Rab5-dependent Trafficking of the m4 Muscarinic Acetylcholine Receptor to the Plasma Membrane, Early Endosomes, and Multivesicular Bodies*

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The m4 subtype of muscarinic acetylcholine receptor regulates many physiological processes and is a novel therapeutic target for neurologic and psychiatric disorders. However, little is known about m4 regulation because of the lack of pharmacologically selective ligands. A crucial component of G protein-coupled receptor regulation is intracellular trafficking. We thus used subtype-specific antibodies and quantitative immunocytochemistry to characterize the intracellular trafficking of m4. We show that following carbachol stimulation, m4 co-localizes with transferrin, and the selective marker of early endosomes, EEA1. In addition, m4 intracellular localization depends on Rab5 activity. The dominant negative Rab5S34N inhibits m4 endocytosis initially following carbachol stimulation, and reduces the size of m4 containing vesicles. The constitutively active Rab5Q79L enhances m4 intracellular distribution, even in unstimulated cells. Rab5Q79L also produces strikingly enlarged vacuoles, which by electron microscopy contain internal vesicles, suggesting that they are multivesicular bodies. m4 localizes both to the perimeter and interior of these vacuoles. In contrast, transferrin localizes only to the vacuole perimeter, demonstrating divergence of m4 trafficking from the pathway followed by constitutively endocytosed transferrin. We thus suggest a novel model by which multivesicular bodies sort G protein-coupled receptors from a transferrin-positive recycling pathway to a nonrecycling, possibly degradative pathway.

Intracellular trafficking has recently emerged as a crucial component of G protein-coupled receptor (GPCR) regulation. GPCR trafficking includes targeting of newly synthesized receptors to the cell surface, endocytosis of activated receptors, recycling back to the plasma membrane, and targeting to lysosomes for degradation (1). A central requirement for understanding the role of trafficking in GPCR regulation is to delineate the intracellular organelles through which the receptors traffic. Recent cell biological studies have identified multiple distinct endocytic compartments involved in cell surface receptor trafficking. These organelles include: 1) early sorting endosomes that contain both recycling proteins and proteins destined for degradation; 2) recycling endosomes; and 3) late endosomes and multivesicular bodies (MVBs) that target proteins for lysosomal degradation (2–4).

The endosomal compartments involved in protein trafficking are morphologically and functionally distinct and can be identified by association with small GTPases called Rabbs. The roles individual Rabbs play in endocytic trafficking have been elucidated primarily based on studies of trafficking of the constitutively endocytosed and recycled transferrin receptor (TfnR) (5). One of the first steps in the endocytic pathway, trafficking of clathrin-coated vesicles from the plasma membrane to early sorting endosomes, is mediated by Rab5. Mutants that alter Rab5 activity affect the targeting of the TfnR from the cell surface to early sorting endosomes and the intracellular distribution of the TfnR within early endosomes (6). Consequently, if GPCR traffic through early sorting endosomes in common with the TfnR, the localization of internalized GPCR within this compartment should depend upon Rab5 activity.

Muscarinic acetylcholine receptors (mACHRs) provide an excellent model for studying GPCR trafficking because of the existence membrane impermeant ligands that identify cell surface receptors and specific antibodies that can identify individual mACHR subtypes. The family of mACHR includes 5 subtypes: Gq-linked m1, m3, and m5, and Gi-linked m2 and m4. The m4 mACHR is one of the principal mACHR subtypes in the brain, yet little is known about its functions due to lack of selective pharmacological agents. However, recent studies indicate that activation of m4 mediates locomotor activity (7), m4 expression is up-regulated in Alzheimer's disease (8), and m4 is a novel target for antipsychotics (9). On a cellular level, m4 regulates adenyl cyclase (10), release of intracellular calcium (11), and calcium channels (12). Therefore, understanding m4 regulation is important for understanding mechanisms that underlie many physiological processes.

The primary goal of this study is to define the early endosomal trafficking of m4 and determine the role of Rab5 in m4 internalization and endocytic trafficking. We used subtype-specific antibodies and immunocytochemistry to selectively quantitate the intracellular trafficking of m4. We chose to examine m4 endogenously expressed in a native system because endogenously expressed GPCRs show different trafficking patterns than receptors transfected and overexpressed in foreign cell lines (13). In addition, because the majority of m4 is expressed in neurons, we studied m4 trafficking in the neuroendocrine PC12 cell line (14). We show that CCh treatment causes m4 to internalize from the cell surface to Tfn- and early
endothelial autoantigen 1 (EEA1)-positive early sorting endosomes. Mutants that alter Rab5 activity produce dramatic effects on m4 cell surface and endosomal localization. The dominant negative Rab5 inhibits m4 internalization and reduces the size of endocytic vesicles containing m4. The constitutively active Rab5 enhances m4 intracellular localization and produces enlarged vacuoles to which m4 is targeted. Ultrastructural analysis of these vacuoles reveals the presence of numerous internal vesicles, suggesting that these structures are MVBs. Interestingly, m4 shows a distinct distribution within the MVB compared with Tfn. These studies thus define the early endosomal trafficking of m4 and identify MVBs as a site of divergence between the m4 mAChR and constitutively recycled cell surface receptors.

MATERIALS AND METHODS

**Cell Culture—** PC12 cells were a gift from Dr. Richard Burry (15). Cells were maintained in DMEM (Mediatech, Herndon, VA) containing 10% heat-inactivated horse serum (Life Technologies, Grand Island, NY), 5% fetal calf serum (HyClone, Logan, UT), and 1% penicillin/streptomycin at 37 °C and 5% CO2. For binding assays, cells were passaged into 6-well culture dishes at 20,000 cells/cm2 3 days before use. For immunocytochemistry experiments, cells were passaged 2 days before use onto Matrigel extracellular matrix (Becton Dickinson, Franklin Lakes, NJ) coated coverslips in 6-well culture dishes at 20,000 cells/cm2.

Construction of rab5 Plasmids and Transfection—Wild type rab5, rab5S34N, and rab5Q79L cDNAs were a gift from Dr. Marino Zerial. To construct the Rab5-GFP fusion proteins, the rab5 cDNA were amplified with polymerase chain reaction with a 5′ oligonucleotide primer that introduced a HindIII restriction enzyme site (5′-TATTTAAGGCTCTAGGGATACGGGTAG-3′) and a 3′ primer that introduced a BamHI restriction enzyme site (5′-TATATTGAGCCTTACATAACAGCTG-3′). The polymerase chain reaction products were digested with HindIII and BamHI and ligated into the pcDNA3 vector using a DNA ligation kit as per manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, IN). Vectors were transformed into ultracompact epieric coli bacteria (Stratagene, La Jolla, CA) and a plasmid preparation was performed using a Maxiprep kit (Qiagen, Valencia, CA). Sequencing confirmed the DNA sequence of the rab5 constructs. For transfections, cells were plated onto 6-well trays at 100,000 cells/cm2 3 days after transfection. The following constructs (1 µg) were transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA): pcEFGP vector (control), wild type rab5, rab5S34N, and rab5Q79L. The next day, cells were rinsed and passaged onto coverslips.

**Drug Treatments—** For all experiments, cells were pretreated with media containing cycloheximide (20 µg/ml) for 30 min to eliminate new mAChR expression (16). Cycloheximide was included in all subsequent treatments. To induce mAChR internalization, cells were continuously treated with media containing carbachol (CCh) (100 µg/ml) for 2.5 h. This loss of both CCh treatment cause a progressive decrease in m4 co-localization with Na+ /K−-ATPase over time (Fig. 1A).

**RESULTS**

**Measurement of mAChR Internalization using [3H]NMS Binding Assays Versus Quantitative Immunocytochemistry—** The neuronotypic PC12 cell line endogenously expresses mACHRs (14) and thus provides an excellent model for studying trafficking in a native system. Because mAChR trafficking may depend on the cell line in which they are expressed (13), we first sought to characterize the extent of CCh-induced loss of cell surface mACHRs in PC12 cells with previously established binding assays using membrane impermeant [3H]NMS. Treatment of PC12 cells with CCh produces a progressive decrease in cell surface binding of mACHR over time (Fig. 1A). This loss in cell surface mACHR is substantial, as ~80% of mACHR internalize by 60 min continuous CCh treatment.

[3H]NMS binds nonspecifically to all mAChRs, and PC12 cells express multiple mAChR subtypes (14). As distinct GPCR subtypes show differences in patterns of intracellular trafficking (19, 20), binding assays are thus inadequate because they do not allow study of individual mAChRs. Therefore, immunocytochemistry using a subtype-selective antibody, confocal microscopy, and image analysis were used to visualize and quantitate the internalization of the m4 subtype of mAChR specifically. In untreated cells, m4 and Na+ /K−-ATPase localize to the cell surface and co-localize extensively (Fig. 1B).

Following 10 min continuous CCh treatment, m4 redistributes from the plasma membrane into large discrete punctate distributions peripherally throughout the cell. After 60 min CCh, m4 moves toward the nucleus. Following both 10 and 60 min CCh treatment, m4 shows minimal co-localization with Na+ /K−-ATPase. To quantitate loss of cell surface m4 in single cells, the percentage of m4 pixels that overlap with Na+ /K−-ATPase pixels in the confocal images was measured. Ten and 60 min CCh treatment cause a progressive decrease in m4 co-localization...
Quantitation of m4 internalization in PC12 cells by [3H]NMS binding and immunocytochemistry. A, binding assays using membrane impermeant [3H]NMS show that cell surface mAChRs are progressively reduced following 10 and 60 min CCh treatment compared with untreated cells (n = 3 experiments). B, in untreated cells, m4 (red) localizes primarily to the cell surface. Na+/K+-ATPase (green) shows an exclusively cell surface distribution and m4 and Na+/K+-ATPase co-localize extensively (yellow). Following 10 min CCh treatment, m4 redistributes from the cell surface to large puncta distributed peripherally throughout the cell. m4 moves to a perinuclear distribution following 60 min CCh. Following 10 and 60 min CCh m4 no longer shows co-localization with Na+/K+-ATPase in the merged images. Scale bar = 10 μm. C, cell surface m4, as measured by the percentage that m4 overlaps with Na+/K+-ATPase is progressively reduced following 10 (n = 24 cells analyzed) or 60 min CCh (n = 24) treatment compared with untreated cells (n = 24). D, data is represented as percentage of untreated cells. Both binding assays (black bars) and quantitation of confocal images (gray bars) show a progressive decrease in cell surface mAChR following 10 and 60 min CCh treatment. Furthermore, the magnitude of internalization measured by both methods is remarkably similar.

Early Endosomal Localization of Internalized m4—We next determined if CCh-induced internalization targets m4 to intracellular compartments in common with the TfnR. To visualize internalization of m4 and the TfnR simultaneously at early time points, cells were prelabeled with the Tfn ligand conjugated to Alexa-633, rinsed, and incubated in CCh. Fig. 2A shows that both internalized m4 and Tfn localize to large puncta distributed throughout the cell. Merged images demonstrate m4 and Tfn co-localization.

The TfnR initially traffics to early sorting endosomes following internalization, but also travels through distinct recycling compartments (2, 3). Therefore localization of the TfnR does not distinguish among segregate endosomal compartments. EEA1 localizes selectively to early sorting endosomes (21). Therefore, to determine whether m4 initially traffics to early sorting endosomes following internalization we measured the extent of m4 co-localization with EEA1. In PC12 cells, EEA1

Rab5-dependent Trafficking of m4

![Diagram of Rab5-dependent Trafficking of m4](http://www.jbc.org/)

Fig. 1D shows that the magnitude of mAChR internalization measured by binding assays and the magnitude of m4 internalization measured by image analysis are very similar. These data are consistent with m4 comprising 95% of total mAChR expressed in PC12 cells (14). Thus, quantitation of confocal images provides a valid method for measuring the extent of internalization following agonist treatment. Furthermore, unlike binding assays, confocal images allow single cell analysis of internalization of the m4 subtype of mAChR specifically and visualization of m4 trafficking to intracellular compartments.

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distributes to large puncta peripherally localized throughout cell. m4 and EEA1 show minimal co-localization in untreated cells (Fig. 2B). Following 10 min CCh, m4 has almost completely redistributed from the cell surface and localizes to large intracellular puncta that co-localize extensively with EEA1. Accordingly, co-localization between m4 and EEA1 increases from ~7.5% in untreated cells to greater than 50% following 10 min CCh (Fig. 2C). These data demonstrate agonist-induced m4 trafficking through EEA1-positive early sorting endosomes.

The Effects of Rab5 on m4 Internalization from the Cell Surface—EEA1 is a Rab5 effector and both molecules direct trafficking of clathrin-coated vesicles from the plasma membrane to early endosomes (21, 22). In addition, previous studies demonstrate that dominant negative Rab5S34N inhibits and constitutively active Rab5Q79L enhances cell surface receptor internalization (6, 23, 24). To determine whether m4 internalization from the cell surface depends on Rab5 activity, the following rab5 constructs were transiently transfected in PC12 cells: wild type rab5, dominant negative rab5S34N which cannot exchange GDP for GTP, and constitutively active rab5Q79L which lacks GTPase activity. Cells transfected with the pEGFP vector alone were included as controls. Transfection efficiency in PC12 cells is low. Thus, the use of established methods, such as [3H]NMS binding, to determine the effects of Rab5 on mAChR trafficking, is not possible. We therefore used confocal microscopy and image analysis to quantify m4 co-localization with a cell surface marker to determine the effects of Rab5 on m4 internalization in single cells.

Expression of wild type Rab5 produces no effect on cell surface m4 as co-localization with Na+/K+-ATPase is equivalent between control cells and wild type Rab5 expressing cells at baseline and throughout the time course of CCh treatment (data not shown). In cells expressing dominant negative Rab5S34N, m4 shows equivalent co-localization with cell surface Na+/K+-ATPase at baseline compared with vector transfected control cells (Fig. 3A). However, cell surface m4 is not reduced following initial (2.5 min) treatment with CCh such that m4 overlap with Na+/K+-ATPase is significantly higher than controls. By 5 min continuous CCh stimulation and throughout the remainder of the time course, m4 co-localiza-

![Image](http://www.jbc.org/doi/fig/2A.png)  
**Fig. 2.** m4 internalization to Tfn and EEA1 positive early sorting endosomes following CCh treatment. A, Tfn was pre-bound to PC12 cells at 4 °C. Cells were rinsed and then warmed to 37 °C in the presence of CCh. m4 (red) and Tfn (green) to localize large puncta distributed throughout the cell that co-localize extensively (yellow, arrows). B, in untreated cells, m4 (red) localizes to the cell surface, EEA1 (green) localizes to large puncta distributed peripherally throughout the cell, and m4 and EEA1 do not co-localize. Following 10 min CCh, m4 shows extensive co-localization (yellow) with EEA1 in the large puncta (arrows). Scale bar = 10 μm. C, quantitative analysis shows that 10 min (n = 6) CCh significantly increases the percentage m4 overlaps with EEA1 compared with untreated cells (n = 13). (t(17) = 17.6) *** indicates a statistically significant difference (p < 0.001) from untreated cells.
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Fig. 3. Effects of mutant Rab5 expression on cell surface m4. A, at baseline, m4 shows equivalent co-localization with Na+/K+-ATPase in control cells and Rab5S34N expressing cells. Following 2.5 min CCh in Rab5S34N expressing cells, cell surface m4 is not decreased and overlap with Na+/K+-ATPase is significantly higher than controls. However, following 5 min CCh stimulation and throughout the remainder of the time course, m4 internalizes from the cell surface such that m4 co-localization with Na+/K+-ATPase is similar between Rab5S34N expressing cells and controls. In cells expressing constitutively active Rab5Q79L, m4 co-localization with Na+/K+-ATPase is significantly decreased compared with controls at baseline. This decreased cell surface m4 is maintained throughout 2.5–10 min CCh treatment. By 60 min CCh stimulation in Rab5Q79L expressing cells, the extent of m4 co-localization with Na+/K+-ATPase is equivalent to control cells. Twenty-four cells were analyzed for each construct at each time point. Asterisks indicate a statistically significant difference from controls (*, p < 0.05; **, p < 0.01; ***, p < 0.001). (F(19,479) = 45.5). B, the data in A is replotted to demonstrate CCh-induced internalization relative to untreated cells. Dominant negative Rab5S34N significantly impairs m4 internalization, but only following 2.5 min CCh stimulation. Expression of Rab5Q79L does not significantly affect the extent of CCh-induced loss of cell surface m4 relative to untreated cells. * indicates a statistically significant difference (p < 0.05) between Rab5S34N expressing cells and vector transfected controls. (F(18,469) = 31.3).

Fig. 4. Effects of mutant Rab5 expression on m4 intracellular distribution at baseline. Wild type Rab5-GFP shows both diffuse and punctate (arrows) staining consistent with localization of wild type Rab5 to both cytosol and membrane. Dominant negative Rab5S34N-GFP shows a mostly diffuse, cytosolic distribution. Expression of constitutively active Rab5Q79L-GFP produces a mostly diffuse, cytosolic distribution. Expression of constitutively active Rab5Q79L-GFP produces large puncta (arrows) and strikingly enlarged vacuoles. In untreated cells, expression of wild type Rab5 or Rab5S34N does not affect cell surface distribution of m4. In untreated cells expressing Rab5Q79L, m4 intracellular distribution is strikingly enhanced and in particular, m4 is targeted to the large vacuole. Scale bar = 10 μm.

In untreated cells, expression of wild type Rab5 or Rab5S34N does not affect the cell surface distribution of m4 compared with controls (Fig. 4). However, m4 shows markedly enhanced intracellular localization in cells expressing constitutively active Rab5Q79L. In particular, m4 is targeted to the large vacuole. The effects of Rab5Q79L are selective for m4 as this Rab5 mutant does not affect cell surface localization of Na+/K+-ATPase (see Fig. 7). Thus, even in unstimulated cells, production of enlarged vacuoles by Rab5-GTP results in dramatic enhancement of intracellular pools of m4.

To determine whether intracellular distribution of m4 following agonist stimulation depends on Rab5 activity, cells were treated continuously with CCh for 10 min and m4 co-localization with EEA1 and Rab5-GFP was analyzed. In cells expressing wild type Rab5, internalized m4, EEA1, and Rab5-GFP internalization in Rab5Q79L-transfected cells is not further increased relative to control cells. Collectively, these results indicate that m4 distribution at baseline and internalization following agonist stimulation are both regulated by Rab5.

Effects of Rab5 on m4 Intracellular Localization—Because Rab5 plays a role in m4 cell surface distribution and m4 traffics to early endosomes following internalization, m4 localization within early endosomes may also depend on Rab5 activity. Therefore, the effects of mutant Rab5 expression on intracellular localization of m4 following CCh treatment were examined. In the GDP bound form, Rab5 localizes to the cytosol while Rab5-GTP attaches to the membrane (25). Accordingly, wild type Rab5 expressed in PC12 cells shows both diffuse staining consistent with cytosolic Rab5-GDP and punctate staining consistent with membrane bound Rab5-GTP (Fig. 4). Localization of dominant negative Rab5S34N-GFP, which does not bind to the membrane is mostly diffuse and cytosolic. Constitutively active Rab5Q79L-GFP produces enlarged puncta and strikingly large vacuoles (Fig. 4) with diameters ranging from ~3.5 to 6.5 μm in ~50% of Rab5Q79L-GFP positive cells.

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co-localize in large puncta (Fig. 5). In cells expressing dominant negative Rab5S34N, the size of m4-positive vesicles are dramatically reduced. EEA1-positive puncta are also decreased in size. m4, EEA1, and Rab5S34N-GFP are widely distributed in the cell and do not co-localize. The two lower rows both illustrate examples of cells transfected with constitutively active Rab5Q79L. Expression of Rab5Q79L produces both large puncta (arrows, third panel from top) and enlarged vacuoles (bottom panel). m4 co-localizes with EEA1 and Rab5Q79L-GFP positive puncta (arrows). In cells in which enlarged vacuoles are produced, m4 localizes to the perimeter where it co-localizes with EEA1 and Rab5Q79L-GFP. However, unlike EEA1 and Rab5, m4 distributes to the interior of the vacuole where it does not co-localize with these markers. Scale bar = 10 μm.

Fig. 5. m4 endosomal localization in cells expressing mutant Rab5. Following 10 min CCh treatment in wild type Rab5 expressing cells, m4 (red) localizes primarily to large puncta that co-localize with EEA1 (blue) and Rab5-GFP positive puncta (green). Co-localization is visualized as white in the merged images. In cells expressing dominant negative Rab5S34N, the size of m4-positive vesicles are dramatically reduced. EEA1-positive puncta are also decreased in size. m4, EEA1, and Rab5S34N-GFP are widely distributed in the cell and do not co-localize. The two lower rows both illustrate examples of cells transfected with constitutively active Rab5Q79L. Expression of Rab5Q79L produces both large puncta (arrows, third panel from top) and enlarged vacuoles (bottom panel). m4 co-localizes with EEA1 and Rab5Q79L-GFP positive puncta (arrows). In cells in which enlarged vacuoles are produced, m4 localizes to the perimeter where it co-localizes with EEA1 and Rab5Q79L-GFP. However, unlike EEA1 and Rab5, m4 distributes to the interior of the vacuole where it does not co-localize with these markers. Scale bar = 10 μm.

Ultrastructural Analysis of Vacuole Produced by Rab5Q79L—Because m4 is a 7-transmembrane spanning receptor it most likely localizes to membranes within the vacuole produced by constitutively active Rab5Q79L. Fig. 6 shows the ultrastructural characteristics of an enlarged vacuole formed by expression of Rab5Q79L. The vacuoles are very large (6 μm diameter, Fig. 6A) and the structures are surrounded by a limiting membrane which encloses luminal material that is more electron dense than the surrounding cytoplasm. The vacuoles often contain several large, irregular membrane profiles (Fig. 6B) and are filled with multiple small, round vesicles (Fig. 6C). The presence of internal vesicles within the lumen of these vacuoles identify these structures as MVBs. Comparison of the ultrastructural appearance of the multivesicular structures with confocal images (see Fig. 5) suggests that m4 localizes both to the limiting membrane and internal vesicles. Therefore, in addition to increasing endosomal size, Rab5Q79L produces enlarged MVBs.

Requirement of m4 Activation for Sequestration within Vacuole Produced by Rab5Q79L—m4 localizes within the large MVBs produced by Rab5Q79L after CCh treatment and in unstimulated cells. At baseline, cell surface m4 could be activated by ACh released by PC12 cells (26, 27) and hence internalized. Substantial levels of choline acetyltransferase activity have been measured and therefore the PC12 cells used in this study synthesize ACh. Thus, sequestration of m4 within large vacuoles formed by Rab5Q79L may depend on m4 stimulation and endocytosis from the plasma membrane. Alternatively, Rab5Q79L expression may target m4 directly from biosynthetic compartments to endosomal compartments, thus preventing m4 from ever reaching the cell surface and causing its intracellular accumulation. To distinguish between these possibilities, the mAChR antagonist, atropine was used to inhibit mAChR activation and consequent internalization. In vector-transfected control cells, prolonged atropine treatment (2.5 h) does not significantly enhance m4 co-localization with Na+/K+-ATPase compared with untreated cells (Fig. 7B). In Rab5Q79L expressing cells, although the enlarged MVBs are still formed, atropine prevents the intracellular accumulation of m4 (Fig. 7A). m4 overlap with Na+/K+-ATPase is significantly increased following atropine treatment compared with untreated cells such that co-localization is equivalent to vector transfected control cells (Fig. 7B). Therefore, Rab5Q79L does not prevent the targeting of m4 to the plasma membrane. By preventing activation and consequent internalization, atropine prevents Rab5Q79L from sequestering m4 intracellularly. Because atropine inhibits m4 endocytosis, and cycloheximide blocks m4 synthesis, only the recycling and degradative pathways remain. Thus, m4 shows increased cell surface localization following 2.5 h atropine treatment compared with untreated cells because m4 either returns to the cell surface or is degraded. In either case, these data demonstrate that m4 must traffic through the endocytic pathway to be delivered to the enlarged MVBs.

Comparison of m4 Intracellular Trafficking to Constitutively Endocytosed and Recycled Tfn—We show that in control cells, m4 traffics through Tfn and EEA1 positive early endosomes (Fig. 2). However, unlike Tfn which completely recycles to the cell surface (6), internalized mAChRs can be targeted for degradation (1). In constitutively active Rab5Q79L expressing cells, EEA1 localizes selectively to the perimeter of the large vacuoles while m4 localizes both to the perimeter and within the vacuoles, suggesting that m4 also traffics to a membrane.

—C. Heilman and A. Levey, unpublished observations.
compartment distinct from early sorting endosomes. Therefore, m4 and Tfn trafficking were compared to determine whether m4 localizes to membrane compartments within the multivesicular structure distinct from a constitutively endocytosed and recycled protein. In Rab5Q79L expressing cells, internalized m4 and Tfn co-localize in some puncta. However, m4 and Tfn show remarkably distinct distributions within the large vacuoles (Fig. 8). Whereas Tfn localization is restricted to the perimeter of the vacuole, m4 is also found in the lumen of vacuole. These data demonstrate that these large vacuoles formed by Rab5Q79L are capable of segregating m4 from constitutively endocytosed and recycled Tfn.

**DISCUSSION**

We show that following agonist stimulated internalization, m4 trafficked to Tfn- and EEA1-positive early sorting endosomes. m4 trafficking from the plasma membrane to early endosomes depends on Rab5 activity as Rab5 mutants produced dramatic effects on m4 localization at cell surface and within early endosomes. The dominant negative Rab5S34N inhibited CCh-induced m4 internalization and the constitutively active Rab5Q79L enhanced m4 intracellular localization even in unstimulated cells. In particular, expression of constitutively active Rab5Q79L resulted in formation of markedly enlarged vacuoles containing vesicles and resembling MVBs, suggesting a surprising new role for Rab5 in MVB biogenesis. While Rab5, EEA1, and Tfn localized selectively to the perimeter of these MVBs, m4 distributed both to the perimeter and the interior of the MVB. Therefore our data suggest a novel model by which MVBs segregate GPCRs away from pathways traveled by constitutively endocytosed and recycled proteins.

To analyze m4 mAChR intracellular trafficking, we developed a method to quantitate changes in the cell surface distribution of m4 in single cells. This method provides several advantages by allowing: 1) analysis of the m4 subtype of mAChR specifically; 2) investigation of the trafficking of an
Constitutively active Rab5Q79L enhanced m4 intracellular distribution even in unstimulated cells and in particular targeted m4 to the enlarged MVBs. m4 targeting to MVBs is consistent with a previous electron microscopy study of m4 trafficking in vivo, showing that, following agonist treatment, m4 localizes to MVBs in medium spiny neurons (17). Therefore, our data highlight that in addition to early endosomes, MVBs are important organelles involved in m4 trafficking. We show that targeting to this vacuole requires m4 activation (i.e. is atropine sensitive) and thus trafficking through an endocytic pathway. Treatment with atropine most likely prevents intracellular accumulation of m4 at baseline in Rab5Q79L expressing cells by inhibiting m4 activation by ACh released by PC12 cells. However, although ACh is synthesized by the clone of PC12 cells used in this study, it is possible that ACh is not released at sufficient levels to stimulate mAChRs. Thus, another mechanism by which atropine prevents intracellular accumulation of m4 is by preventing a conformational change in m4 that allows the receptor to internalize.

In Rab5Q79L expressing cells, m4 localization by confocal microscopy suggests that m4 is present on the MVB limiting membrane and internal vesicles within the MVB (see Fig. 5). Tfn, however, localizes selectively to the perimeter of the MVB. Because Tfn is a typical marker of the recycling pathway, m4 on the limiting membrane may be able to recycle, while m4 that localizes to the internal vesicles may be targeted for degradation. Although our study did not address the fate of the m4 receptors localized to the MVB, previous cell biological studies provide intriguing clues that MVB may segregate recycling receptors from those targeted for degradation in lysosomes. For example, the TfnR localizes to the limiting membrane of MVBs while lysosomally targeted EGFR are found within internal vesicles (29). In addition, unlike wild type EGFR, a kinase-deficient EGFR internalizes, is not degraded, and recycles to the plasma membrane. This EGFR mutant localizes selectively to the perimeter of the MVB (30).

As discussed in a recent review (31), controversy exists in the literature regarding the organelles responsible for sorting GPCRs from recycling pathways to lysosomal/degradative pathways. We propose that following agonist activation, GPCRs initially internalize into early sorting endosomes in common with Tfn. GPCRs and Tfn also travel to the limiting membrane of MVBs and can recycle from both early endosomes and the limiting membrane. However, GPCR and Tfn trafficking diverge, as GPCRs localize to internal vesicles of the MVBs. Similar to the EGFR, we suggest that GPCRs in internal vesicles are targeted to lysosomes for degradation.

In conclusion, we show that following activation and internalization, endogenously expressed m4 traffics through an endocytic pathway in common with Tfn. The cell surface and early endosomal localization of m4 depends on Rab5 activity as mutants affecting Rab5 activity produce striking changes in m4 intracellular localization. Moreover, our results also reveal MVBs as important organelles involved in GPCR trafficking and suggest that MVBs segregate recycling GPCRs from those targeted to a nonrecycling, possibly degradative pathway. By determining the organelles through which GPCR traffic, we can begin to elucidate the mechanisms that sort them to recycling or to degradative pathways, and thus better understand how the cell controls the cellular responsiveness to ligand.

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