The mechanism by which muscarinic receptors internalize upon agonist exposure is poorly understood. To determine the endocytic pathways responsible for muscarinic receptor internalization, we have stably transfected human embryonic kidney (HEK 293) cells with the Hm1 (human muscarinic subtype 1) receptor tagged at the amino terminus with the epitope EYMPME. The subcellular location of the receptor was visualized by immunofluorescence confocal microscopy and quantified with the use of binding studies. The receptor redistributed into intracellular compartments following agonist treatment. This process was reversible upon removal of agonist and inhibited by antagonist. Acid treatment of the cells, which disrupts internalization via clathrin-coated vesicles, inhibited carbachol-stimulated internalization. Phorbol 12-myristate 13-acetate, on the other hand, which inhibits caveola-mediated endocytosis, had no effect on carbachol-induced endocytosis. Double-labeling confocal microscopy was used to characterize the intracellular vesicles containing Hm1 receptor following agonist treatment. The Hm1 receptor was shown to be colocalized with clathrin and α-adaptn, a subunit of the AP2 adaptor protein which links endocytosed proteins with clathrin in the intracellular vesicles. In addition, endosomes containing Hm1 also contained the transferrin receptor, which internalizes via clathrin-coated vesicles. In contrast, caveolin, the protein that comprises caveolae, did not colocalize with Hm1 in intracellular vesicles following agonist treatment, indicating that caveolae are not involved in the agonist-induced internalization of Hm1. These results indicate that agonist-induced internalization of the Hm1 receptor occurs via clathrin-coated vesicles in HEK cells.

Upon agonist treatment, many cell surface receptors undergo endocytosis into compartments inaccessible to extracellular ligands. This process is known as receptor internalization. The mechanism of internalization and its role in receptor regulation and function are largely unknown. Internalization may be a possible mechanism of receptor desensitization (i.e. reduction in agonist-induced activity), or alternatively, as recently suggested by Pippig et al. (1), resensitization. The endocytosed receptors may be either recycled back to the cell surface (2) or transported to lysosomes where receptors are subsequently degraded (2), a process known as receptor downregulation.

Cell surface proteins may internalize into clathrin-coated vesicles (3), caveolae (4), or noncoated vesicles (5, 6). Little is known about the mechanism of internalization for the family of G protein-coupled receptors. Studies thus far suggest that the mechanism may vary with the receptor type. Raposo et al. (7) showed that noncoated vesicles appear to be involved in the endocytosis of muscarinic receptors in CCL137 fibroblast cells. On the other hand, Silva et al. (8) have shown muscarinic receptor activity in purified clathrin-coated vesicles from bovine brain. Using electron microscopy, β2-adrenoreceptors have been shown, by Raposo et al. (9), to internalize via noncoated vesicles in A431 cells. In contrast to the report by Raposo et al. (9), von Zastrow and Kobilka (10), using immunofluorescence microscopy, have demonstrated that in HEK 293 cells the β2-adrenoreceptor is endocytosed and recycled into endosomes carrying the constitutively recycling transferrin receptor, which undergoes endocytosis via clathrin-coated vesicles (3, 11-13). In addition, Fonseca et al. (14) have shown that the α1B-adrenoreceptor colocalizes with transferrin receptor after agonist treatment. Moreover, the human chorionic gonadotropin hormone/leutinizing hormone receptor (2) and gastrin releasing peptide receptor (15) and neurokinin 1 and 2 receptors (16) also appear to be endocytosed via clathrin-coated vesicles. Hence, the mechanism of endocytosis of this family of receptors remains controversial.

Endocytosis via caveolae is another possible mechanism by which internalization may occur. The folate receptor, for example, which is involved in the transfer of folate into cells, has been shown to be localized in caveolae (17, 18). Although caveolae may be involved in receptor-mediated endocytosis, these cellular compartments have also been implicated in intracellular signaling (17-20). A number of molecules implicated in various cellular signaling pathways have been found associated with caveolae. These molecules include an inositol-1,4,5-trisphosphate-sensitive calcium pump (21), src-like protein kinases (22, 23), heterotrimeric G proteins (α and β subunits) (22), Rap GTPases (19, 20), and ATP-dependent calcium pump (21). In addition, Chun et al. (24) have shown colocalization of the endothelin receptor with caveolin in the absence of any agonist.

Details of the cellular trafficking of the Hm1 receptor remain unknown. In the present study, we determined the role of clathrin-coated vesicles and caveolae in carbachol-induced internalization of the Hm1 receptor transfected into HEK 293 cells using immunofluorescence confocal microscopy. Our results suggest that in this cell system, carbachol-induced internalization of the Hm1 receptor occurs via clathrin-coated vesicles.
EXPERIMENTAL PROCEDURES

Materials—Fish gelatin, poly-L-lysine, and carbochab were purchased from Sigma, and Fluoromount G was obtained from Fisher Scientific (Pittsburgh, PA). The monoclonal antibody to the epitope EYMPME (anti-E) was purchased from Onyx Inc. (Richmond, VA). The Cy5 (indocarbocyanine) labeling kit and Cy3 (indocyanine)-conjugated goat anti-mouse IgG antibodies were obtained from Biological Detection Systems Inc. (Pittsburgh, PA). FITC-conjugated goat anti-mouse IgG antibody was purchased from Cappel Technika (Durham, NC). Monoclonal antibody to transferrin receptor was purchased from Amersham. Monoclonal and polyclonal antibodies to chlathrin heavy chain and monoclonal antibody to a-adaptin were generous gifts from Dr. Frances Brodsky, UCSF. Monoclonal and polyclonal antibodies to caveolin were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibody to the C terminal of the Hm1 receptor was a generous gift from Drs. Stefan Nahorski and Andrew Tobin, University of Leicester, UK.

Epitope Tag and Stable Expression of Hm1—The gene encoding the human muscarinic cholinergic subtype I receptor (Hm1) was obtained from a human placental genomic library as described previously (25). An epitope tag with the sequence EYMPME was added to the amino-terminal of the receptor using the polymerase chain reaction. The 5' primer with sequence TGAATTCACCATGGAATACATGCCAATG-GAAAACACTTCAGCCCCACCTGCTGTC was synthesized which contained the following components: an EcoRI restriction site, a 3'-nucleotide spacer, an initiating methionine, the sequence coding for the tag, and a portion of Hm1 sequence. The 3' primer with sequence TTG-GCCGCTGTCCGTCTGTCTGCCCGTA contained a BamHI restriction site. The double-digested polymerase chain reaction product was ligated into pSG5, and the construct was used to transfect DH53a by electroporation (Bio-Rad Gene Pulser, Hercules, CA). The sequence of a selected clone was verified and the plasmid was co-transfected with pRSMG into human embryonic kidney (HEK 293) cells by the calcium phosphate precipitation method. Clonal cell lines expressing EE-Hm1 sites were selected in Dulbecco's modified Eagle's medium/H-16/F-12 containing 10% fetal calf serum, 1% penicillin/streptomycin, and 400 mg/ml G418 in 5% CO2 and maintained in this medium with 200 mg/ml G418.

Inhibition Studies of Caveolin, Clathrin, and AP2—Cells stably transfected with EE-tagged or untagged Hm1 receptors were seeded onto 12-well cell culture dishes, allowed to attach overnight, and treated with 1 mM carbachol for varying times. After washing with ice-cold PBS, cell surface receptor binding was assessed using the hydrophilic ligand [3H]NMS. Each point is the average of quadruplicate points. Error bars represent standard deviation.

RESULTS

Cells grown overnight on chamber slides were pretreated with either 5 mM acetic acid in HEPES (pH 5.0) for 5 min (27) or 1 mM phorbol 12-myristate 13-acetate (PMA) for 30 min at 37°C (28). Following pretreatment, 1 mM carbachol or buffer was added, and cells were incubated for an additional 30 min at 37°C. Cells were permeabilized, stained, and visualized as described above.

Colocalization Studies of EE-Hm1 Receptor Internalization—For double labeling studies, we directly conjugated a fluorophore (Cy5) to the anti-EE mouse monoclonal antibody. Detection of the second protein of interest was carried out sequentially with primary antibody and secondary antibody conjugated to a second fluorophore (Cy3). This allowed us to use two mouse primary antibodies to localize the proteins of interest without the possibility of interaction of the secondary antibody with both primary antibodies. The colocalization assay was carried out as follows: cells grown for 1 or 2 days on poly-L-lysine-treated chamber slides were treated with specified times with 1 mM carbachol or buffer in serum-free medium at 37°C. Cells were washed, fixed, and permeabilized as above, then incubated for 1 h with monoclonal antibody to clathrin, AP2, transferrin receptor, or caveolin. After four more washes with PBS, cells were incubated with Cy3-labeled goat anti-mouse secondary antibody for 30 min followed by four washes with PBS prior to incubation with Cy5-conjugated anti-EE monoclonal antibody. After washing, cells were mounted with mounting media and visualized as above. When polyclonal antibodies to clathrin or cavinolin were used, the cells were sequentially incubated with primary antibody to dextrin or cavinolin, followed by Cy3-labeled secondary antibody (donkey anti-rabbit) and then with anti EE-antibody followed by Cy5-labeled goat anti-mouse secondary antibody. For the polyclonal Hm1 receptor antibody, Cy3-conjugated donkey anti-rabbit secondary antibody was used to label anti-Hm1 antibody, followed by primary monoclonal antibody to dextrin, a-adaptin and Cy5-conjugated goat anti-mouse secondary antibody. Cy3/Cy5 double emission was detected using a C1/C2 filter block combination (29). Images of a misalignment of two from different photomultiplier tubes were collected simultaneously and then superimposed to identify areas of colocalization. When the images are merged, Hm1 is arbitrarily colored red, the other proteins (clathrin, adaptin, transferrin receptor, or caveolin) are colored green, and areas of colocalization appear yellow.

All data represented are from a representative experiment repeated three times or more unless indicated otherwise.

Internalization of Tagged and Untagged Hm1 Receptor Measured by Binding Assay—Cells stably transfected with the tagged or untagged Hm1 receptor were treated with or without 1 mM carbachol in serum-free medium for varying times. After carbachol treatment, cells were washed, and [3H]NMS binding was measured. The time course of receptor internalization of the tagged and untagged receptors (Fig. 1) was similar.
Internalization of Tagged Hm1 Receptor by Confocal Microscopy—Carbachol-induced internalization of the tagged Hm1 receptor could be visualized by immunofluorescence confocal microscopy using the antibody to the epitope tag followed by a secondary antibody conjugated to a fluorophore (FITC, Cy3, or Cy5). In the absence of carbachol, receptors resided predominantly at the cell surface (Fig. 2A), although in some cells a small degree of intracellular staining could also be observed. Part of theintracellular staining was hazy nonspecific staining, while in some cells vesicular intracellular staining could also be observed. These vesicles in the unstimulated cells could represent Hm1 in transit to the surface following synthesis or receptors continuously cycling from the cell surface into the cell and back to the cell surface. After 30 min of carbachol treatment, receptors were redistributed to endosomes within the cell interior (Fig. 2B), and the cell surface staining of the receptor was substantially reduced. Atropine, a muscarinic antagonist, did not cause redistribution of the Hm1 receptors from the cell surface (Fig. 2C) and blocked the internalization of Hm1 induced by carbachol (Fig. 2D). Internalization was reversible following the removal of carbachol and incubation of the cells in agonist-free medium for 1 h (Fig. 2E).

Effect of PMA and Acetic Acid on Hm1 Receptor Internalization—To determine whether or not carbachol-stimulated internalization involves caveolae, cells were pretreated with 1 mM PMA for 30 min prior to a 30-min treatment with 1 mM carbachol (4). Visualization of the receptors by confocal microscopy revealed that PMA had no effect on carbachol-induced internalization (Fig. 3, lower panels).

To disrupt clathrin-mediated endocytosis, cells were pretreated with acetic acid as described previously (27). Cells were pretreated for 5 min with 5 mM acetic acid at 37°C prior to treatment with 1 mM carbachol for 30 min. Confocal microscopy indicated that acid pretreatment completely blocked carbachol-induced internalization (Fig. 3, upper panel), indicating that internalization may be occurring via clathrin-coated vesicles.

Colocalization Studies with Hm1 and Clathrin—To further investigate the potential role of clathrin in Hm1 internalization, colocalization studies using antibodies to clathrin were performed. Cells were treated with carbachol for times varying between 0 and 20 min to determine the time course of Hm1 colocalization with clathrin. Before agonist treatment, although some intracellular staining is observed, the majority of the receptors are diffusely distributed at the plasma membrane. Double-labeling studies with a monoclonal anti-clathrin antibody showed a high degree of colocalization of Hm1 and clathrin at the cell surface prior to carbachol treatment (Fig. 4A). After 5–10 min of carbachol treatment, Hm1 staining was shifted from the plasma membrane to the cell’s interior resulting in a strong increase in the number of receptor-containing intracellular endosomes, the majority of which were also found to contain clathrin (Fig. 4, B–D, yellow vesicles). Colocalization of Hm1 with clathrin using a polyclonal antibody to clathrin gave the same results (data not shown).

In the above study, we used directly labeled monoclonal antibody to the epitope tag on the receptor along with monoclonal antibody to clathrin. In order to rule out the possibility of any false positive signal resulting from cross-reactivity of secondary antibody with directly conjugated antibody, double-labeling studies of clathrin were carried out using a polyclonal antibody to the C tail of the Hm1 receptor. Before agonist treatment, Hm1 was diffusely located at the cell surface, where it was colocalized with clathrin (data not shown). Following 10 min of carbachol treatment, cell surface staining of Hm1 was dramatically decreased, and the receptor was translocated into intracellular vesicles containing clathrin (Fig. 4E). The high degree of intracellular colocalization of Hm1 with clathrin is still observed (Fig. 4E, lower panel, yellow vesicles) indicating that the observed colocalization is in fact real and not due to any cross-reactivity of antibodies.

We also studied the effect of treatment with the muscarinic antagonist atropine on the colocalization pattern of the Hm1 receptor. When double-labeling studies were performed in the presence of atropine alone or in combination with carbachol, little vesicular Hm1 staining was observed intracellularly (as seen in Fig. 2, C and D), and Hm1 receptors at the cell surface colocalized with clathrin (Fig. 4F). Thus, the antagonist blocks carbachol-induced redistribution of the receptor into intracellular vesicles but not its association with clathrin at the plasma membrane.

Colocalization Studies with Hm1 and β-Adaptin—To confirm the role of clathrin-coated vesicles in the internalization of Hm1, colocalization studies were also carried out with β-adaptin. β-Adaptin appeared in a punctate pattern throughout the interior of the cell (Fig. 5A, top panel). The AP2 complex has
been shown to be localized to vesicles in the proximity of the plasma membrane (26); thus, the rather evenly distributed punctate staining of the \( \alpha \)-adaptin indicates that the optical section chosen for visualization is located above the cell nucleus. This staining did not dramatically change after carbachol treatment (Fig. 5, B–E, top panels). Hm1 receptors were localized primarily at the cell surface before any treatment (Fig. 5A, middle panel). Again, a small degree of intracellular staining was present in some cells prior to agonist treatment; however, part of the intracellular labeling represented a hazy background staining. This background staining was accentuated in the color photographs as compared to the black and white micrographs (Figs. 2 and 3). The vesicular intracellular staining again possibly corresponded to receptors coming to the cell surface after synthesis or representative of continuously cycling receptors. Following agonist treatment, plasma membrane staining of Hm1 markedly shifted to the intracellular vesicles (Fig. 5, B–E, middle panels), resulting in a strong increase in the intracellular and subsurface staining of the receptor in the form of intracellular vesicles and a parallel decrease in cell surface staining. The Hm1 receptor and \( \alpha \)-adaptin were colocalized at the cell surface prior to any agonist treatment (Fig. 5A, lower panel) as shown by the yellow color. Although some intracellular vesicles containing Hm1 were present prior to agonist treatment, \( \alpha \)-adaptin was absent from some of these vesicles (Fig. 5A, lower panel, red vesicles). After several minutes of agonist treatment, (Fig. 5, B–D, lower panels), a marked relocation of the receptor from the cell surface to the cell’s interior occurred. Many intracellular vesicles containing both the receptor and \( \alpha \)-adaptin (yellow vesicles) appeared, and receptor-\( \alpha \)-adaptin colocalization at the cell membrane strongly decreased. To confirm these results, we carried out experiments with the monoclonal antibody to Hm1. The staining prior to agonist treatment was similar to that observed with the monoclonal antibody (data not shown). After agonist treatment, once again a dramatic shift of the receptor staining from the cell surface to the intracellular vesicles was observed (Fig. 5E), and vesicles containing both receptor and \( \alpha \)-adaptin predominated (yellow vesicles). In all these cells (Fig. 5, lower panels), some of the intracellular vesicles appeared red, suggesting that a population of intracellular receptors exists that are not colocalized with these adaptor proteins. We propose that Hm1 receptors not colocalized with the AP2 protein either are being transported from the Golgi to the cell surface following synthesis or recycled back to the cell surface following internalization. This recycling mechanism is important in HEK cells where no receptor down-regulation occurs (30). Either transport process would account for an association with clathrin, which is involved in a number of transport processes (31, 32) but not the AP2 protein, which is exclusively involved in the endocytic pathway.

Colocalization Studies with Hm1 and Transferrin Receptors—In addition to clathrin and \( \alpha \)-adaptin, we also performed double-labeling studies with anti-transferrin receptor antibody to further confirm the role of clathrin-coated vesicles in internalization of Hm1. As expected, transferrin receptor was present at the cell periphery and throughout the cell’s interior (Fig. 6, A and B, top panel). Hm1 was present primarily at the cell surface prior to agonist treatment (Fig. 6A, middle panel) as seen by strong plasma membrane staining. After agonist treatment, this peripheral staining was replaced by predominantly intracellular, vesicular staining (Fig. 6B, middle panel). The merged images of transferrin receptor and Hm1 (lower panel) indicated a colocalization primarily at the cell surface prior to carbachol treatment (Fig. 6A), with a shift to intracellular vesicles after agonist treatment (Fig. 6B). Contrary to results with \( \alpha \)-adaptin, most of these vesicles were colored yellow (i.e. stained with both antibodies), suggesting that the majority of

Fig. 4. Colocalization of EE-Hm1 with clathrin. HEK cells expressing EE-Hm1 were treated with 1 mM carbachol for varying times. After fixing and permeabilizing, cells were sequentially labeled with monoclonal antibody to clathrin followed by Cy3-labeled goat anti-mouse secondary antibody. Then Hm1 was labeled with Cy5-labeled anti-EE antibody (A–D) or polyclonal antibody to the carboxyl terminus of Hm1 followed by Cy5-labeled donkey anti-rabbit secondary antibody (E). The green color indicates the localization of clathrin (top panel), red is the localization of Hm1 (middle panel), and yellow is indicative of colocalization of the two proteins in the merged image (lower panel). A, no carbachol treatment; B, 5 min of carbachol; C, 10 min of carbachol; D, 20 min of carbachol; E, 10 min of carbachol with polyclonal antibody to Hm1; F, 10 min of carbachol (1 mM) + atropine (10 \( \mu \)M). Images from a midsection of the cells are shown. Arrows point to representative areas of colocalization.
Hm1 receptors located intracellularly were localized to the same compartments as the constitutively recycling transferrin receptors. The presence of a few intracellular vesicles containing both Hm1 and transferrin receptors in the absence of any carbachol treatment supports the notion that a small population of the Hm1 receptors recycle in and out of the cell at the resting state. However, there is a marked difference in the degree of punctate intracellular staining before and after carbachol treatment (compare Fig. 2, A and B). It should be emphasized that the nature of these observations is primarily qualitative. Conclusions were based on observations of the extent of colocalization as indicated by yellow color and the location of the colocalization apparent in intracellular vesicles.

Colocalization Studies with Hm1 and Caveolin—To confirm our preliminary conclusion that caveolae are not involved in carbachol-induced Hm1 internalization, we performed double-labeling studies using a monoclonal antibody to caveolin, a major protein component of caveolae. Cells were treated in the presence or absence of 1 mM carbachol, fixed, and sequentially labeled with antibody to caveolin and Hm1 receptor. Caveolin staining (green) was primarily at the cell surface with some hazy intracellular staining also present. In the absence of carbachol, some degree of colocalization was apparent at the surface of the cells (Fig. 7A). The shift of Hm1 staining (red) from the cell surface to the cell interior was dramatic, as described above. After carbachol treatment, almost no colocalization of Hm1 and caveolin was observed (Fig. 7, B–E). Most intracellular vesicles were stained only with antibody to the tagged Hm1 receptor and appeared red. Intracellular regions appeared yellow only when an area of dense staining was present in the cell, suggesting that the colocalization is coincidental. Wherever the vesicles were evenly distributed (see Fig. 7, C versus D and E), no colocalization was observed (no yellow vesicles). Results from separate experiments using a polyclonal antibody to caveolin support these findings (data not shown). In addition, these negative results further confirm that the colocalization of Hm1 with clathrin, α-adaptin, and transferrin receptor is not due to the cross-reactivity of the antibodies.

**DISCUSSION**

Receptor regulation remains a poorly understood phenomenon for the G protein-coupled receptors in general and for Hm1 specifically. Agonist-induced receptor internalization, as a mechanism of receptor regulation, remains to be clarified. The first step toward a clearer understanding of the mechanism of internalization of Hm1 is to define the pathway by which the receptor is transported into the interior of the cell. In this study we report the first comprehensive work describing the pathway of internalization of Hm1 using immunofluorescence confocal microscopy.

We have shown that the Hm1 receptor internalizes into intracellular vesicles following agonist treatment. This process is inhibited by the antagonist atropine and is reversible. Our results suggest that carbachol-stimulated internalization of Hm1 receptors in HEK cells occurs via clathrin-coated vesicles, consistent with an earlier study that demonstrated the presence of muscarinic receptors in bovine brain-coated vesicles (8) and with a recent study that showed inhibition of muscarinic receptor internalization in SH-SY5Y cells following perturbation of clathrin distribution (33). The fact that internalization...
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is blocked by acetic acid, which prevents internalization by clathrin-coated vesicles, but not by PMA, which inhibits caveolae formation, provides indirect evidence that clathrin-coated vesicles, and not caveolae, are involved in Hm1 internalization. Double-labeling studies of Hm1 with clathrin, α-adaptin, transferrin receptor, and caveolin provide further evidence in support of this conclusion. The colocalization after carbachol treatment of Hm1 and clathrin in intracellular vesicles confirms that carbachol-induced internalization involves the interaction of Hm1 receptors with clathrin. The apparent colocalization at the cell surface before and after agonist treatment may be due to the association of these two proteins at the plasma membrane, i.e., the presence of Hm1 in clathrin-coated pits. This prelocalization in coated pits has been shown previously by electron microscopy for the human chorionic gonadotropin hormone/leutinizing hormone receptor (2). The preassociation of Hm1 with clathrin in the absence of agonist may be indicative of a population of Hm1 receptors recycling at the steady state level, as was demonstrated for the β2 adrenoreceptor (10). In addition, the presence of an intracellular population of Hm1 at the steady state level suggests that a small population of the receptor might be cycling in and out of the cell without any agonist treatment. A similar phenomenon has previously been observed for the β2 adrenoreceptor (34) and the α2-adrenoreceptor (35) using electron microscopy. Thus, it is possible that this basal internalization is a common feature of these receptors, which is generally not detected by the less sensitive methods such as binding. The use of confocal microscopy and electron microscopy, however, allows us to focus on individual cells enabling us to observe this phenomenon.

Adaptor proteins are proteins associated with clathrin coated vesicles that mediate the interaction of receptors with the clathrin triskelion. Two populations of adaptor proteins exist in the cells. The plasma membrane adaptor protein (AP2) associates with clathrin during endocytosis, and the Golgi adaptor protein (AP1) associates with clathrin during transport of protein from the Golgi to the cell surface (31). Double-labeling studies with α-adaptin, a subunit of AP2, again implicate a clathrin-mediated mechanism of Hm1 internalization. Following carbachol treatment, Hm1 receptors clearly interact with α-adaptin in intracellular vesicles. The presence of two populations of Hm1 receptors, one which colocalizes with α-adaptin and one which does not, suggests that some Hm1 receptors are either being recycled back to the cell surface, transported to the cell surface following synthesis, or both, in addition to being internalized into AP2-containing intracellular compartments. Since AP2 complex is only involved in the endocytosis of the receptors, we would not expect to observe this complex in the recycling endosome or the endosome involved in secretion. This is in contrast to clathrin staining, since clathrin has been shown to be involved in all three processes of endocytosis, together with AP2 complex (31), protein secretion from the Golgi, together with AP1 complex (31) and receptor recycling from the endosome to the cell surface, together with an as yet unidentified adaptor protein (32). Thus, we observe a greater colocalization of Hm1 with clathrin and transferrin receptor compared to α-adaptin.

Furthermore, our studies indicate that Hm1 receptors are associated with transferrin receptors during the entire endocytic pathway. The continuous recycling of transferrin receptor is known to be clathrin-mediated (3, 11, 12, 36, 37). The observation that Hm1 receptors in intracellular vesicles are completely colocalized with transferrin receptors is consistent with the idea that Hm1 receptors are being internalized and recycled to the cell surface by an identical pathway as the constitutively recycling transferrin receptors. This observation is consistent with those of other investigators who have shown colocalization of a G protein-coupled receptor with transferrin receptor (10, 16) or transferrin (14, 15).

In order to address the possibility that other mechanisms of receptor-mediated endocytosis may be involved in Hm1 internalization in HEK cells, we investigated the colocalization of Hm1 with caveolin, a major protein in caveolae. To date, the exact role of caveolae in the cells has not been clarified. While these compartments have been shown to be involved in pinocytosis (4), they have also been implicated in cellular signaling (19, 20, 24). Our double-labeling studies with caveolin confirm the preliminary conclusion that caveolae are not involved in agonist-stimulated internalization of Hm1 receptors. There is some colocalization at the cell surface prior to agonist treatment. This colocalization might be an artifact stemming from the proximity of both proteins at the cell surface. On the other hand, the surface colocalization could indicate that the Hm1 receptor is in contact with caveolae for other receptor functions such as signaling. Chun et al. (24) have reported colocalization of the endothelin receptor with caveolin in the absence of agonist, indicating that this G protein-coupled receptor might in fact be in contact with caveolae to mediate signaling. Our results indicate that the same could also be true for the Hm1 receptor. More detailed experiments are necessary to investigate this phenomenon.

Receptor-mediated endocytosis could also occur via non-coated vesicles. Raposo et al. (7) have reported that muscarinic receptors internalize into noncoated vesicles in CCL137 cells.
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iestononcoated vesicles were not available to us. Furthermore, cell line. In this study, we did not address the possibility of a different pathway of endocytosis of Hm1 is functional in each cell line. Thus, it is possible that since the fate of Hm1 is different in HEK cells and CCL137 cells, different pathways are functional in its trafficking. Another possible explanation is that the two cell lines contain different proteins such as G proteins and other proteins involved in internalization so that a different pathway of endocytosis of Hm1 is functional in each cell line. In this study, we did not address the possibility of colocalization with noncoated vesicles, mainly because antibodies to noncoated vesicles were not available to us. Furthermore, Roettger et al. (38) have suggested that the noncoated vesicles shown to be associated with muscarinic receptors in CCL137 cells in Raposo's study may in fact be caveolae.

From the data presented in this paper, the following main conclusions can be drawn. First, Hm1 receptor internalizes into intracellular vesicles after agonist treatment, and this process is reversible following removal of carbachol from the medium and capable of being inhibited by the antagonist atropine. Second, the results indicate that internalization of Hm1 occurs by a clathrin-mediated pathway. This is the first time internalization of a G protein-coupled receptor has directly been shown to involve clathrin-coated vesicles. Examination of the internalization of other G protein-coupled receptors has yielded evidence of colocalization of these receptors with transferrin (15) or the transferrin receptor (10, 14, 16) and hence inferred the involvement of a clathrin-mediated pathway of endocytosis. Third, the results also suggest that a population of Hm1 receptors may be constitutively recycling in the absence of any agonist and that treatment with the agonist increases the rate of receptor internalization. Finally, our results do not exclude the interaction of the Hm1 receptor with caveolae and the involvement of caveolae in receptor signaling, but strongly suggest that caveolae are not involved in agonist-induced endocytosis of Hm1 receptor.

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