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Agonist-Dependent Delivery of M_2 Muscarinic Acetylcholine Receptors to the Cell Surface after Pertussis Toxin Treatment

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ABSTRACT

The internalization of the M_2 muscarinic cholinergic receptor (mAChR) proceeds through an atypical pathway that is independent of arrestin and clathrin function and shows a unique sensitivity to dynamin when the receptor is expressed in human embryonic kidney 293 cells. In this report we demonstrate that the internalization of the M_2 mAChR was modulated by activation of heterotrimeric G proteins, because treatment with pertussis toxin, which ADP-ribosylates G proteins of the $G_{i/o}$ family, caused a significant delay in the onset of internalization of the M_2 mAChR. The effects of pertussis toxin could not be explained by alteration of the agonist-dependent phosphorylation of the M_2 mAChR. The modulation of internalization by pertussis toxin was revealed to be due to recruitment of intracellular receptors to the cell surface upon agonist treatment. Pretreatment with pertussis toxin also greatly increased both the rate and extent of recovery of M_2 mAChRs to the cell surface after agonist-mediated internalization. These results demonstrate a novel aspect involved in the regulation of GPCRs. As with the tightly controlled internalization of GPCRs, the delivery of GPCRs to the cell surface is also highly regulated.

A major mechanism involved in the regulation of G protein-coupled receptors (GPCR) involves their removal from the cell surface by a process known as internalization or endocytosis (for reviews, see Krupnick and Benovic, 1998; Lefkowitz, 1998; Bunemann and Hosey, 1999). Internalization of GPCRs is a complex process that may be achieved via several pathways. A large number of GPCRs have been demonstrated to internalize through clathrin-coated pits by a process that is also dependent on arrestin and dynamin (for reviews, see Krupnick and Benovic, 1998; Lefkowitz, 1998; Bunemann and Hosey, 1999). However, other pathways also participate in the endocytosis of GPCRs. Some receptors, such as the angiotensin AT_{1A} receptor, the dopamine D2 receptor, and the M_2 mAChR can internalize by an unidentified pathway that shows an atypical sensitivity to dynamin (Zhang et al., 1996; Pals-Rylaarsdam et al., 1997; Vickery and von Zastrow, 1999; Werbonat et al., 2000).

The M_2 mAChR is a $G_{i/o}$-coupled GPCR that internalizes in HEK293 cells by endogenous endocytic machinery that is independent of arrestin function (Pals-Rylaarsdam et al., 1997) and shows an atypical sensitivity to dynamin (Werbonat et al., 2000). Although a dominant-negative isoform of dynamin that inhibits internalization of other GPCRs has no effect on internalization of the M_2 mAChR, other dominant negative isoforms of dynamin are capable of inhibiting the internalization of the M_2 mAChR (Pals-Rylaarsdam et al., 1997; Werbonat et al., 2000). This suggests that the endocytic pathway involved in internalization of the M_2 mAChR exhibits a dynamin dependence that differs from that involved in arrestin- and clathrin-dependent pathways. In our attempts to characterize this as yet unidentified pathway of internalization of the M_2 mAChR, we asked whether signaling through G proteins was necessary, even though activation of heterotrimeric G proteins by GPCRs is not believed to be universally necessary for internalization of the receptors. For example, the thyrotropin-releasing hormone receptor is incapable of signaling in cells lacking the α-subunit of the $G_{q/11}$ family, but is still able to undergo normal agonist-induced internalization (Yu and Hinkle, 1999). The β_2-adrenergic receptor has also been demonstrated to internalize normally in S49 lymphoma cells that either express a mutant form of the α_s-subunit (Mahan et al., 1985), or lack the α_s-subunit (Clark et al., 1985). Thus, it has been demonstrated that for some GPCRs, activation of heterotrimeric G proteins is not necessary for agonist-induced internalization of the receptors.

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ABBREVIATIONS: GPCR, G protein-coupled receptor; mAChR, muscarinic acetylcholine receptor; NMS, *N*-methyl scopolamine; QNB, quinuclidinyl benzilate; PBCM, propylbenzilylcholine mustard; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; CCh, carbachol; TBS, Tris-buffered saline; GIRK, G protein-regulated inwardly rectifying potassium channel; GRK, G protein-coupled receptor kinase; PAR, protease activated receptor; HEK, human embryonic kidney.

One method that can be used to examine the role of G proteins in the internalization of the M_2 mAChR is treatment with pertussis toxin. Pertussis toxin is a bacterial toxin that ADP-ribosylates heterotrimeric G proteins of the $G_{i/o}$ family and uncouples them from their receptors. Interestingly, pertussis toxin has been previously demonstrated to have a number of different effects on trafficking events in various cell types. The secretion of vesicles and trafficking of proteins from intracellular sites to the cell surface have been reported to be both stimulated and inhibited by pertussis toxin (Stow et al., 1991; Brunskill et al., 1996; Foti et al., 1997; Kobayashi et al., 1998; Valenti et al., 1998). Pertussis toxin has also been demonstrated to have effects on endocytosis of proteins, such as CD4 and albumin, from the cell surface (Brunskill et al., 1996; Foti et al., 1997). Although the effects of pertussis toxin indirectly implicate G proteins of the $G_{i/o}$ family in the affected cellular trafficking events, G proteins have also been shown to be directly involved in many of these processes (Stow et al., 1991; Brunskill et al., 1996; Denker et al., 1996; Kobayashi et al., 1998; Valenti et al., 1998). In the studies presented here we have examined the effects of pertussis toxin on the internalization and trafficking of the M_2 mAChR, as well as other mAChR subtypes in HEK293 cells.

Experimental Procedures

Materials. Cell culture reagents were purchased from Mediatech, Inc. (Herndon, VA). The muscarinic receptor antagonists [^{3}H]*N*-methyl scopolamine ([^{3}H]NMS) and [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) were purchased from PerkinElmer Life Sciences (Boston, MA). Propylbenzilylcholine mustard (PBCM) was a generous gift from Dr. J. M. Young (Cambridge, UK). Pertussis toxin was from Sigma (St. Louis, MO) or Calbiochem (La Jolla, CA). The monoclonal rat anti-M_2 mAChR and mouse anti-Golgi zone antibodies were purchased from Chemicon International, Inc. (Temecula, CA), and the affinity-purified polyclonal rabbit anti-M_2 mAChR antibody was from Alomone Labs (Jerusalem, Israel). The TRITC-conjugated goat anti-rat and FITC-conjugated goat anti-mouse antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The rabbit anti-α-mannosidase II antibody was a generous gift of Dr. Marilyn Farquhar (University of California, San Diego). The pcDNA3.1 myc-tagged α2,6-sialyltransferase was a generous gift from Dr. Karen Colley (University of Illinois, Chicago). The enhanced chemiluminescence reagents and the UltraLink Immobilized Protein G were from Pierce (Rockford, IL). [^{32}P]Orthophosphate was from Amersham Co. (Arlington Heights, IL). Phosphate-free DMEM was from Life Technologies (Baltimore, MD). Other reagents were purchased from Sigma.

Cell Culture and Transfection. HEK293 cells stably expressing the wild-type M_2 mAChR have been described previously (Pals-Rylaarsdam et al., 1995; Pals-Rylaarsdam and Hosey, 1997). HEK293 cells stably expressing M_1, M_3, or M_4 mAChRs were prepared as follows. HEK293 cells were transfected with 10 μg of pcDNA3 M_1 mAChR, pcDNA3 M_3 mAChR, or pcDNA3 M_4 mAChR using the calcium phosphate precipitation method as described previously (Pals-Rylaarsdam et al., 1995; Pals-Rylaarsdam and Hosey, 1997). Cells were diluted in media containing G418 24 h post-transfection, and individual clones were selected and analyzed for receptor expression using radioligand binding as described in the internalization assay. The receptor expression levels were as follows: cell surface M_2 mAChRs, 200 to 300 fmol receptor/mg protein; total cellular M_2 mAChRs, 400 to 600 fmol receptor/mg protein; cell surface M_1 mAChR, 1000 to 1400 fmol receptor/mg protein; cell surface M_3 mAChR, 750 to 2000 fmol receptor/mg protein; and cell surface M_4 mAChR, 100 to 400 fmol receptor/mg protein. Where indicated, cells were treated with pertussis toxin (50 ng/ml overnight) or brefeldin A (50 μM for 1 h) before the addition of the muscarinic agonist carbachol (CCh). Also, where indicated, hypertonic sucrose (400 mM) was added immediately before the addition of CCh. Unless otherwise indicted, cells were treated with 1 mM CCh for varying times at 37°C.

Receptor Internalization and Recycling Assays. Internalization assays were performed as described previously (Roseberry and Hosey, 1999), by assessing the observed change in the number of mAChRs located at the cell surface with the radiolabeled, hydrophilic antagonist [^{3}H]NMS (1.0–2.0 nM), which is membrane impermeable. Briefly, cells were incubated with CCh, washed with ice-cold PBS, and subjected to radioligand binding with saturating concentrations of [^{3}H]NMS. Data were expressed as a percentage of the [^{3}H]NMS binding observed in untreated cells or cells treated with pertussis toxin alone. The reappearance of receptors at the cell surface after agonist-induced internalization was assayed as described previously (Roseberry and Hosey, 1999). Briefly, internalization was initiated by CCh treatment, the agonist was removed, and receptors were allowed to recover for various periods of time, followed by [^{3}H]NMS binding. For evaluation of the constitutive internalization and recycling of M_2 mAChRs, receptors at the plasma membrane were irreversibly alkylated with the muscarinic receptor antagonist PBCM as described (Roseberry and Hosey, 1999). Briefly, cells were alkylated with activated PBCM, washed with PBS containing sodium thiosulfate, and reincubated in fresh media for the indicated times followed by measurement of cell surface and total receptor number.

In Vivo Phosphorylation of M_2 mAChR in Intact HEK293 Cells. Confluent 100-mm plates of HEK293 cells stably transfected with the M_2 mAChR were labeled with 0.25 mCi/ml of ^{32}P-labeled orthophosphate in phosphate-free DMEM for 4 h after pretreatment with or without 50 ng/ml pertussis toxin overnight. Cells were then stimulated with CCh for the indicated times and membrane fractions prepared. Cells were washed with 5 ml of PBS and resuspended in homogenization buffer (20 mM $NaPO_4$, 20 mM NaP_7O_4, 50 mM NaF, 5 mM EDTA, 5 mM EGTA) followed by homogenization on ice with a Brinkman polytron PTA 10TS probe at setting 7. The homogenate was centrifuged at 100,000*g* for 30 min at 4°C. The resulting crude particulate fraction was resuspended using a 22-gauge needle and syringe and solubilized in 1 ml of solubilization buffer (homogenization buffer + 1% Triton X-100) for 1 h on ice. The solubilized proteins were separated from the nonsoluble fraction by centrifugation at 100,000*g* for 30 min at 4°C. The solubilized M_2 mAChRs were then immunoprecipitated overnight at 4°C with a polyclonal anti-M_2 mAChR antibody coupled to UltraLink immobilized Protein G beads. The immunoprecipitates were washed 5 to 6 times with solubilization buffer to remove nonspecifically adsorbed proteins. The immunoprecipitated proteins were then loaded onto SDS-acrylamide gels (containing 8% acrylamide) and run at a constant current of 65 mA. Proteins were then transferred to 0.2-μm nitrocellulose filters and blocked with 5% milk in Tris-buffered saline for 1 h. The filters were then incubated with the monoclonal anti-M_2 mAChR antibody diluted 1:2000 in 5% milk/TBS at room temperature for 4 h. The filters were then rinsed with TBS and incubated with secondary antibodies diluted 1:2500 in 5% milk/TBS for 1 h at room temperature. The filters were rinsed with TBS for 30 min and enhanced chemiluminescence was used to detect the expressed proteins. Autoradiography was performed to detect phosphorylated proteins using a Molecular Dynamics Storm PhosphorImager. Densitometric analysis was performed on both the autoradiograms and the Western blots for quantitation of the amount of phosphorylation of M_2 mAChRs observed. Phosphorylation was expressed as a ratio of the radioactivity incorporated per amount of receptor protein present. Data were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescent Staining of Cells. HEK293 cells stably expressing the M_2 mAChR were seeded to poly-L-lysine–coated cov-

erslips and incubated at 37°C overnight. Before staining, cells were treated with appropriate drugs, and subsequently washed with PBS for 5 min at room temperature followed by fixation with precooled methanol/acetone (1:1) at 4°C for 10 min. Fixed cells were treated with blocking buffer (10% normal goat serum, 0.1% bovine serum albumin, in PBS) for 45 min at 37°C to decrease nonspecific binding. Primary antibodies were diluted in labeling buffer (0.1% bovine serum albumin in PBS) and incubated with cells for 2 h at 37°C, or overnight at 4°C. Antibodies were diluted as follows: anti-M_2 mAChR-1:200, anti-Golgi zone-1:200. For double labeling experiments, two primary antibodies were used simultaneously. After incubation with primary antibodies, cells were washed three times with room temperature PBS. Secondary antibodies (TRITC-conjugated goat anti-rat IgG and FITC-conjugated goat anti-mouse IgG) were subsequently diluted 1:100 in labeling buffer and incubated with the cells for 1 to 2 h at 37°C. Coverslips were mounted onto slides and viewed on a laser-scanning confocal microscope using a 63× objective (Zeiss, Oberkochen, Germany). Images are representative of an entire population of cells. Negative control experiments were performed using secondary antibodies alone to control for nonspecific staining. No nonspecific staining was observed under these conditions (data not shown).

Electrophysiological Measurement of G Protein-Regulated Inwardly Rectifying Potassium Channel (GIRK) Currents. GIRK currents were measured in HEK293 cells stably expressing the M_2 mAChR and transiently expressing GIRK1 and GIRK4 as described previously (Bunemann and Hosey, 1998).

Results

Does Pertussis Toxin Affect Internalization of the M_2 mAChR? Internalization of the M_2 mAChR in HEK293 cells is independent of arrestin and clathrin function (Pals-Rylaarsdam et al., 1997; Vogler et al., 1999), but shows a unique sensitivity to dynamin (Werbonat et al., 2000). Because very little is known about the preferred pathway operating for internalization of the M_2 mAChR, we sought to examine whether G protein-dependent signaling modulated receptor internalization. Pretreatment of HEK293 cells stably expressing the M_2 mAChR with pertussis toxin, which results in the uncoupling of G proteins of the $G_{i/o}$ family from GPCRs, caused a significant delay in the onset of agonist-induced internalization of the M_2 mAChR, but had no effect on the overall extent of internalization observed (Fig. 1A). Under these conditions pertussis toxin totally inhibited the activation of GIRK currents in response to CCh treatment (Fig. 1B). The effects of pertussis toxin on internalization of the M_2 mAChR seemed to be specific for agonist-induced internalization, because the muscarinic inverse agonist atropine had no effect on trafficking of M_2 mAChRs either in the presence or absence of pertussis toxin (data not shown). The striking effect of pertussis toxin on internalization of the M_2 mAChR is a novel and interesting finding, because activation of G proteins has not been shown to be necessary for internalization of other GPCRs (Clark et al., 1985; Mahan et al., 1985; Yu and Hinkle, 1999).

Does Pertussis Toxin Affect the Agonist-Dependent Phosphorylation of the M_2 mAChR? It has been demonstrated previously that internalization of the M_2 mAChR in HEK293 cells is modulated by the agonist-dependent phosphorylation of the receptors (Pals-Rylaarsdam et al., 1995; Pals-Rylaarsdam and Hosey, 1997). We examined whether pertussis toxin caused the observed effects on internalization of the M_2 mAChR by altering the agonist-dependent phosphorylation of the receptors. Because release of the $\beta\gamma$-subunits of heterotrimeric G proteins has been proposed to be necessary for recruitment and activation of GRK2 and 3 (Pitcher et al., 1992, 1995; DebBurman et al., 1996), it was possible that inactivation of G proteins with pertussis toxin affected the GRK-mediated phosphorylation of the M_2 mAChR. To test this hypothesis, the effect of pertussis toxin on the phosphorylation of the M_2 mAChR was examined in intact HEK293 cells. Stimulation of HEK293 cells stably expressing the M_2 mAChR with CCh for either 5 or 30 min resulted in an approximately 3-fold increase in receptor phosphorylation relative to untreated cells (Fig. 2). Pretreatment with pertussis toxin had no effect on the level of phosphorylation observed after either 5-or 30-min CCh treament (Fig. 2). This suggested that the effects of pertussis toxin on internalization of the M_2 mAChR were not mediated through alteration of the phosphorylation of the M_2 mAChR and that the release of $\beta\gamma$-subunits from $G_{i/o}$ proteins was not required for full phosphorylation of the M_2 mAChR.

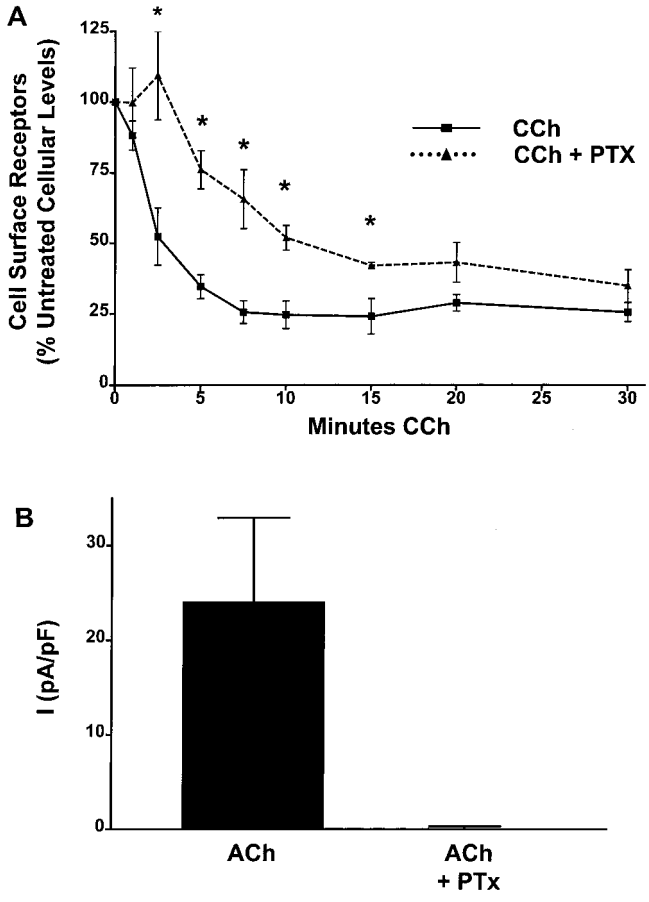

Fig. 1. Internalization of M_2 mAChRs after pertussis toxin pretreatment. A, HEK293 cells stably expressing M_2 mAChRs were treated with (▲) or without (■) 50 ng/ml pertussis toxin overnight before the addition of CCh for the times indicated. Cell surface receptor levels were measured using the hydrophilic antagonist [^{3}H]NMS (1.0–2.0 nM). Results shown are means ± S.E.M. of three to seven independent experiments. The number of cell surface receptors in the absence of agonist treatment in both control cells and cells treated with pertussis toxin alone were normalized to 100%. (*$p < 0.05$ versus control). B, pertussis toxin inhibited M_2 mAChR-activated GIRK currents. GIRK1 and GIRK4 were transiently expressed in HEK293 cells stably expressing M_2 mAChRs. Inward $I_{K(ACh)}$ (holding potential of −90 mV, E_K ~50 mV) was activated by superfusion of the cells with 2 μM acetylcholine (ACh) for 15 s after pretreatment with or without pertussis toxin (PTx).

How Does Pertussis Toxin Modulate the Trafficking of M_2 mAChRs after Agonist Treatment? We next visualized the effects of pertussis toxin on the trafficking of the M_2 mAChR through the use of immunocytochemical techniques and confocal laser-scanning microscopy. The M_2 mAChR exhibited intense cell surface staining (denoted by open arrows in Fig. 3, A, D, and F) as well as a significant amount of intracellular staining in untreated cells (denoted by closed arrows in Fig. 3A), as has been described previously (Roseberry and Hosey, 1999). Due to the rapid internalization of the M_2 mAChR at 37°C (~80% internalization within 5 min; Fig. 1) reduced incubation temperatures were used to slow the rate of internalization (Fig. 4B), to better assess the effects of pertussis toxin on the agonist-induced trafficking of the M_2 mAChR. Incubation of the cells with CCh for either 2.5 or 5 min at 15°C resulted in only a minor loss of cell surface staining (Fig. 3, C and E). However, in cells pretreated with pertussis toxin, treatment with CCh resulted in an increase, rather than a decrease, in the intensity of staining of M_2 mAChRs at the cell surface compared with untreated cells (Fig. 3, D and F). Interestingly, treatment with pertussis toxin also seemed to cause a strong loss of the intracellular staining of the M_2 mAChRs upon CCh treatment (note closed arrows in Fig. 3, D and F). The increase in the intensity of the cell surface staining (Fig. 3, D and F, open arrows), as well as the loss of intracellular staining (Fig. 3, D and F, closed arrows) suggested that intracellular M_2 mAChRs were trafficked to the cell surface after agonist treatment in cells that had been treated with pertussis toxin. These effects were entirely agonist-dependent, because pertussis toxin alone caused no change in the distribution of the M_2 mAChRs (Fig. 3B), in agreement with the biochemical findings that the proportion of cell surface/total receptors was unchanged by pertussis toxin (Table 1).

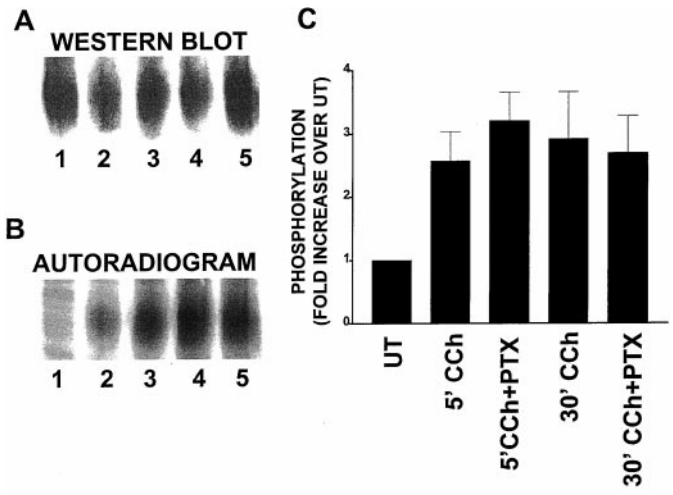

Fig. 2. In vivo phosphorylation of the M_2 mAChR after pertussis toxin pretreatment. HEK293 cells stably expressing M_2 mAChRs were treated with vehicle or 50 ng/ml pertussis toxin overnight. Cells were then labeled with [^{32}P]orthophosphate in phosphate-free DMEM for 4 h followed by stimulation with CCh for the indicated times. M_2 mAChRs were immunoprecipitated, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters for Western blotting and autoradiography. A, representative immunoblot of the M_2 mAChR detected with anti-M_2 mAChR antibodies. B, representative phosphorimage of the phosphorylated M_2 mAChR. The lanes in A and B are as follows: 1, untreated cells; 2, 5 min CCh; 3, 30 min CCh; 4, 5 min CCh + pertussis toxin; and 5, 30 min CCh + pertussis toxin. C, average increase in phosphorylation in response to agonist stimulation. Data are presented as the fold increase in receptor phosphorylation relative to the receptor phosphorylation in untreated cells, which was normalized to a value of 1. Results shown are means ± S.E.M. of three independent experiments.

To test the hypothesis that M_2 mAChRs were trafficking to the cell surface in response to agonist after pertussis toxin treatment, we used hypertonic sucrose to inhibit internalization of the M_2 mAChR, and tested the effects of pertussis toxin on changes in cell surface receptor number. As demonstrated previously (Roseberry and Hosey, 2001) treatment of cells with hypertonic sucrose resulted in the total inhibition of internalization of the M_2 mAChR (Fig. 4A). When cells were treated with both pertussis toxin and hypertonic sucrose, a striking increase in cell surface receptor number was observed upon addition of CCh (Fig. 4A). This increase occurred at the earliest time point tested (2.5 min) and remained constant throughout the duration of the experiment. Similar results were obtained for internalization of the M_2 mAChR in the presence of sucrose and pertussis toxin at 15°C (Fig. 4B), whereas the inclusion of sucrose alone had no effect on cell surface M_2 mAChR levels due to the low internalization observed at this temperature (data not shown). These results confirm the results of the immunofluorescent staining observed in Fig. 3, and demonstrate that internal M_2 mAChRs were delivered to the cell surface upon agonist treatment in cells that had been pretreated with pertussis toxin. The time courses of the delivery of M_2 mAChRs to the cell surface and the changes in the loss of receptors from the cell surface during internalization seemed to differ slightly. As such, it is difficult to determine whether the effects on internalization were solely due to delivery of receptors to the cell surface due to the dynamic nature of trafficking of receptors to and from the cell surface. However, it is apparent that the pretreatment with pertussis toxin did allow for an agonist-dependent delivery of M_2 mAChRs to the cell surface, and this delivery would be expected to have effects on internalization.

Delivery of M_2 mAChRs to the cell surface was also demonstrated by immunofluorescent staining of cells treated with pertussis toxin and hypertonic sucrose. In the absence of agonist treatment, pretreatment with pertussis toxin and sucrose had no effect on the staining pattern of M_2 mAChRs (compare Fig. 5, A and B). Although treatment with CCh for either 2.5 min (Fig. 5C) or 5 min (Fig. 5F) induced strong internalization of the M_2 mAChR, the inclusion of hypertonic sucrose totally ablated internalization (Fig. 5, D and G). Cells treated with hypertonic sucrose and pertussis toxin showed a strong loss of intracellular receptor staining and an increase in the intensity of staining at the cell surface upon stimulation with CCh after both 2.5-min (Fig. 5E) and 5-min (Fig. 5H) CCh treatment. The results from Figs. 3 to 5 support the hypothesis that pretreatment with pertussis toxin allowed for an agonist-dependent delivery of M_2 mAChRs to the cell surface. Thus, the apparent effect of pertussis toxin on internalization (Fig. 1A) was most likely due to agonist-dependent delivery of M_2 mAChRs to the cell surface, causing the observed net decrease in the agonist-induced loss of M_2 mAChRs from the cell surface at the early time points, although it is possible that pertussis toxin may have had direct effects on the internalization process itself.

Does Pertussis Toxin Increase the Recovery of M_2 mAChRs to the Cell Surface after Agonist-Mediated Internalization? The recovery of the M_2 mAChR to the cell

surface after agonist-mediated internalization is a very slow process that requires approximately 4 h for cell surface receptor levels to recover to a new steady state that is only ~70% of untreated cell surface receptor levels (Roseberry and Hosey, 1999). Because pertussis toxin caused delivery of intracellular M_2 mAChRs to the cell surface in an agonist-dependent manner, we asked whether pertussis toxin could increase the rate of recovery of receptors to the cell surface after their internalization. Cells were treated with CCh for 30 min after pretreatment with or without pertussis toxin, the CCh was removed, and cells were allowed to recover for various periods of time followed by measurement of cell surface receptor levels. Pertussis toxin pretreatment caused both a rapid increase in the rate of recovery of M_2 mAChRs to the cell surface as well as an increase in the overall extent of recovery observed over the time course of the experiments (Fig. 6). This suggested that pertussis toxin treatment also facilitated the recovery of receptors to the cell surface after agonist-induced internalization. Thus, it seemed that pretreatment with pertussis toxin facilitated delivery of intracellular M_2 mAChRs to the cell surface under a number of different conditions.

Does Pertussis Toxin Pretreatment Affect the Constitutive Internalization and Recycling of the M_2 mAChR? Internalization of GPCRs is a dynamic, rather than a static process, whereby receptors are constantly trafficking between intracellular sites and the cell surface (Koenig and Edwardson, 1994a,b, 1996, 1997; Roseberry and Hosey, 1999). This process has been shown to be very slow for M_2 mAChRs expressed stably in HEK293 cells (Roseberry and Hosey, 1999). Because pertussis toxin was able to affect the agonist-dependent delivery of receptors to the cell surface, the effects of pertussis toxin on the rate of constitutive, agonist-independent, internalization and recycling of the M_2 mAChR were tested. Alkylation of cell surface M_2 mAChRs with the irreversible alkylating muscarinic antagonist PBCM allowed for examination of the rate of delivery of unalkylated receptors to the cell surface, which is a reflection of the rate of constitutive internalization and recycling of the receptors. Treatment of HEK293 cells stably expressing the M_2 mAChR with PBCM resulted in inactivation of approximately 90% of cell surface M_2 mAChRs (Fig. 7, zero time point). The rate of delivery of nonalkylated receptors to the cell surface after inactivation of the cell surface receptors, which has been demonstrated to be independent of synthesis of new M_2 mAChRs (Roseberry and Hosey, 1999), was not affected by pertussis toxin (Fig. 7). Similarly, no changes in the distribution of receptors between the cell surface and intracellular sites (as deduced by the measurement of total receptor density with the hydrophobic radioligand [^{3}H]QNB) were detected in the presence or absence of pertussis toxin (data not shown). Thus, it seemed that pertussis toxin did not affect the constitutive internalization and recycling of M_2 mAChRs, but only affected the agonist-dependent internalization and delivery of receptors to the cell surface.

Is the Agonist-Dependent Delivery of M_2 mAChRs to the Cell Surface after Pertussis Toxin Pretreatment Elicited by Activation of other GPCRs? Because the pertussis toxin-dependent delivery of M_2 mAChRs to the cell surface required agonist stimulation of the M_2 mAChR, we next examined whether stimulation of endogenous $G_{i/o}$-, G_q-, or G_s-coupled GPCRs could also affect delivery of the M_2 mAChR to the cell surface after pertussis toxin pretreatment. HEK293 cells have previously been demonstrated to contain endogenous G_s-coupled β_2-adrenergic receptors and G_q-coupled receptors for ATP (Mundell et al., 1999; Mundell and Benovic, 2000). In addition we found that stimulation of HEK293 cells with sphingosine-1-PO_4 was able to activate GIRK currents through the activation of pertussis toxin-sensitive $G_{i/o}$ proteins (M. Bünemann, A. G. Roseberry, and

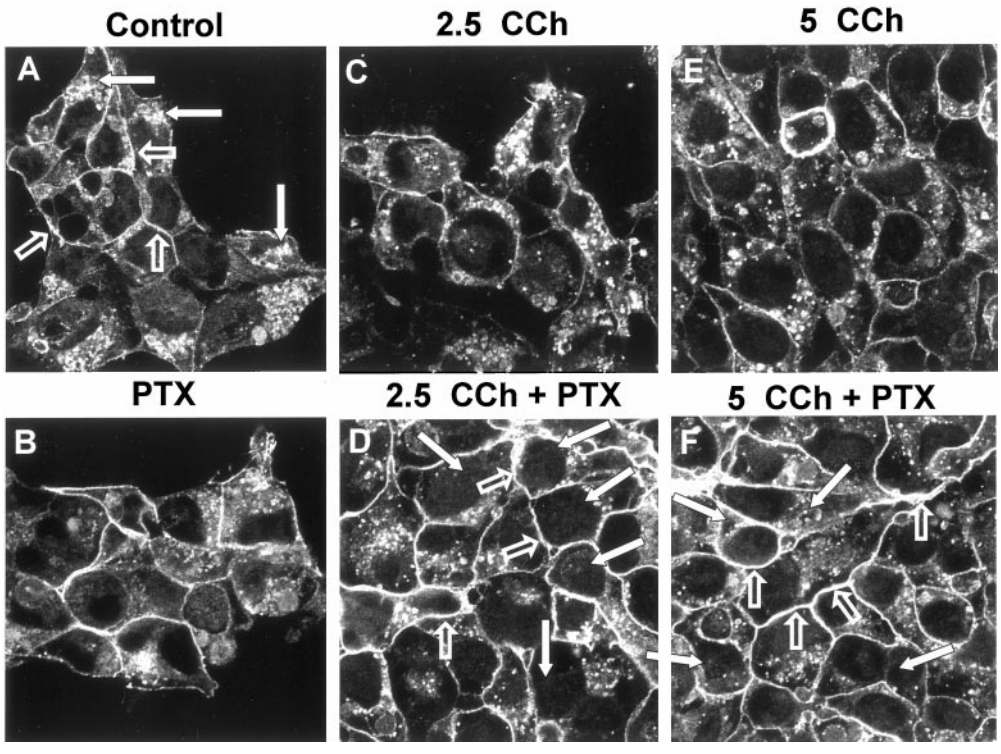

Fig. 3. M_2 mAChR staining in HEK293 cells after pertussis toxin pretreatment. HEK293 cells stably expressing M_2 mAChRs were either untreated (A, C, and E) or treated with pertussis toxin (B, D, and F) before the addition of CCh for 2.5 min (C and D) or 5 min (E and F) at 15°C. Cells were fixed and stained with anti-M_2 mAChR antibodies and visualized with TRITC-conjugated anti-rat antibodies on a confocal laser-scanning microscope. Images are representative of an entire population of cells. Arrows in A indicate intracellular staining in untreated cells. Arrows in D and F indicate cells with loss of intracellular staining. Open arrows denote plasma membrane staining.

TABLE 1

Changes in cell surface and total M_2 mAChR levels after pertussis toxin treatment

Cell surface and total receptor number were measured using [^{3}H]NMS and [^{3}H]QNB respectively, in the absence or presence of pertussis toxin pretreatment as described under *Experimental Procedures*. The percentage of receptors at the cell surface was determined by the ratio of bound [^{3}H]NMS to bound [^{3}H]QNB (% = [^{3}H]NMS/[^{3}H]QNB × 100). Values are expressed as mean ± S.E.M.

	Increase in Cell Surface Receptors	Increase in Total Receptors	% Receptors at the Cell Surface
− Pertussis toxin	0	0	48.1 ± 4.7%
+ Pertussis toxin	21.0 ± 3.0%	19.1 ± 12.8%	46.3 ± 3.0%

M. M. Hosey, unpublished observations). Interestingly, after pertussis toxin pretreatment, stimulation with sphingosine-1-PO_4, but not ATP or isoproterenol, was able to elicit a small but significant increase (14.7 ± 6.6%) in cell surface M_2 mAChR levels. This suggested that the agonist-dependent delivery of M_2 mAChRs to the cell surface after pertussis toxin pretreatment could be achieved by stimulation of other GPCRs, but was not due to cross-coupling to G_q or G_s.

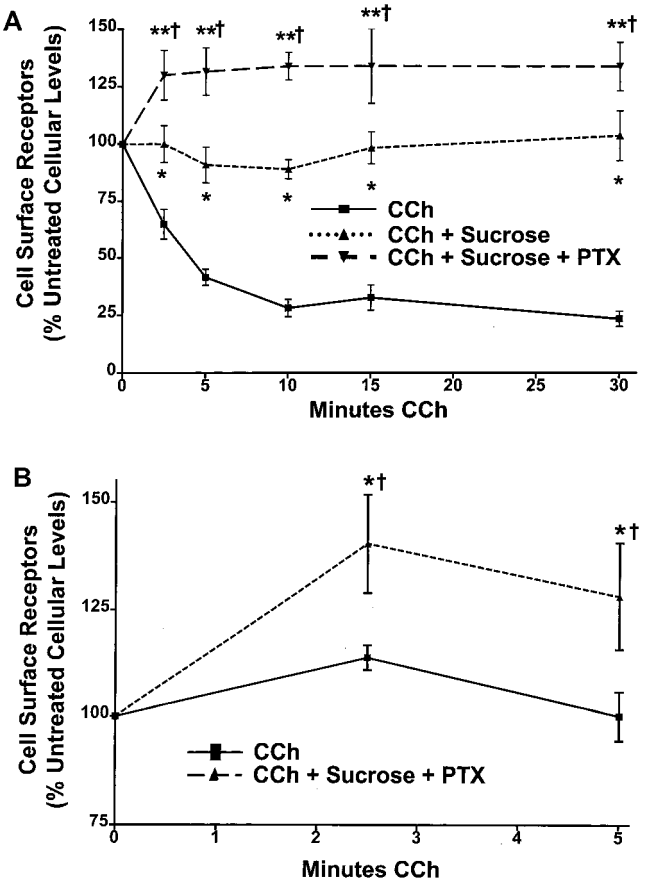

Fig. 4. Internalization of M_2 mAChRs after pertussis toxin pretreatment in the presence of hypertonic sucrose. A, HEK293 cells stably expressing M_2 mAChRs were untreated (■), treated with 400 mM hypertonic sucrose (▲), or treated with 400 mM hypertonic sucrose after pretreatment with pertussis toxin (▼), before the addition of CCh for the times indicated at 37°C. Cell surface receptor levels were measured using the hydrophilic antagonist [^{3}H]NMS (1.0–2.0 nM). Results shown are means ± S.E.M. of five independent experiments. (*$p < 0.05$ CCh + sucrose versus CCh; **$p < 0.05$ CCh + pertussis toxin + sucrose versus CCh + sucrose; †$p < 0.05$ versus zero time point). B, cells were incubated at 15°C and were untreated (■) or treated with 400 mM hypertonic sucrose after pertussis toxin pretreatment (▲) before incubation with CCh for the times indicated. (*$p < 0.05$ versus control, †$p < 0.05$ versus zero time point).

Do M_2 mAChRs Colocalize with Markers for the Golgi Zone in HEK293 Cells? Because it seemed that intracellular M_2 mAChRs were delivered to the cell surface in an agonist-dependent manner after pertussis toxin pretreatment, we sought to examine where the intracellular M_2 mAChRs were located. The staining pattern of intracellular M_2 mAChRs in both untreated and agonist-treated cells (Figs. 3 and 5) was reminiscent of the staining pattern of the Golgi zone; therefore we examined whether the internal M_2 mAChRs colocalized with a marker for the Golgi zone. In untreated cells there was significant staining of the M_2 mAChR both at the cell surface and at a perinuclear site (Fig. 8A) that strongly colocalized with a marker (a mouse anti-Golgi zone antibody from Chemicon International, Inc.) for the Golgi zone (Fig. 8, B and C). In addition, colocalization of the M_2 mAChR with both α-mannosidase II and α2,6-sialyltransferase, both of which have been widely used as markers for the Golgi zone (Roth et al., 1985; Velasco et al., 1993), was observed in untreated cells (data not shown). Similar results were observed in the presence of cycloheximide (data not shown), demonstrating that the intracellular receptors were not newly synthesized receptors waiting to traffic to the cell surface. Thus, it seemed that a significant intracellular pool of M_2 mAChRs was located in the Golgi zone in HEK293 cells.

Does Brefeldin A Inhibit Trafficking of M_2 mAChRs from the Golgi to the Cell Surface? The above-mentioned results led us to examine whether brefeldin A, which has been demonstrated to lead to Golgi disruption and inhibit trafficking from the Golgi to the cell surface (Chardin and McCormick, 1999), could inhibit delivery of M_2 mAChRs from their intracellular locale to the cell surface. Brefeldin A had no effect on the agonist-induced internalization of the M_2 mAChR (Fig. 9A), but caused extensive inhibition of the recovery of receptors to the cell surface after the agonist-induced internalization (Fig. 9B). Although it has been shown previously that the recovery of M_2 mAChRs to the cell surface is partially dependent on protein synthesis, recovery is independent on the synthesis of new M_2 mAChRs (Roseberry and Hosey, 1999). This demonstrates that brefeldin A was not inhibiting the delivery of newly synthesized receptors to the cell surface, but was indeed inhibiting delivery of previously existing receptors to the cell surface.

Because intracellular receptors in untreated cells seemed to be located in the Golgi zone, we next examined whether brefeldin A could also inhibit the constitutive internalization and recycling of the M_2 mAChR. PBCM treatment resulted in inactivation of ~85% of the cell surface receptors, so that at the beginning of the experiment there were ~15% of the receptors on the cell surface that could be detected with [^{3}H]NMS (Fig. 9C). The appearance of receptors at the cell surface then proceeded at a very slow rate (Fig. 9C), as previously demonstrated (Roseberry and Hosey, 1999). Brefeldin A significantly inhibited the appearance of new receptors at the cell surface (Fig. 9C), indicating that it inhibited constitutive recycling of the M_2 mAChR. Brefeldin A did not affect total receptor number, measured with the hydrophobic antagonist [^{3}H]QNB (data not shown). Thus, brefeldin A inhibited delivery of M_2 mAChRs to the cell surface during both the constitutive recycling of M_2 mAChRs and the recycling of receptors after agonist-mediated inter-

nalization, suggesting that M_2 mAChRs traffic from the Golgi to the cell surface during these processes.

Does Pertussis Toxin Pretreatment Affect the Trafficking of Other $G_{i/o}$-Coupled GPCRs? We next examined whether pretreatment with pertussis toxin could also affect the trafficking of other $G_{i/o}$-coupled GPCRs. Because the M_4 mAChR is also coupled to $G_{i/o}$, we examined the effects of pertussis toxin pretreatment on internalization of the M_4 mAChR. As was observed with the M_2 mAChR, pretreatment with pertussis toxin resulted in a strong inhibition of internalization of the M_4 mAChR at early time points, but also showed significant inhibition of internalization at later time points as well (Fig. 10A). Because this pattern of internalization was similar to that observed for the M_2 mAChR, we next examined whether the inhibition of internalization of the M_4 mAChR was also due to delivery of intracellular receptors to the cell surface upon agonist treatment. Although sucrose was unable to completely inhibit internalization of the M_4 mAChR, significant inhibition of internalization was observed (Fig. 10B). Pretreatment with pertussis toxin in combination with sucrose treatment resulted in an increase in cell surface receptor number that was significantly different from cells treated with sucrose alone (Fig. 10B). This demonstrates that, as with the M_2 mAChR, pertussis toxin pretreatment resulted in the agonist-dependent delivery of intracellular M_4 mAChRs to the cell surface.

Because the delivery of the M_4 mAChR to the cell surface after pretreatment with pertussis toxin seemed to be regulated similar to that of the M_2 mAChR, we next examined whether the recovery of M_4 mAChRs to the cell surface after agonist-mediated internalization was also affected by pertussis toxin. Recovery of the M_4 mAChR to the cell surface after treatment with CCh for 30 min was greatly accelerated by pertussis toxin (Fig. 10C). In pertussis toxin-pretreated cells recovery of the M_4 mAChR was nearly complete within 20 min after agonist removal, whereas in the absence of pertussis toxin pretreatment full recovery required nearly 4 h (Fig. 10C). Thus, it seemed that pertussis toxin was able to facilitate delivery of the M_4 mAChR to the cell surface under a number of different conditions as well.

Does Pertussis Toxin Pretreatment Affect the Trafficking of Non-$G_{i/o}$-Coupled GPCRs? Because pertussis

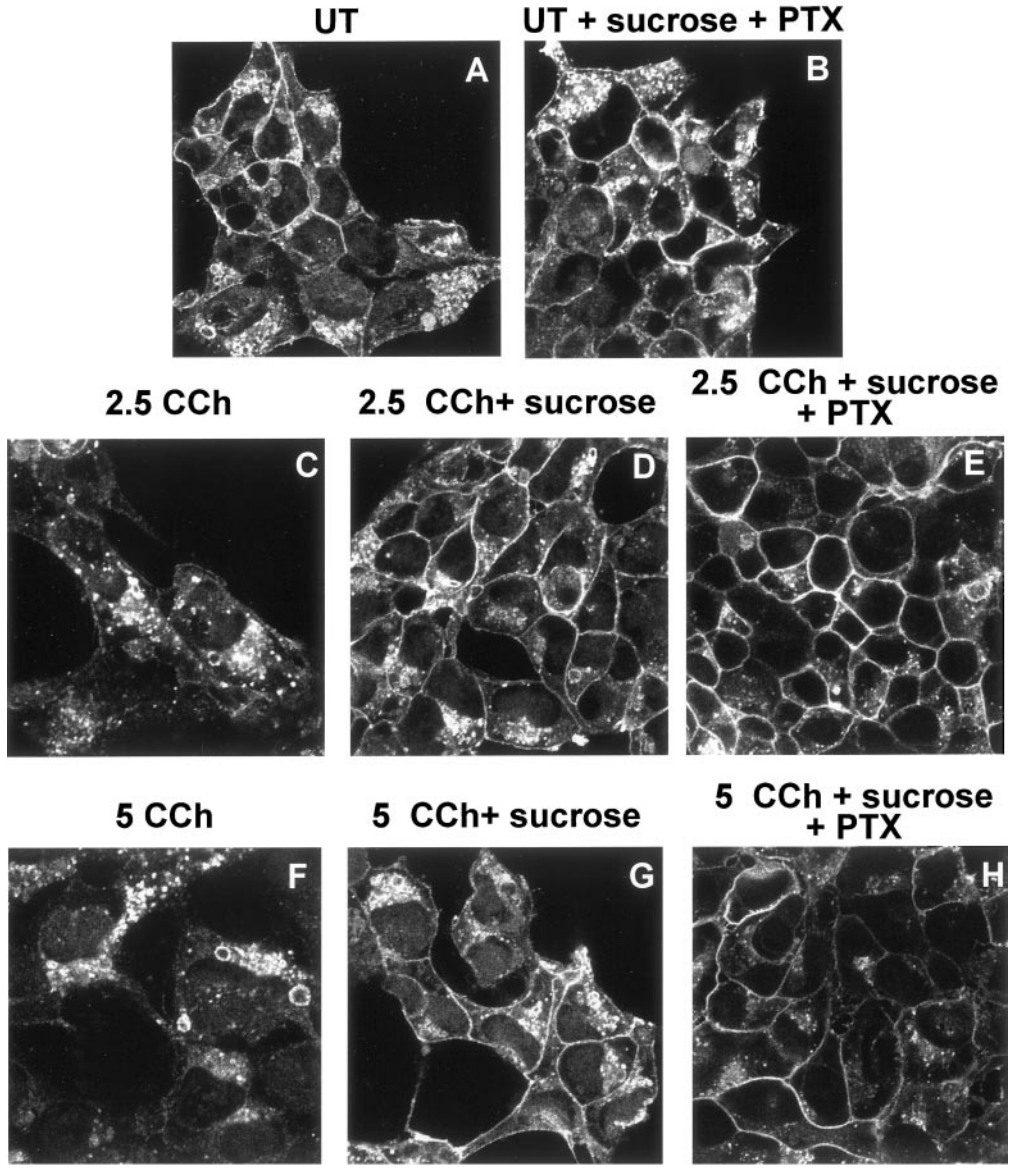

Fig. 5. M_2 mAChR staining in HEK293 cells in the presence of pertussis toxin and hypertonic sucrose. HEK293 cells stably expressing M_2 mAChRs were untreated (A, C, and F), treated with 400 mM hypertonic sucrose (D and G), or treated with hypertonic sucrose after pretreatment with pertussis toxin (B, E, and H). Cells were then left untreated (A and B) or treated with CCh for 2.5 min (C–E) or 5 min (F–H) at 37°C. Cells were fixed and stained with anti-M_2 mAChR antibodies and visualized with TRITC-conjugated anti-rat antibodies on a confocal laser-scanning microscope. Images are representative of an entire population of cells.

toxin was able to affect the trafficking of the $G_{i/o}$-coupled M_2 and M_4 mAChRs, we next examined whether pretreatment with pertussis toxin could affect trafficking of non-$G_{i/o}$-coupled GPCRs. Because both the M_1 and M_3 mAChRs activate G proteins of the G_q family, which will not be affected by pertussis toxin, we hypothesized that the trafficking of these receptors would be unaffected by pretreatment with pertussis toxin. As expected, pertussis toxin had no effect on the internalization of the M_1 and M_3 mAChRs (Fig. 10D; data not shown). To ensure that pertussis toxin was not having an effect on trafficking of the M_1 and M_3 mAChRs that was not apparent when examining internalization, we inhibited internalization with hypertonic sucrose in the presence or absence of pertussis toxin pretreatment. Interestingly, although the inclusion of pertussis toxin and sucrose did not lead to an increase in cell surface M_3 mAChR levels compared with sucrose alone (data not shown), pretreatment with pertussis toxin and sucrose led to an agonist-dependent increase in the number of M_1 mAChRs at the cell surface (Fig. 10E). Because the delivery of M_1 mAChRs to the cell surface was similar to what was observed with the M_2 and M_4 mAChRs (Figs. 4 and 10B), we next examined whether recovery of the M_1 mAChR after agonist-dependent internalization was affected by pertussis toxin, as was observed for both the M_2 and M_4 mAChRs. After internalization of the M_1 mAChR for 30 min, pretreatment with pertussis toxin increased the rate of recovery of M_1 mAChRs to the cell surface, but had no overall effect on the extent of recovery of receptors to the cell surface (Fig. 10F). Thus, it seemed that pretreatment with pertussis toxin was not only able to affect delivery of the $G_{i/o}$-coupled M_2 and M_4 mAChRs to the cell surface, but could also affect delivery of the G_q-coupled M_1 mAChR to the cell surface. However, pertussis toxin treatment was unable to effect delivery of M_3 mAChRs to the cell surface.

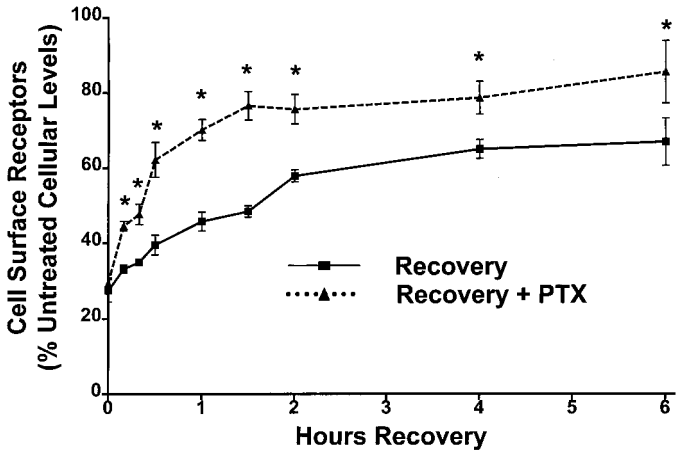

Fig. 6. Recovery of cell surface M_2 mAChR binding after pertussis toxin pretreatment. HEK293 cells stably expressing M_2 mAChRs were treated with or without pertussis toxin overnight before the addition of CCh for 30 min. The CCh was removed by washing in PBS, and the cells were then incubated at 37°C in fresh media with (▲) or without (■) pertussis toxin for the times indicated. Cell surface receptor levels were then determined using [^{3}H]NMS, and are shown as a percentage of the levels observed in untreated cells. Results shown are means ± S.E. of four to seven independent experiments. (*$p < 0.05$ versus control).

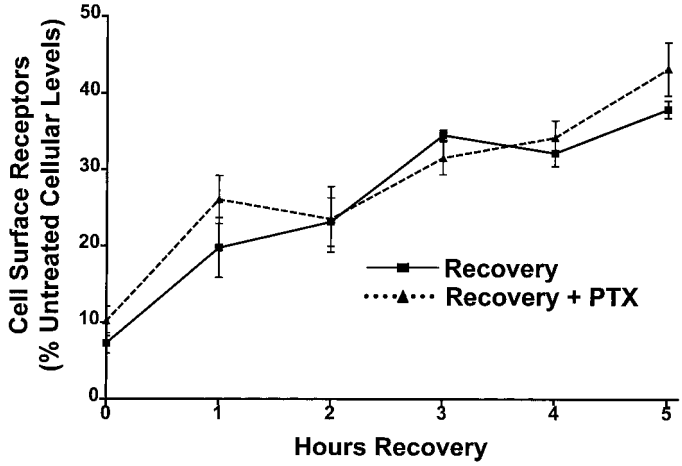

Fig. 7. Changes in M_2 mAChR levels after irreversible alkylation of cell-surface receptors after pertussis toxin pretreatment. Cells were untreated or treated with pertussis toxin overnight followed by treatment with PBCM for 30 min at room temperature to irreversibly alkylate cell-surface receptors. Recovery of cell-surface M_2 mAChRs in the presence (▲) or absence (■) of pertussis toxin at the indicated times after PBCM removal was measured using the hydrophilic antagonist [^{3}H]NMS. Cell surface receptor levels are shown as a percentage of the levels observed in untreated cells. Results shown are means ± S.E.M. of three independent experiments.

Discussion

In these studies we have demonstrated a novel aspect of the regulation of GPCRs. Pretreatment of cells with pertussis toxin, which ADP-ribosylates G proteins of the $G_{i/o}$ family, and thus inhibits signaling through $G_{i/o}$-coupled GPCRs, allowed for delivery of intracellular M_2 mAChRs to the cell surface in an agonist-dependent manner. This delivery of M_2 mAChRs to the cell surface caused an apparent delay in the onset of internalization, but had little or no overall effect on the extent of internalization. Pretreatment with pertussis toxin did not modify receptor trafficking through effects on the agonist-dependent phosphorylation of the M_2 mAChR, but did accelerate the otherwise slow recovery of receptors to the cell surface after agonist-mediated internalization. We also identified an intracellular pool of M_2 mAChRs that was located in the Golgi. Trafficking of M_2 mAChRs from this intracellular pool to the cell surface was sensitive to treatment with brefeldin A, further supporting the contention that the pool of receptors was located in the Golgi. We hypothesize that the effect of pertussis toxin was due to the neutralization of an intracellular retention signal that normally regulates the agonist-dependent delivery of M_2 mAChRs to the cell surface from the Golgi. Conceivably, the retention signals that normally cause slow recovery of M_2 mAChRs to the cell surface and those regulating delivery of M_2 mAChRs to the cell surface after agonist stimulation may be related. Furthermore, pretreatment with pertussis toxin was also able to facilitate the agonist-dependent delivery of M_4 and M_1 mAChRs to the cell surface, and led to an increase in the rate of recovery of these receptors after agonist-mediated internalization. This suggests that the mechanisms regulating delivery of M_2, M_4, and M_1 mAChRs to the cell surface may be related.

The mechanism by which pertussis toxin pretreatment facilitates the agonist-dependent delivery of M_2 mAChRs to the cell surface is unclear. One possible mechanism is that there may be a G protein located in an intracellular location, such as the Golgi, that exerts a tonic inhibition of delivery of M_2 mAChRs to the cell surface. Although heterotrimeric G

proteins are most typically localized to the plasma membrane, G proteins of the $G_{i/o}$ family have been widely demonstrated to be located in intracellular sites such as the Golgi (Denker et al., 1996; Jamora et al., 1997, 1999), and to have various effects on trafficking events in cells (Stow et al., 1991; Brunskill et al., 1996; Helms et al., 1998; Kobayashi et al., 1998; Valenti et al., 1998). In addition, delivery of certain proteins, such as heparan sulfate proteoglycan, from intracellular sites to the cell surface has been demonstrated to be dependent on the α-subunit of the $G_{i/o}$ family (Stow et al., 1991). In one study, overexpression of an $\alpha_{i/o}$-subunit inhibited delivery of heparan sulfate proteoglycan to the cell surface, and this inhibition was relieved by pertussis toxin treatment (Stow et al., 1991). Thus, it seems possible that intracellular G proteins may regulate delivery of M_2 mAChRs to the cell surface under various conditions.

This hypothesis would also predict that this inhibitory G protein may contribute to the very slow rates of recovery of M_2 mAChRs to the cell surface after internalization (Roseberry and Hosey, 1999). In this scenario, prevention of the interaction of receptors with G proteins by pertussis toxin would allow for the observed agonist-dependent recruitment of receptors to the cell surface as well as the faster rate of recovery of receptors to the cell surface after agonist-induced internalization. Because the roles of intracellular G proteins are poorly understood, it is possible that their ADP-ribosylation by pertussis toxin could inhibit one or more functions of these intracellular G proteins, either through uncoupling from GPCRs, or through some alternative unknown mechanism.

It needs to be emphasized that the delivery of M_2 mAChRs to the cell surface after pertussis toxin pretreatment seems to occur only after agonist treatment. Delivery of M_2 mAChRs to the cell surface during internalization was only observed after agonist stimulation, because there was no change in the distribution of M_2 mAChRs between intracellular sites and the cell surface in cells treated with pertussis toxin in the absence of agonist. In addition, the recovery of M_2 mAChRs to the cell surface after agonist-dependent internalization was increased by pertussis toxin, whereas recovery from constitutive, agonist-independent internalization was unaffected by pertussis toxin. However, it does not seem that the agonist-dependent delivery of M_2 mAChRs to the cell surface is stimulated by coupling of the M_2 mAChR to G_q or G_s, because stimulation of these G proteins had no effect on the trafficking of the M_2 mAChR after pertussis toxin treatment. It is possible that the agonist-dependent event arises from coupling of the M_2 mAChR to G_{12} or G_{13}, or to some as yet unidentified signaling protein. These possibilities need to be examined in more detail.

Pertussis toxin has been shown to have direct effects on internalization of various proteins in different cell types (Brunskill et al., 1996; Foti et al., 1997). Thus, we cannot exclude the possibility that pertussis toxin may have had an effect on the internalization process itself, in addition to the effects on delivery of receptors to the cell surface. However, pertussis toxin would have to affect multiple endocytic pathways directly, because the internalization of the M_2 mAChR proceeds through an unidentified, clathrin-independent pathway (Roseberry and Hosey, 2001), whereas the internalization of the M_1 and M_4 mAChRs proceeds through clathrin-coated pits (Vogler et al., 1999). Further experiments will test whether pertussis toxin may have a direct effect on the internalization process itself, in addition to its effects on the delivery of receptors to the cell surface.

The identification of an intracellular pool of M_2 mAChRs located to the Golgi shows similarities to the protease-activated receptors (PARs). PARs are irreversibly activated by cleavage of their extracellular N terminus, and after their removal from the cell surface, are targeted to lysosomes for degradation (Hoxie et al., 1993; Hein et al., 1994). Intracellular pools of nonactivated PARs exist in the Golgi, and are delivered to the cell surface after stimulation of cell surface receptors to replenish the supply of receptors at the cell surface (Hein et al., 1994; Dery et al., 1999). This seems to function in an analogous manner to the recycling of other GPCRs to the cell surface after their internalization. Thus, the function of the intracellular pool of PARs is to allow for recovery of the capacity of the cell to signal through PARs, which is not achievable by recycling of the irreversibly activated receptors. Unlike the PARs, M_2 mAChRs do not seem to be degraded upon their internalization, because these re-

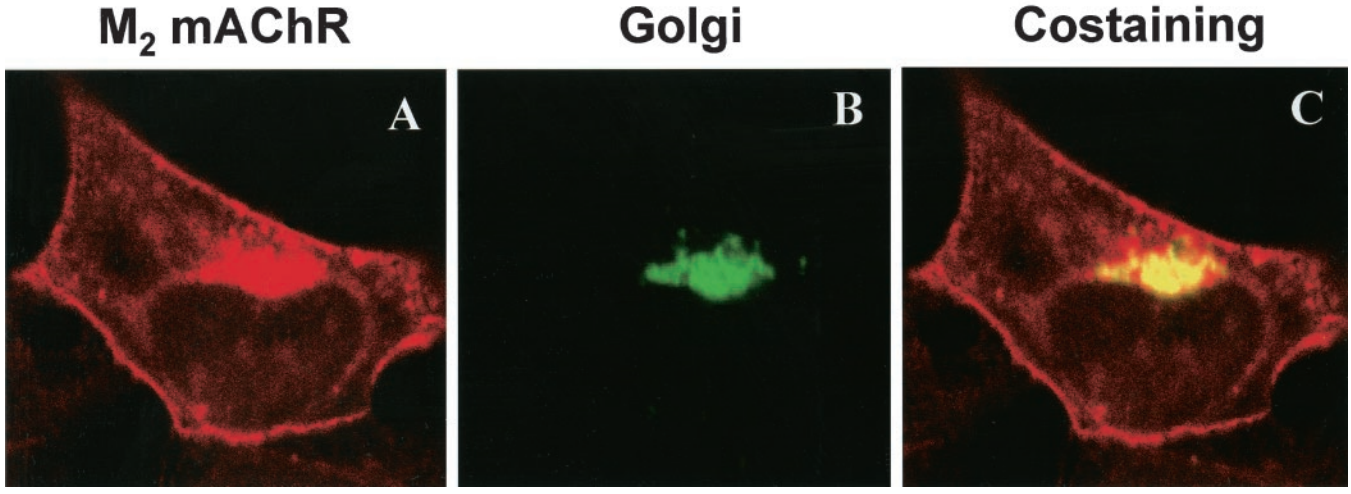

Fig. 8. M_2 mAChR and Golgi zone staining in HEK293 cells. HEK293 cells stably expressing M_2 mAChRs were fixed and stained with anti-M_2 mAChR antibodies (A) and anti-Golgi zone antibodies (B) and visualized with TRITC-conjugated anti-rat antibodies and FITC-conjugated anti-mouse antibodies. C, an overlay of the corresponding M_2 mAChR and Golgi zone images, and yellow color indicates colocalization.

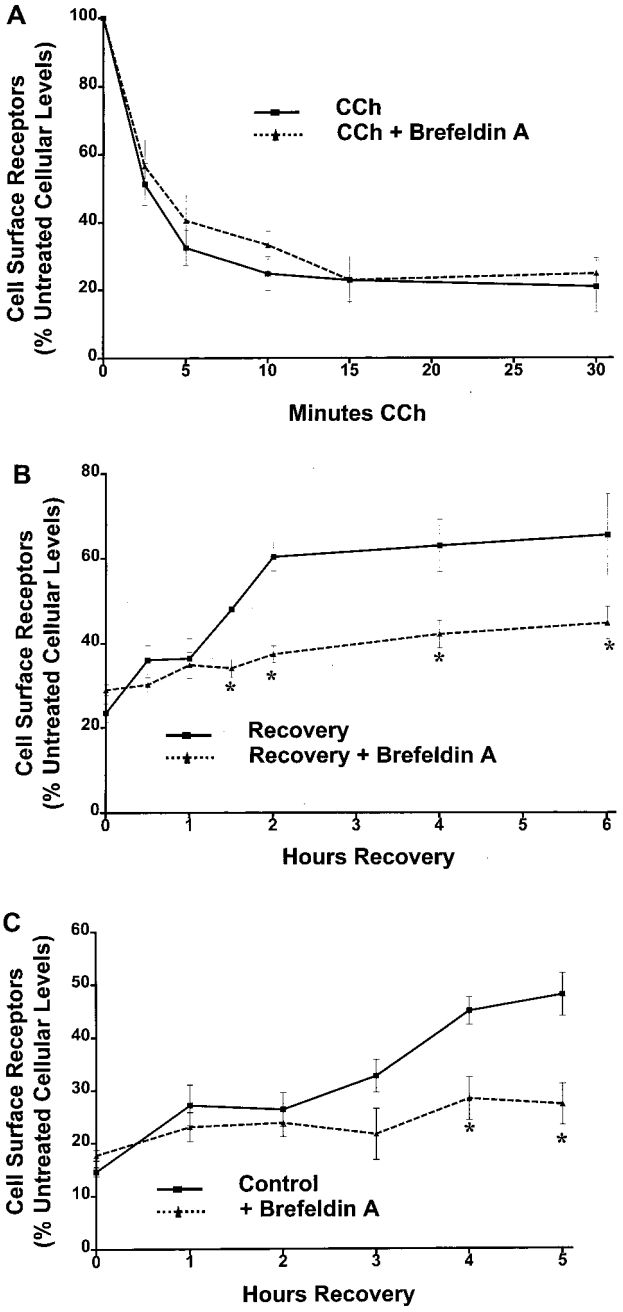

Fig. 9. Internalization and recycling of the M_2 mAChR after brefeldin A pretreatment. A, internalization of M_2 mAChRs. HEK293 cells stably expressing M_2 mAChRs were treated with (▲) or without (■) 50 μM brefeldin A for 1 h before the addition of CCh for the times indicated. Cell surface receptor levels were measured using the hydrophilic antagonist [^{3}H]NMS. B, recovery of M_2 mAChRs from agonist-dependent internalization. HEK293 cells stably expressing M_2 mAChRs were treated with (▲) or without (■) 50 μM brefeldin A for 1 h before the addition of CCh for 30 min. The CCh was removed by washing in PBS, and the cells were then incubated at 37°C in fresh media with (▲) or without (■) brefeldin A for the times indicated. Cell surface receptor levels were then determined using [^{3}H]NMS and are shown as a percentage of the levels observed in untreated cells. Results shown are means ± S.E. of four to six independent experiments. (*$p < 0.05$ versus control). C, changes in M_2 mAChR levels after irreversible alkylation of cell-surface receptors with PBCM. Cells were untreated or treated with 50 μM brefeldin A for 1 h at 37°C followed by treatment with PBCM for 30 min at room temperature to irreversibly alkylate cell-surface receptors. Recovery of cell-surface M_2 mAChR binding at the cell surface in the presence (▲) or absence (■) of brefeldin A was measured at the indicated times after PBCM removal. Cell surface receptor levels are shown as a percentage of the levels observed in untreated cells. Results shown are means ± S.E. of three to four independent experiments. (*$p < 0.05$ versus control).

ceptors do not down-regulate in HEK293 cells (Roseberry and Hosey, 1999). However, because there is an intracellular pool of M_2 mAChRs located in the Golgi, and the recovery of M_2 mAChRs to the cell surface after their internalization is sensitive to brefeldin A, it seems that the intracellular pool of receptors may function in the recovery of receptors to the cell surface after internalization. Because recovery of receptors to the cell surface is independent of the synthesis of new receptors (Roseberry and Hosey, 1999), this suggested that the intracellular receptors that were located in the Golgi were not newly synthesized receptors waiting to be delivered to the cell surface. Furthermore, the inclusion of cycloheximide had no effect on the colocalization of the M_2 mAChR with the Golgi markers (data not shown). These findings, in combination with the identification of heterotrimeric G proteins in the Golgi in a number of cell types, supports the hypothesis that the delivery of M_2 mAChRs to the cell surface may be regulated by heterotrimeric G proteins.

We have also demonstrated that pretreatment with pertussis toxin allowed for the agonist-dependent delivery of the $G_{i/o}$-coupled M_4 mAChRs to the cell surface and increased the rate of recovery of M_4 mAChRs to the cell surface after internalization. This suggests that this level of regulation of the trafficking of the M_2 mAChR is used by other GPCRs as well. It is tempting to speculate that the hypothesized regulation of delivery of mAChRs to the cell surface may be related to the role of internalization in desensitization of mAChRs. It has been demonstrated that internalization of the M_4 mAChR serves to prolong its desensitization (Bogatkewitsch et al., 1996). However, for other GPCRs, internalization is hypothesized to lead to resensitization of the receptors (Pippig et al., 1995; Zhang et al., 1997; Yang et al., 1999). The very slow recovery of M_2 mAChRs to the cell surface is incompatible with a role in rapid resensitization, and suggests that internalization may serve to prolong desensitization of this receptor subtype as well. Thus, it seems possible that regulation of the delivery of receptors to the cell surface by heterotrimeric G proteins may be a mechanism used to slow recovery of receptors to the cell surface and to prolong desensitization.

We also observed that pertussis toxin had no effect on the trafficking of the G_q-coupled M_3 mAChR as expected, but did allow for the agonist-dependent delivery of M_1 mAChRs to the cell surface, in addition to increasing the rate of recovery of M_1 mAChRs from internalization. This finding may seem to be contrary to the hypothesis that intracellular $G_{i/o}$ proteins may regulate delivery of GPCRs to the cell surface, because the M_1 mAChR is a G_q-coupled GPCR. However, regulation by $G_{i/o}$ proteins may not require coupling to the receptors themselves, and could simply function at a level independent of interactions with the receptors. This seems likely, because the trafficking of other nonreceptor proteins has been shown to be regulated by G proteins of the $G_{i/o}$ family. Furthermore, if the regulation of delivery to the cell surface is a mechanism involved in desensitization of GPCRs, this mechanism may operate for many GPCRs, regardless of the class of G proteins to which they couple. It is interesting that the agonist-dependent delivery of M_1 mAChRs to the cell surface after pertussis toxin pretreatment had no apparent effect on the internalization of the M_1 mAChR as it did for both the M_2 and M_4 mAChRs. This cannot be explained at this point, and requires further study.

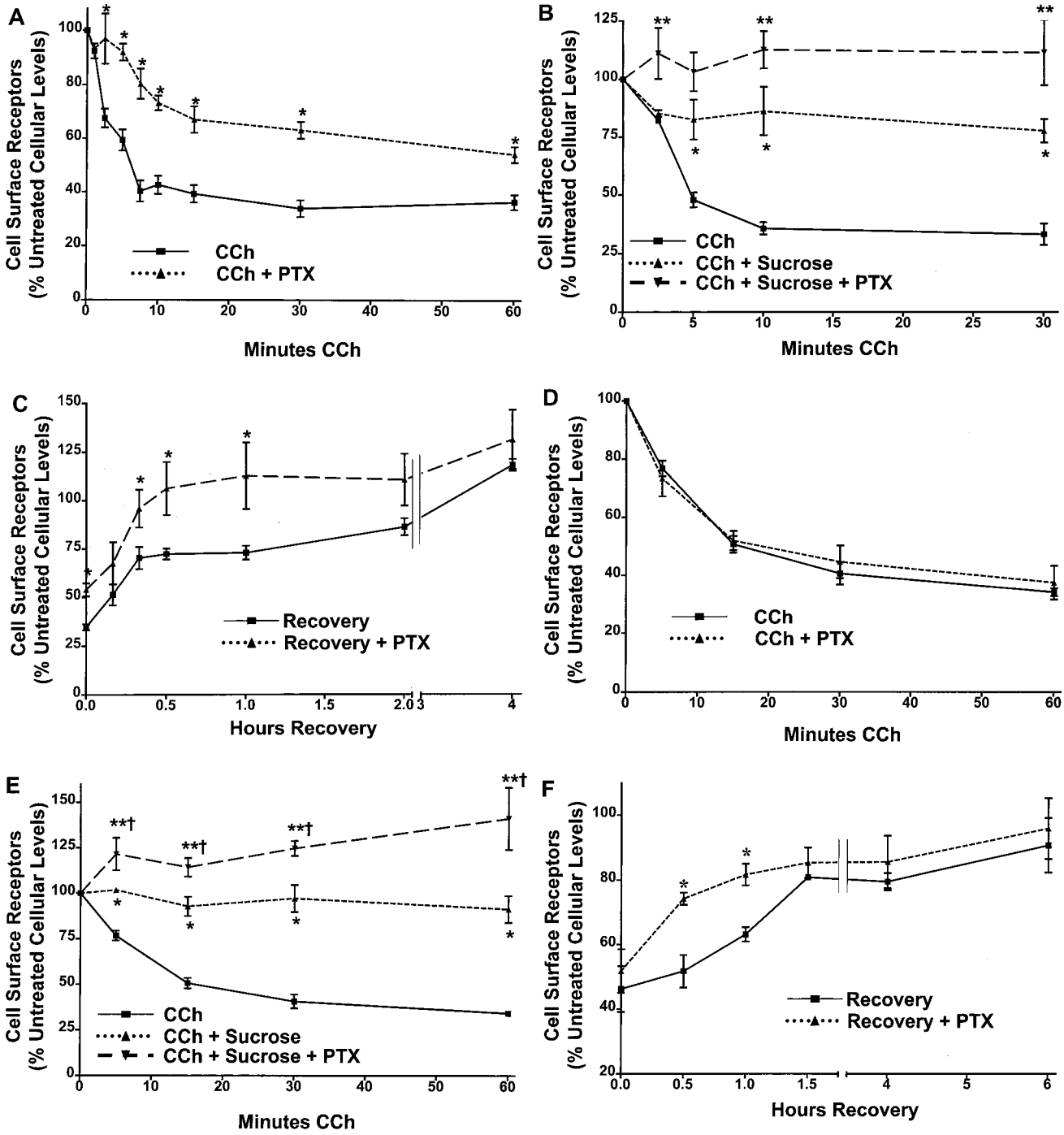

Fig. 10. Internalization and recovery of M_4 and M_1 mAChRs after pertussis toxin pretreatment. A, internalization of M_4 mAChRs after pertussis toxin pretreatment. HEK293 cells stably expressing M_4 mAChRs were treated with (▲) or without (■) pertussis toxin before the addition of CCh for the times indicated. Cell surface receptor levels were measured using [^{3}H]NMS. (*$p < 0.05$ versus control). B, internalization of M_4 mAChRs after pretreatment with sucrose and pertussis toxin. Cells were untreated (■), treated with 400 mM hypertonic sucrose (▲), or treated with 400 mM hypertonic sucrose after pretreatment with pertussis toxin (▼), before the addition of CCh for the times indicated. Cell surface receptor levels were measured using [^{3}H]NMS. Results shown are means ± S.E.M. of three to nine independent experiments. (*$p < 0.05$ CCh + sucrose versus CCh; **$p < 0.05$ CCh + pertussis toxin + sucrose versus CCh + sucrose). C, recovery of M_4 mAChRs after pertussis toxin pretreatment. Cells were treated with CCh for 30 min at 37°C after pretreatment with (▲) or without (■) pertussis toxin, and recovery to the cell surface was measured as described under *Experimental Procedures* and Fig. 6. Results shown are means ± S.E.M. of three to nine independent experiments. (*$p < 0.05$ versus control). D, internalization of M_1 mAChRs after pertussis toxin pretreatment. HEK293 cells stably expressing M_1 mAChRs were treated with (▲) or without (■) pertussis toxin before the addition of CCh for the times indicated. Cell surface receptor levels were measured using [^{3}H]NMS. E, internalization of M_1 mAChRs after pretreatment with sucrose and pertussis toxin. Cells were untreated (■), treated with 400 mM hypertonic sucrose (▲), or treated with 400 mM hypertonic sucrose after pretreatment with pertussis toxin (▼), before the addition of CCh for the times indicated. Cell surface receptor levels were measured using [^{3}H]NMS. (*$p < 0.05$ CCh + sucrose versus CCh; **$p < 0.05$ CCh + pertussis toxin + sucrose versus CCh + sucrose; †$p < 0.05$ versus zero time point). F, recovery of M_1 mAChRs after pertussis toxin pretreatment. Cells were treated with CCh for 30 min at 37°C after pretreatment with (▲) or without (■) pertussis toxin, and recovery to the cell surface was measured as described under *Experimental Procedures* and Fig. 6. Results shown are means ± S.E.M. of three to four independent experiments. (*$p < 0.05$ versus control).

The observation that pertussis toxin treatment allowed for the delivery of intracellular mAChRs to the cell surface in response to agonist stimulation brings up a number of interesting questions. What is the mechanism by which pertussis toxin is achieving these effects? What is the function of this delivery of receptors to the cell surface? Another interesting question concerns the nature of the signal arising from the receptors to tell intracellular receptors to traffic to the cell surface in response to agonist, because the M_2 mAChR is unable to signal through its normal subset of G proteins. In other studies we have observed that M_2 mAChRs can inhibit G protein-activated inwardly rectifying K^+ channels after pertussis toxin pretreatment (Bunemann et al., 2000). Taken together with the present results, these data demonstrate that the M_2 mAChR can signal through at least two independent pathways (K^+ channel inhibition and protein trafficking) in the absence of functional $G_{i/o}$ proteins. Obviously, further studies are necessary to identify the nature of the signals generated. It will also be important to determine whether pertussis toxin affects the internalization process itself, or whether the effects are limited to delivery of receptors to the cell surface. Furthermore, it will be of interest to ascertain whether pertussis toxin can cause these effects on a multitude of GPCRs. More studies are needed to attempt to answer these questions and to examine this process further.

Many aspects of the trafficking of GPCRs have been widely studied. Internalization of GPCRs is one aspect of the trafficking of GPCRs that has been widely examined, and although many aspects of the regulation of internalization remain to be elucidated, there is much that is understood about the regulation of internalization. In these studies we have demonstrated a previously unappreciated aspect of the regulation of the trafficking of GPCRs through the identification of the regulation of the delivery of GPCRs to the cell surface. These studies provide new insight into the trafficking of GPCRs from intracellular sites to the cell surface, and may lead to a better understanding of the role of internalization in modulating desensitization of GPCRs.

Acknowledgments

We thank Dr. Katharine Lee for helpful discussions during the course of this work.

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