Internalization is an important mechanism regulating the agonist-dependent responses of G-protein-coupled receptors. The internalization of the M2 muscarinic cholinergic receptors (mAChR) in HEK293 cells has been demonstrated to occur by an unknown mechanism that is independent of arrestins and dynamin. In this study we examined various aspects of the trafficking of the M2 mAChR in HEK293 cells to characterize this unknown pathway of internalization. Internalization of the M2 mAChR was rapid and extensive, but prolonged incubation with agonist did not lead to appreciable down-regulation (a decrease in total receptor number) of the receptors. Recovery of M2 mAChRs to the cell surface following agonist-mediated internalization was a very slow process that contained protein synthesis-dependent and -independent components. The protein synthesis-dependent component of the recovery of receptors to the cell surface did not appear to reflect a requirement for synthesis of new receptors, as no changes in total receptor number were observed either in the presence or absence of cycloheximide. Phosphorylation of the M2 mAChR did not appear to influence the rate or extent of the recovery of receptors to the cell surface, as the recovery of a phosphorylation-deficient mutant M2 mAChR, the N,CAla-8 mutant, was similar to the recovery of the wild type M2 mAChR. Finally, the constitutive, nonagonist-dependent internalization and recycling of the M2 mAChR was very slow and also contained protein synthesis-dependent and -independent components, suggesting that a similar pathway controls the recovery from agonist-dependent and -independent internalization. Overall, these data demonstrated a variety of previously unappreciated facets involved in the regulation of M2 mAChRs.

G-protein coupled receptors (GPCRs) are heptahelical transmembrane receptors that mediate cellular responses to various stimuli such as hormones and neurotransmitters. Stimulation of GPCRs by their agonists results in activation of heterotrimeric G-proteins leading to the regulation of a variety of signal transduction events in the cell. One of the major mechanisms modulating the cellular response to agonists is regulation of the GPCRs themselves. There are multiple processes involved in the regulation of GPCRs in response to agonist stimulation, and these processes are very complex and not well understood (for reviews see Refs. 1 and 2).

Desensitization of GPCRs is believed to occur very rapidly following their activation by agonist. Agonist stimulation leads to phosphorylation of GPCRs by specific G-protein-coupled receptor kinases on serine and threonine residues (1–3). This phosphorylation then allows for interaction of the GPCRs with proteins termed arrestins (4). Arrestin binding is believed to cause desensitization by uncoupling the receptors from their G-proteins, thus preventing further signaling by the receptors (3).

Following receptor G-protein uncoupling many GPCRs are removed from the cell surface by a process known as receptor internalization or endocytosis. For some receptors, such as the beta2-adrenergic receptor (beta2AR), arrestin binding to the receptors also serves to target the receptors to clathrin-coated pits for internalization (5, 6). This function is dependent on the C termini of nonvisual arrestins, which contain a clathrin-binding domain (7). This pathway of internalization has been demonstrated to be dependent on the hydrolysis of GTP by the GTPase dynamin, which is believed to cause fission of the endocytic vesicles from the plasma membrane (8).

The roles of internalization in desensitization of GPCRs are not well understood. It has been demonstrated that internalization serves to allow for resensitization of certain GPCRs, such as the beta2AR (9, 10). Following their internalization, beta2ARs are delivered to endosomes, where a specific GPCR phosphatase associates with and dephosphorylates the receptors (11). After the receptors have been dephosphorylated and “resensitized,” they are then recycled to the plasma membrane where they are once again capable of responding to agonist stimulation (9, 10). In contrast, it has been demonstrated that internalization served to prolong desensitization of the M2 mAChR (12).

Down-regulation of GPCRs, a decrease in the total number of receptors in a cell, is another type of regulation observed following activation of GPCRs (13). The mechanisms and functions of down-regulation are not well understood for most GPCRs. It is possible that down-regulation serves to decrease the responsiveness of a cell to a certain stimulus by decreasing the total number of receptors present in the cell. Down-regulation also serves to terminate signaling and to remove receptors that are only activated once, such as the protease-activated receptors in mammalian cells and the pheromone receptors in yeast (14–16). Following activation, these receptors are internalized and targeted for degradation rather than being recycled to the cell surface.

The M2 mAChR is a member of the GPCR superfamily that is responsible for various physiologically important regulatory functions in tissues such as heart, smooth muscle, and brain (17). It has been demonstrated that the agonist-dependent internalization of the M2 mAChR expressed in HEK293 cells occurs through a novel pathway that has yet to be identified.

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1 The abbreviations used are: GPCR, G-protein-coupled receptor; beta2AR, beta2 adrenergic receptor; PBCM, propylbenzilycholine mustard; CCh, carbachol; [3H]NMS, [3H]-methylscopolamine; [3H]QNB, [3H]quinuclidinyl benzilate; PBS, phosphate-buffered saline; mAChR, muscarinic cholinergic receptor.
The agonist-dependent internalization of the M2 mAChR by the endogenous machinery in HEK293 cells is independent of arrestins and dynamin (19). Agonist-dependent phosphorylation of the M2 mAChR does appear to play a key role in the internalization of these receptors, as phosphorylation-deficient mutants exhibit a reduction in the rate and extent of internalization (20).

In this report we examined characteristics of the trafficking of the M2 mAChR in HEK293 cells to further define the properties of the novel pathway responsible for the agonist-dependent internalization of the M2 mAChR and its role in receptor regulation. We observed that the recovery of M2 mAChRs to the cell surface following agonist treatment was a slow process that contained both protein synthesis-dependent and -independent components. We also demonstrated that recovery to the cell surface was not modulated by receptor phosphorylation and that the M2 mAChR does not appreciably down regulate in HEK293 cells. Finally we found that the constitutive, nonagonist-dependent internalization and recycling of the M2 mAChR in HEK293 cells does occur and was also very slow and contained both protein synthesis-dependent and -independent components.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Mediatech, Inc. (Herndon, VA). G418 was purchased from Agri-bio, Inc. (North Miami, FL). The muscarinic receptor antagonists [3H]N-methylscopolamine ([3H]NMS) and [3H]quinuclidinyl benzilate ([3H]QNB) were purchased from NEN Life Science Products. Propylbenzilylcholine mustard (PBCM) was a generous gift from Dr. J. M. Young (Cambridge, UK). Other reagents were purchased from Sigma.

Cell Culture and Transfection—HEK293 cells stably expressing the wild type M2 mAChR or the N,CAla-8 mutant have been described previously (20) and were used for all experiments described within the text. The expression levels were as follows: cell-surface M2 mAChRs or N,CAla-8, 200–300 fmol of receptor/mg of protein; total cellular M2 mAChRs or N,CAla-8, 400–600 fmol of receptor/mg of protein.

Receptor Internalization and Down-regulation Assays—The internalization assay was performed as described (19) by assessing the observed change in the number of M2 mAChRs located at the cell surface as determined by the binding of a radiolabeled, hydrophilic antagonist, [3H]NMS, which cannot cross cellular membranes. Down-regulation assays were performed by assessing the observed change in the total number of M2 mAChRs as determined by the binding of a radiolabeled, hydrophobic antagonist, [3H]QNB, which is able to cross cellular membranes and bind to all receptors in the cell. HEK293 cells stably transfected with the M2 mAChR were passed from 100% confluent 100-mm plates to 60-mm plates at a 1:5 or 1:6 dilution. Cells were then grown to confluency in the 60-mm plates before use. The cells were incubated with carbamylcholine (CCh) and any other drugs as indicated for the specified times. Unless otherwise specified, the concentration of CCh was 1 mM, and cycloheximide was used at 20 μg/ml. At the end of the incubation times, cells were washed three times with 3 ml of ice-cold PBS. Cells were then resuspended in ice-cold HEPES-buffered Dulbecco’s modified Eagle’s medium:F12 and subjected to radioligand binding with saturating concentrations of [3H]NMS (1.0–2.0 nM) for 2 h at 4 °C or [3H]QNB (1.0 nM) for 90 min at 37 °C. Non-specific binding was measured in the presence of 10 μM atropine. Protein assays were performed on an aliquot of cells from each plate to control for differences in cell density. Data were expressed as a percentage of the [3H]NMS or [3H]QNB binding observed in untreated cells.

Assay for Recovery of Receptors to the Cell Surface—The recovery assay was performed by observing the reappearance of receptors at the cell surface using the hydrophilic antagonist [3H]NMS following agonist-induced internalization. Cells were incubated with CCh +/- cycloheximide for the indicated times followed by washing 5 times with 3 ml PBS. Fresh medium +/- cycloheximide was added, and the cells were incubated at 37 °C for the indicated times. At the end of the incubation time, binding of [3H]NMS was performed as described above. Data for the recovery of receptors to the cell surface were expressed as a percentage of the [3H]NMS binding observed in cells that had not been exposed to carbamylcholine. Thus, in the recovery experiments, the zero time point represents the amount of receptors remaining at the cell surface after the indicated time of exposure to carbamylcholine. For measurement of total receptor number, [3H]QNB was used as described in the down-regulation protocol.

Alkylation of Cell-surface Receptors with PBCM—Muscarinic receptors at the plasma membrane were irreversibly alkylated with the muscarinic receptor antagonist PBCM. The irreversibly acting form of GABA was prepared as described previously (21). PBCM (10 mM in ethanol) was diluted in PBS to 100 μM and incubated for 1 h at room temperature to allow it to cyclize. The reaction was stopped by diluting the PBCM to 10 nM in PBS. The activated PBCM was then kept on ice until used. Activated PBCM was used within 2 h of activation to prevent inactivation. HEK293 cells stably transfected with the M2 mAChR were washed with PBS followed by incubation with activated PBCM (10 mM) for 30 min at room temperature. The PBCM was then removed, and the cells were washed with PBS containing 1 mM sodium thiosulfate to quench any residual, unreacted PBCM.

Assay for Constitutive Internalization and Recycling of Receptors—HEK293 cells stably expressing the M2 mAChR were either untreated or pretreated with cycloheximide for 2 h, then alkylated with activated PBCM, washed with PBS containing sodium thiosulfate, and reincubated in fresh medium +/- cycloheximide for the indicated times. Cell-surface and total receptor levels were then determined through incubation with [3H]NMS or [3H]QNB as described in the receptor internalization and down-regulation assay methods.

RESULTS

The M2 mAChR Does Not Appreciably Down Regulate in HEK293 Cells—We initially sought to examine whether the M2 mAChR down regulates (undergoes a net decrease in total cellular receptor number) in response to prolonged agonist exposure when expressed in HEK293 cells. It has been previously demonstrated that other GPCRs, such as the β2AR, down regulate in HEK293 cells (22). However, because the pathway of internalization of the M2 mAChR in HEK293 cells differs from that of the β2AR (19), we sought to determine whether the M2 mAChR down regulates as the β2AR does or whether it differs from the β2AR in this aspect as well. For these studies we used a cell line of HEK293 cells stably expressing the M2 mAChR (18). In this cell line, 50–60% of the receptors are expressed on the cell surface, whereas the remainder are present in an intracellular compartment. In previous studies, we demonstrated that the internalization of the M2 mAChR is very extensive and rapid in this cell line (18). Approximately 80% of the cell-surface receptors were internalized within 5–10 min of CCh treatment (Fig. 1A). In this study we asked if the rapid loss in cell-surface receptor number was accompanied by a loss in total receptor number. When the total (cell surface and intracellular) receptor number was determined by the binding of the cell permeable antagonist, [3H]QNB, following prolonged CCh treatment for up to 8 h, only a small change in total receptor number was observed. In other experiments, we also observed only minimal down-regulation after a 24-h treatment with CCh (not shown). Thus the M2 mAChR did not down regulate substantially in response to agonist treatment when expressed in HEK293 cells.

The Reappearance of M2 mAChRs at the Cell Surface following Agonist Treatment Was Slow and Was Partially Dependent on Protein Synthesis—Because the M2 mAChR did not down regulate but did rapidly internalize, we next sought to examine the characteristics of the recovery of M2 mAChRs to the cell surface following agonist pretreatment. After treatment of the M2 mAChR-HEK293 cells with CCh for 30 min, the cells were washed with PBS and placed in fresh medium without CCh for various periods of time. The reappearance ("recovery") of receptors at the cell surface following agonist pretreatment was very slow, requiring nearly 4 h for the cells to return to a new steady state in which cell-surface receptor density was equivalent to approximately 80% of untreated cell-surface receptor levels (Fig. 2A). Treatment with the protein synthesis inhibitor cycloheximide showed an interesting pattern of recovery (Fig. 2A). In the presence of cycloheximide the initial phase of recovery through the first 2 h was identical to cells not treated with cyclohexi-
mide. However, in cells pretreated with cycloheximide, no further recovery was observed beyond this level, whereas in the absence of cycloheximide, the density of receptors at the cell surface continued to increase. This suggested that there were two components to the reappearance of M2 mAChRs at the cell surface following agonist-induced internalization. There was an initial protein synthesis-independent component responsible for recovery to approximately 50–60% of untreated cell-surface receptor levels. This was followed by a protein synthesis-dependent component that was responsible for further recovery to the cell surface. Initially, this suggested that the initial phase of recovery reflected recycling of internalized receptors to the cell surface and that the protein synthesis-dependent component might be because of synthesis of new receptors that were delivered to the cell surface. However, as demonstrated earlier (Fig. 1B), the M2 mAChR did not appreciably down-regulate in HEK293 cells. Furthermore, this lack of down-regulation was also observed in experiments performed in the presence of cycloheximide (data not shown), suggesting that synthesis of new receptors was not required to maintain receptor density. This observation also suggested that synthesis of new receptors was not responsible for the protein synthesis-dependent component of recovery of receptors to the cell surface.

To further characterize the process of reappearance of M2 mAChR to the cell surface, we determined whether the length of agonist treatment affected the rate or extent of the recovery process. The recovery of internalized M2 mAChRs to the cell surface following a 10-min CCh treatment was similar to the recovery following a 30-min CCh treatment (Fig. 2). There appeared to be both protein synthesis-independent and -dependent components to the recovery following a 10-min CCh treatment. Interestingly, the protein synthesis-dependent component of recovery was smaller following a 10-min CCh treatment than for a 30-min CCh treatment. Thus, it appeared that the length of agonist treatment did not significantly affect the pattern of recovery of M2 mAChRs to the cell surface following agonist treatment.

To visualize the trafficking of the M2 mAChR to and away from the cell surface, we used immunofluorescent confocal microscopy. Immunostaining of the M2 mAChR showed heavy labeling of the cell surface that appeared to be continuous but did contain some punctate sites of staining. Significant intracellular staining located primarily at a perinuclear site was also detected (Fig. 3A, arrows). This intracellular staining reflected the finding from biochemical studies that approximately 40% of the total M2 mAChRs are intracellular in this cell line. Treatment with CCh for 30 min led to a striking redistribution of the M2 mAChR staining from the cell surface to a perinuclear site (Fig. 3B). Inclusion of cycloheximide with CCh had no effect on the internalization of M2 mAChRs (Fig. 3C), as was observed in the biochemical experiments (Fig. 2A, 0 time
untreated cells (compare Fig. 3A versus 3F). The staining of the 
M2 mAChR following 6 h of recovery appeared to be much more  
punctate and clustered at or near the cell surface, as opposed to  
the more continuous cell surface labeling in untreated cells.  
Overall, the results obtained with the immunofluorescent  
staining of the M2 mAChR directly reflected the results from  
the biochemical experiments performed on the recovery of  
internalized M2 mAChRs and demonstrated discrete phases in  
trafficking of the receptors to the cell surface.

Recruitment of M2 mAChRs to the Cell Surface following Agonist  
Treatment Was Independent of Receptor Phosphorylation—It  
has been reported recently that phosphorylation of the V2  
vasopressin receptor serves as an intracellular retention signal  
(23). The V2 receptor did not recycle back to the cell surface  
following agonist treatment unless the receptor was made into  
a phosphorylation-deficient mutant (23). Because we had  
previously demonstrated that phosphorylation of the M2 mAChR  
facilitated the internalization of this receptor (20), we asked  
whether phosphorylation served to regulate the recovery of the  
M2 mAChR to the cell surface. Two clusters of serine and  
threonine residues in the third intracellular loop of the M2  
mAChR have been identified as sites of agonist-dependent  
phosphorylation (20). When these residues were mutated to  
alanines in the N,C Ala-8 mutant, a total loss of agonist-induced  
phosphorylation of the M2 mAChR was observed (20). We  
utilized this N,C Ala-8 mutant to test whether phosphorylation of  
the M2 mAChR had an effect on its recovery from agonist-  
mediated internalization. The N,C Ala-8 mutant does not internalize  
as rapidly or as extensively as the wild type M2 mAChR (20).  
Note that after a 60-min treatment with CCh only 30–35% of  
the cell-surface receptors have been internalized (Fig. 4,  
0 time recovery). The pattern of recovery of the N,C Ala-8 mutant  
appeared to be generally similar to the recovery of the wild type  
M2 mAChR, as the recovery of the N,C Ala-8 mutant was relatively  
slow and was not affected by cycloheximide during the first  
2 h of recovery (Fig. 4). However, in contrast to the wild  
type M2 mAChR, there was no statistical difference in the  
recovery curves of the N,C Ala-8 mutant through 6 h of recovery.  
Nevertheless, there appeared to be a trend in the individual  
experiments of a small protein synthesis-dependent phase for  
the recovery of the N,C Ala-8 receptor but an accurate analysis of  
this component was confounded by the low amount of internal- 
ization observed for this mutant receptor compared with its  
wild type counterpart. Overall, the results observed with this  
phosphorylation-deficient mutant suggested that phosphoryla- 
tion did not significantly affect the recovery of the M2 mAChR  

FIG. 4. Recovery of cell-surface N,CAla-8 mutant binding follow- 
ing a 60-min CCh treatment. Cells stably expressing the N,CAla-8  
mutant were treated with CCh for 60 min in the presence (▲) or absence  
(■) of cycloheximide. Recovery of receptors to the cell surface was then  
measured as in Fig. 2. Results shown are mean ± S.E. of 4–10 inde- 
pendent experiments.
The appearance of nonalkylated receptors on the cell surface was detected with the cell impermeable ligand $[^3H]$NMS and should reflect the delivery to the cell surface of receptors that were located at internal sites during the alkylation. This measurement should also reflect the constitutive internalization of receptors that were alkylated on the cell surface. The appearance of nonalkylated receptors on the cell surface following alkylation was very slow, as cell-surface receptor levels returned to approximately 40% of untreated cell-surface levels within 5 h post-alkylation (Fig. 5A). Incubation with cycloheximide showed a pattern of appearance of cell-surface $M_2$ mACHRs that was reminiscent of the recovery of cell-surface receptors that was observed following agonist treatment. There appeared to be a small amount of recovery from constitutive recycling that was independent of protein synthesis, whereas the majority of the recovery was dependent on protein synthesis (Fig. 5A).

To test whether synthesis of new $M_2$ mACHRs was responsible for the protein synthesis-dependent component of constitutive internalization and recycling, we measured total receptor number during the alkylation experiments using the hydrophobic antagonist $[^3H]$QNB. There was no change in total receptor number over the course of the recovery following alkylation, both in the absence and presence of cycloheximide (Fig. 5B). This suggested that new receptor synthesis was not involved in the protein synthesis-dependent component of the appearance of new receptors at the cell surface following alkylation. Rather, the results suggested a requirement for the synthesis of another protein that was necessary for the trafficking of the receptors.

**DISCUSSION**

This study presents new insights into the molecular mechanisms involved in the regulation of $M_2$ mACHRs by the ill-defined pathway that is responsible for the internalization of these receptors in HEK293 cells. The results demonstrated that the $M_2$ mACHR did not appreciably down regulate in HEK293 cells and that the reappearance of $M_2$ mACHRs at the cell surface following agonist-mediated internalization was a slow process. This latter process was independent of receptor phosphorylation but contained both protein synthesis-dependent and -independent components. Furthermore, the constitutive, nonagonist-dependent internalization and recycling of $M_2$ mACHRs was an even slower process that also contained protein synthesis-dependent and -independent components.

That the $M_2$ mACHR did not appreciably down regulate when stably expressed in HEK293 cells was very surprising, as down-regulation is a regulatory mechanism thought to be utilized by most GPCRs, and has been shown to occur for the $M_2$ mACHR when it is expressed in other cell types (28). A general defect in down-regulation of GPCRs in HEK293 cells can be ruled out by the demonstration of down-regulation of the $\beta_2$AR in HEK293 cells (22). The lack of appreciable down-regulation of the $M_2$ mACHR represents yet another piece of evidence demonstrating that the $M_2$ mACHR is regulated uniquely in HEK293 cells (22). The lack of appreciable down-regulation of the $M_2$ mACHR when it is expressed in other cell types (28). A general defect in down-regulation of GPCRs in HEK293 cells can be ruled out by the demonstration of down-regulation of the $\beta_2$AR in HEK293 cells (22). The lack of appreciable down-regulation of the $M_2$ mACHR represents yet another piece of evidence demonstrating that the $M_2$ mACHR is regulated uniquely in HEK293 cells (22).
The experiments addressing the reappearance of internalized M₂ mAChRs to the cell surface demonstrated a novel aspect of recovery in that the process was very slow and contained two separate components. This is the first known demonstration of this pattern of recovery of a GPCR following internalization. The initial phase of recovery for the M₂ mAChR was independent of protein synthesis, whereas the later component was dependent on protein synthesis. The protein synthesis-dependent component did not appear to require synthesis of new receptors because there was a down-regulation of M₂ mAChRs (Fig. 2) and there was no change in total receptor number over the course of the recovery experiments (data not shown). More likely, there was an accessory protein that was required for delivery of the M₂ mAChR to the cell surface following internalization. We hypothesized that the turnover of this protein was relatively rapid and that cellular levels of this protein were sufficient for the protein synthesis-independent component of recovery, but synthesis of a new accessory protein was necessary for full recovery of M₂ mAChRs to the cell surface. Because the magnitude of the protein synthesis-dependent component appeared to be reduced in cells that had been treated with agonist for 10 versus 30 min, it is possible that stimulation of the M₂ mAChR resulted in the activation of the synthesis of the required accessory protein. Stimulation of the M₂ mAChR for 30 min may have allowed for more production of the accessory protein and a resultant increase in the protein synthesis-dependent component of recovery. This hypothesis was ruled out by experiments employing cycloheximide during the CCh pretreatment period only and not during the subsequent recovery, or conversely, by adding cycloheximide during the recovery period only and not during the CCh pretreatment. If the production of the hypothesized accessory protein was stimulated by CCh, incubation with cycloheximide during the CCh pretreatment and not the recovery should show only the protein synthesis-independent component of recovery. Conversely, if the production of the accessory protein is independent of CCh stimulation, incubation with cycloheximide only during the recovery period should show only the protein synthesis-independent component of recovery. Incubation with cycloheximide during the CCh pretreatment only showed both protein synthesis-independent and -dependent components, whereas incubation with cycloheximide during the recovery period only showed just the protein synthesis-independent component (data not shown). This suggests that the production of the accessory protein is independent of CCh stimulation. Thus, it appears that the necessary accessory protein is quite labile and has a high turnover rate.

Phosphorylation of the M₂ mAChR did not appear to affect the recovery of M₂ mAChRs to the cell surface following agonist treatment. Phosphorylation has previously been demonstrated to act as a retention signal for the V2 vasopressin receptor following its internalization (23). However, the phosphorylation-deficient M₂ mAChR mutant, the NCA₃₁₅ Mutant (20), displayed a recovery to the cell surface similar to that of the wild type M₂ mAChR.

The constitutive, nonagonist-dependent internalization and recycling of the M₂ mAChR reflected the pattern of recovery of the M₂ mAChR following agonist-mediated internalization. However, whereas a small amount of constitutive internalization and recycling was observed in the absence of protein synthesis, a much larger and slower component of the constitutive recycling was dependent on protein synthesis. Measurement of total receptor number following alkylation of cell-surface receptors showed that there was no change in total receptor number over the time course of the recovery experiments. This suggested that an accessory protein was required for recycling of receptors to the cell surface during the constitutive, nonagonist-dependent internalization and recycling. These results suggested that similar mechanisms could be operating for trafficking of M₂ mAChRs to the cell surface following both agonist-dependent and constitutive, nonagonist-dependent internalization.

In summary, we have demonstrated previously unappreciated aspects of the trafficking of M₂ mAChRs. When stably expressed in HEK293 cells the M₂ mAChR internalized extensively but did not appreciably down regulate in response to prolonged CCh treatment. The recovery of M₂ mAChRs to the cell surface following both agonist-induced internalization as well as constitutive, nonagonist-dependent internalization was slow and contained both protein synthesis-dependent and -independent components. We hypothesize that the protein synthesis-dependent component involved an accessory protein necessary for trafficking of M₂ mAChRs to the cell surface. Finally, the data indicated that recovery from agonist-induced internalization is independent of receptor phosphorylation. These experiments provide needed insights into the novel pathways mediating the internalization of M₂ mAChRs and their recycling to the cell surface.

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