. While the lack of effect of the constitutively active Rab22 in the current study is surprising in light of the inhibition caused by Rab22WT, these results together suggest that Rab22 may be involved in the fusion of M₂ containing early endosomes to late endosomes. This is also consistent with the increased internalization at only the 15 min time point as the increased fusion would speed up the endocytosis but not necessarily increase the extent of endocytosis. As expected, neither Arf6 nor Rab22 are important for M₄ endocytosis.

The last small G-protein that we analyzed in this study is RhoA, which has also been implicated in clathrin-independent endocytosis and more specifically has been shown to affect the non-preferred pathway for M₂ internalization in HEK293 cells (Vogler et al., 1999a,b). Our results here show that RhoA is not involved in the endocytosis of either M₂ or M₄ in JEG-3 cells.

Transfection of the various G-proteins, when effective on mAChR internalization, attenuated but did not completely inhibit receptor internalization. This is consistent with previous observations that overexpression of small G-proteins could partially but not completely inhibit the internalization of a variety of G-protein coupled receptors (Seachrist et al., 2000; Volpicelli et al., 2001; Delaney et al., 2002; Moore et al., 2004). While the effects of overexpression can be dependent on the level of endogenous G-protein expression in the cell, a differential effect of a given G-protein construct on the regulation of the M₂ and M₄ receptors provides strong evidence for a differential involvement in receptor internalization. The specificity of the effects on mAChR internalization reported here is supported by the fact that G-protein constructs which affect M₂ internalization did not affect M₄

internalization, and constructs affecting M₄ internalization did not affect M₂ internalization.

Our immunocytochemical studies confirm that M₂ and M₄ internalize through distinct pathways. After stimulation M₂ and M₄ are largely contained in separate vesicles. Even at the 30 min time point, only ~50% of the cells observed had significant co-localization of the receptors. These results, which are the first description of simultaneous localization of the M₂ and M₄ receptors during agonist-induced internalization, are striking because even though the receptors can be found in the same areas on the cell surface, they are able to separate into distinct endocytotic vesicles and employ internalization pathways which do not overlap. These results suggest that the M₂ and M₄ receptors do not interact or form heterodimers during the early stages of agonist-induced internalization. Interestingly, approximately half of the cells exhibited significant co-localization of the two internalized receptors at 30 min. These results indicate that while the M₂ and M₄ receptors initially utilize different endocytotic pathways, the receptors are beginning to share the same trafficking pathway at 30 min. This convergence of initially distinct trafficking pathways is consistent with the results of Delaney et al. (2002), who reported that the M₂ receptor and transferrin receptor initially were internalized in distinct endocytotic vesicles and subsequently showed partial overlap in the same intracellular compartments. These studies have identified an additional small G-protein that appears to be involved in the M₂ endocytic pathway and demonstrate that the M₂ and M₄ mAChR internalize through distinct pathways regulated by separate subsets of small G-proteins.

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