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Mutation of Carboxyl-Terminal Threonine Residues in Human m3 Muscarinic Acetylcholine Receptor Modulates the Extent of Sequestration and Desensitization

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SUMMARY

We previously reported that a mutant human m3 muscarinic acetylcholine receptor in which threonine residues at positions 550, 553, and 554 in the carboxyl terminus had been substituted with alanines showed a significant blockage of receptor down-regulation when expressed in Chinese hamster ovary-K1 cells. Because Chinese hamster ovary cells showed little receptor sequestration, in the present study we investigated further the effects of these mutations on sequestration and desensitization in human embryonic kidney (HEK) 293 cells. Wild-type and mutant receptors were transiently transfected into HEK 293 cells. The level of m3 muscarinic acetylcholine receptor expression was ~300 fmol/mg protein, and the transfection efficiency was ~30% for all receptors. Also, wild-type and mutant receptors induced similar 4-fold increases in phosphoinositide (PPI) hydrolysis and showed similar Ca^{2+} responses after carbachol (CCh) treatment. However, the sequestration of wild-type receptors, determined as the difference between the extent of binding of lipophilic and hydrophilic ligands, occurred in a time- and dose-dependent manner to a maximum of ~40% of total receptors. In contrast, sequestration was almost totally blocked in cells expressing $Ala^{550,553}$ or $Ala^{550,553,554}$ mutant receptors. To determine the functional significance of sequestration and investigate its relationship to receptor desensitization, cells were preincubated with CCh and then washed free of agonist and restimulated with CCh. Desensitization was manifest as a time- and concentration-dependent decrease in the ability of the second stimulation to increase PPI hydrolysis. One-hour pretreatment with 1 mM CCh decreased PPI hydrolysis by 24% for wild-type receptors but had no effect on the ability of the mutant receptors to respond to a second CCh challenge. Furthermore, inhibition of wild-type receptor sequestration by treatment with conconavalin A also blocked desensitization to a 1-hr treatment with CCh. These results suggest that sequestration may be directly involved in m3 receptor desensitization at early times. More prolonged CCh treatment (3–9 hr) reduced the PPI hydrolysis response of the mutant and the wild-type receptors, indicating that the mechanism of m3 receptor desensitization at later times involves multiple components.

mAChR belongs to the G protein-coupled receptor family (1). Desensitization, the reduction in cellular response to agonist after prolonged exposure, is a common feature of this receptor family and may have multiple components, including receptor phosphorylation, uncoupling from G proteins, sequestration, and down-regulation (2). Despite numerous efforts to understand these cell functions and the relationships among them, the mechanistic and structural bases of these phenomena remain in large part unknown. In the best-studied example, the βAR system, the major component of desensitization appears to be the uncoupling of the receptor from its G protein (3). Receptor sequestration, the movement of the receptor from the cell surface to a compartment accessible to hydrophobic but not hydrophilic ligands, has been implicated as a potential component of desensitization (4) or, more recently, of resensitization (5, 6). Receptor down-regulation, the loss of receptors, occurs over a longer time period and is not believed to be important for rapid desensitization (2). Sequestration and down-regulation both likely involve receptor internalization. However, βAR sequestration and down-regulation appear to be regulated by different mechanisms that may or may not involve the activation of G proteins or receptor phosphorylation (7–9). It also is not clear whether receptor

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; HEK, human embryonic kidney; PKC, protein kinase C; PKA, protein kinase A; β_2AR, β_2-adrenergic receptor; βAR, β-adrenergic receptor; CHO, Chinese hamster ovary; CCh, carbachol; BBS, bombesin; [^{3}H]NMS, [^{3}H]*N*-methylscopolamine; PPI, phosphoinositide; PBS, phosphate-buffered saline; $[Ca^{2+}]_i$, intracellular calcium concentration; Con A, conconavalin A; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

sequestration serves as a prerequisite for receptor down-regulation or whether the two processes are independent.

We previously found that threonine residues at positions 550, 553, and 554 in the human m3 mAChR carboxyl terminus are required for receptor down-regulation in CHO-K1 cells and that the loss of down-regulation reduces receptor desensitization (10). Unfortunately, we could not study further the role of receptor sequestration in the same system because m3 mAChRs did not show significant agonist-induced sequestration in CHO cells. In the present study, we examined the regulation of wild-type and mutant human m3 receptors in HEK 293 cells, which are known to rapidly sequester mAChR (11). We found that the mutations that were previously found to block down-regulation in CHO cells also blocked sequestration in 293 cells. We also found that sequestration may be a component of receptor desensitization at early times but that other components were involved in receptor desensitization at later times.

Experimental Procedures

Materials. [^{3}H]NMS (81.5 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). [^{3}H]Scopolamine (83 Ci/mmol) was custom-synthesized by Amersham Corp. (Arlington Heights, IL) and generously supplied by Dr. S. K. Fisher (University of Michigan, Ann Arbor, MI). *myo*-[^{3}H]Inositol (19.1 Ci/mmol) was obtained from Amersham Corp. Atropine and carbachol were obtained from Sigma Chemical Co. (St. Louis, MO). Conconavalin A was obtained from Calbiochem (La Jolla, CA). Analytical-grade Dowex 1-X8 (AGE-X8, 100–200 mesh) was obtained from Bio-Rad (Rockville Center, NY). Fura-2/AM was obtained from Molecular Probes (Eugene, OR). Lipofectin, lipofectamine, Dulbecco's modified Eagle's medium, Dulbecco's PBS, trypsin-EDTA, and fetal bovine serum were purchased from GIBCO (Grand Island, NY). Tissue culture plasticware was obtained from Costar (Cambridge, MA).

Construction of vectors expressing human m3 mAChRs and mutant receptors. The construction of wild-type and mutant human m3 receptors in the mammalian expression vector pTEJ-8 was described previously (10). As previously reported, mutant receptors were generated with the use of site-directed mutagenesis to replace threonines 550, 553, and 554 with alanines ($Ala^{550,553,554}$, $Ala^{553,554}$, Ala^{550}, and Ala^{553}) and cysteines 561 and 563 with glycines ($Gly^{561,563}$).

Cell culture and transfections. Native human kidney HEK 293 cells were obtained from Dr. D. Ginsberg (University of Michigan, Ann Arbor, MI). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum in an environment of 5% CO_2. Transfections of HEK 293 cells were carried out by the lipofectin or lipofectamine method. Briefly, 2×10^6 HEK 293 cells were plated onto 10-cm tissue culture dishes. After a 24-hr culture, a mixture of 10 μg DNA with 50 μl of either lipofectin or lipofectamine was added to the cells cultured in the media without fetal bovine serum. After 6 hr of incubation at 37°, cells were changed to a fresh complete media and cultured for an additional 48 hr. Cells were then plated onto 24-well dishes at a density of 2×10^5 and allowed to attach overnight. Subsequently, receptor binding and PPI hydrolysis assays were performed. For stable transfectants, cells were treated similarly except that selection was carried out with G418. G418-resistant clones were chosen and tested for receptor expression with a radioligand binding assay.

Radioligand binding and sequestration assays. HEK 293 cells plated onto 24-well culture dishes were incubated with the radioactive antagonist [^{3}H]NMS or [^{3}H]scopolamine at 4° for 24 hr. The concentration of radioligands used for binding studies was 2 nM. The cells were incubated in 1 ml buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, and 30 mM Na-HEPES buffer, pH 7.4). Nonspecific binding determined in the presence of 10 μM atropine was less than 10% of the total binding. For the study of receptor sequestration, cells were preincubated with indicated concentrations of carbachol for different time periods at 37°. The cells were detached and washed three times with PBS, after which binding assays with either [^{3}H] NMS or [^{3}H]scopolamine were performed at 4°. The binding reactions were terminated by centrifugation, and the cells were subsequently washed four times with 2 ml ice-cold 0.9% NaCl. Cells were lysed with 0.5 ml of 0.1 M NaOH, and radioactivity was determined in 5 ml Bio-Safe II scintillation fluid (Research Products International Corp., Mount Prospect, IL). Protein concentrations were determined according to the method of Bradford (12) with a Bio-Rad protein assay kit.

Measurement of $[Ca^{2+}]_i$. Transfected HEK 293 cells were allowed to attach to a coverslip overnight. These cells were incubated with 1 μM Fura-2/AM at an ambient temperature for 30 min and then washed with fresh physiological saline solution. For measurement of $[Ca^{2+}]_i$, Fura-2-loaded cells were transferred to a closed 100-μl chamber, mounted onto the stage of a Zeiss Axiovert inverted microscope, and continuously superfused at 1 ml/min with physiological saline solution at 37°. Solution changes were rapidly accomplished with a valve attached to an eight-chamber superfusion reservoir. Measurement of $[Ca^{2+}]_i$ was made with an Attofluor digital imaging system (Rockville, MD) (13). In brief, excitation of cells at 334 and 380 nm (10 nm bandpass) was accomplished with a computer-selectable filter-and-shutter system. Resultant emission at 510 nm was monitored with an intensified CCD camera and subsequently digitized. Calibration of $[Ca^{2+}]_i$ signals was as described previously (14). The excitation of the cells was induced by applying 100 μM CCh through the perfusion chamber for 4 min. Cells showing a response to CCh within 20 sec consisting of an increase in $[Ca^{2+}]_i$ of more than 30 nM were counted as positive, indicating that they were transfected cells. Untransfected cells showed no response. Each experiment included data from at least 10 cells.

PPI hydrolysis assays and desensitization studies. HEK 293 cells transiently transfected with wild-type and mutant receptor cDNA were plated at a density of 2×10^5/well in 24-well culture dishes and labeled with *myo*-[^{3}H]inositol (3 μCi/ml) in culture media for 24 hr at 37°. For studies in which we measured the time course of desensitization, cells were incubated with *myo*-[^{3}H]inositol for 24 hr with or without 1 mM CCh for different time periods. For desensitization dose-response studies, cells were treated with the indicated concentration of CCh for 9 hr. For studies on the effects of treatment with Con A, cells were treated with 0.25 mg/ml Con A in buffer A minus glucose at 37° for 20 min. Cells were then washed two times with PBS and once with buffer A containing 20 mM LiCl and then incubated in 1 ml of this buffer with or without 1 mM CCh for an additional hour at 37°. After stimulation, reactions were terminated by the addition of 10% trichloroacetic acid. Total inositol phosphates were extracted and isolated following the method of Berridge *et al.* (15).

Effects of pretreatments on the total PPI precursor pool were measured with the use of chloroform-methanol extraction (16) after [^{3}H]inositol labeling of cells. For these control studies, we used HEK 293 cells stably transfected with wild-type and $Ala^{550,553,554}$ m3 mAChRs. It was necessary to use stably expressing cells because changes in the precursor pool would be expected only in cells expressing the receptors. The stably transfected cells showed identical properties to the transients in terms of binding, coupling, sequestration, and desensitization (data not shown). Briefly, cells treated with or without 1 mM CCh for 9 hr were pelleted and resuspended in 4 ml of chloroform/methanol (2:1) containing 0.25% (v/v) HCl. After incubation on ice for 20 min, 0.8 ml of 1 N HCl was added, and the samples were centrifuged for 5 min. Two milliliters of the lower phase were taken and dried under N_2. The dried lipids were dissolved in 120 μl of chloroform/methanol (2:1), and 50 μl was applied to a thin layer chromatography plate. PI, PIP, and PIP2 bands were visualized with the use of autoradiography after spraying with Enhance (New Eng-

land Nuclear); bands were then scraped and counted in 10 ml of liquid scintillation cocktail as well as the remaining 70 μl of total PI, PIP, and PIP2 lipids.

Statistical analysis. Specific receptor binding was obtained by subtracting nonspecific binding in the presence of atropine from the amount bound in its absence. All values are given as mean ± standard error. Student's *t* test was used for statistical analysis of the data.

Results

Wild-type and mutant m3 mAChR expression and signal transduction measurement. Wild-type and mutant receptors were transiently transfected into HEK 293 cells. The expression of receptors was measured by binding of the antagonist [^{3}H]NMS at a concentration that saturates high affinity receptors; wild-type receptor expression was approximately 270 fmol/mg protein (Table 1). Ala^{550,553,554}, Ala^{553,554}, Ala^{550}, Ala^{553}, and Gly^{561,563} mutants exhibited similar expression levels as the wild-type receptor. To investigate the transfection efficiency, we measured the agonist-induced increase in intracellular Ca^{2+} for many transfected and native HEK 293 cells with the use of digital imaging. Transfected cells showed a rapid increase in $[Ca^{2+}]_i$ after stimulation with 0.1 mM CCh for both wild-type and Ala^{550,553,554} receptors (Fig. 1). No significant differences between cells expressing wild-type or Ala^{550,553,554} receptors were noted in either peak increase (wild-type, 161 ± 10 nM, 5 experiments) ; Ala^{550,553,554}, 156 ± 8 nM, 3 experiments) or plateau increase over basal (wild-type, 30 ± 4 nM; Ala^{550,553,554}, 27 ± 4 nM) $[Ca^{2+}]_i$ responses. Approximately 30% of the cells were transfected in these assays and no significant difference was seen in transfection efficiency between wild-type and Ala^{550,553,554} (Table 1). On the basis of a 30% transfection efficiency, the overall receptor level of 270 fmol/mg of protein, and ~2.2 × 10^6 HEK 293 cells/mg of protein, an estimation can be made of 243,000 receptors per transfected cell. None of the untransfected HEK 293 cells showed $[Ca^{2+}]_i$ increase in response to the CCh stimulation.

In addition to analysis of the receptor induced calcium signaling, we measured agonist-induced PPI hydrolysis in transfected cells. The increase in total [^{3}H]inositol phosphates was linear for up to 90 min of CCh stimulation in both wild-type and alanine mutant receptor-bearing cells (data not shown). Untransfected HEK 293 cells displayed no significant increase in PPI hydrolysis on CCh addition (Fig. 2). Wild-type receptor transfected cells (n = 5) showed a 4.1 ± 0.8-fold increase in response to 1 mM CCh. The alanine (n = 5) and glycine (n = 4) mutants showed a similar level of agonist-induced PPI hydrolysis as wild-type receptor, with 3.6 ± 0.5- and 4.5 ± 0.9-fold increases, respectively. No significant differences were noted in the basal levels of PPI hydrolysis in unstimulated cells expressing the different receptors (data not shown). These results indicated that similar to what was found in CHO cells (10), mutated residues in the m3 mAChR carboxyl terminus were not involved in receptor binding or signal transduction in HEK 293 cells. The PPI responses in HEK 293 cells were smaller than those previously found in the stable transfected CHO cell clones, which showed an 8–9-fold increase (10), but are similar when adjusted to the 30% receptor transfection rates. In addition to the similar maximum PPI response to CCh, the wild-type and mutant receptors displayed the same CCh concentration dependence (Fig. 3); the EC_{50} values for the increase in inositol phosphates induced by wild-type and mutant receptors were 8 and 10 μM, respectively. These results indicated that the binding and coupling of mutant receptors to signal

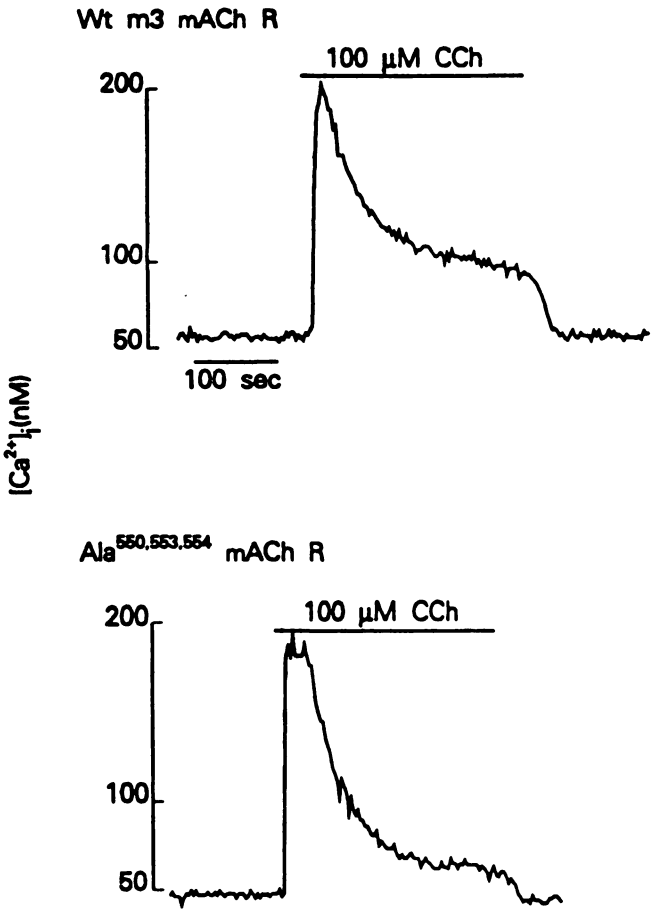

Fig. 1. CCh-induced Ca^{2+} signaling in wild-type (*Wt*) and Ala^{550,553,554} m3 mAChR transfected HEK 293 cells. At 48 hr after transfection, cells were plated onto coverslips for overnight attachment and then loaded with Fura-2 and imaged. Cells were stimulated with 100 μM CCh for 4 min. Each individual experiment involved one to four slips. The figure represents a representative single-cell Ca^{2+} response.

TABLE 1

Expression efficiency of wild-type and mutant m3 mAChR in HEK 293 cells

We plated 10^6 HEK 293 cells onto 10-cm culture dishes and transfected them the next day with 50 μl lipofectin and 10 μg of either wild-type or mutant receptor plasmids. After 48-hr incubation, cells were split onto 24-well dishes, and radioligand binding assays were performed 24 hr later with a saturating concentration of 2 nM [^{3}H]NMS and incubation for 24 hr at 4°. To measure Ca^{2+} signaling, cells were allowed to grow on coverslips. After overnight attachment, cells were loaded with Fura-2 and stimulated with 100 μM CCh for 4 min. Each individual experiment contained one to four slides, from which at least 10 cells were recorded, and data are given as percentage of the total cells responding to the CCh stimulation. All values are given as mean ± standard error of the indicated numbers of experiments. ND = not determined.

	Receptor expression	Ca^{2+} signaling
	fmol/mg protein	*% cells responding*
Wild-type	270 ± 38 (n = 8)	31 ± 2 (n = 5)
Ala^{550,553,554}	255 ± 52 (n = 5)	28 ± 4 (n = 3)
Ala^{553,554}	286 ± 30 (n = 3)	ND
Ala^{550}	325 ± 40 (n = 4)	ND
Ala^{553}	341 ± 11 (n = 4)	ND
Gly^{561,563}	266 ± 77 (n = 4)	ND

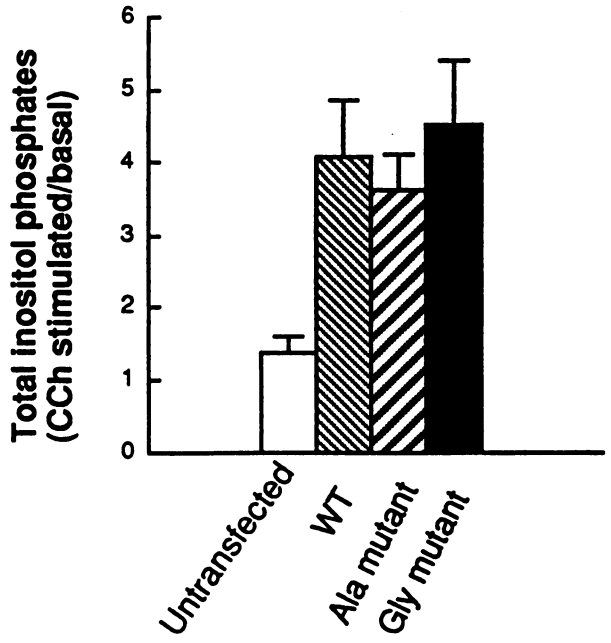

Fig. 2. CCh-induced PPI hydrolysis of wild-type (*WT*) and mutant m3 mAChRs. Wild-type, Ala^{550,553,554}, and Gly^{561,563} expression plasmids were transiently transfected into HEK 293 cells. After 48 hr, cells were plated onto 24-well culture dishes and incubated with 3 μCi/ml *myo*-[^{3}H]inositol for an additional 24 hr. Cells were then washed and stimulated with 1 mM CCh for 1 hr at 37° in the presence of 20 mM LiCl. Inositol phosphates were extracted and compared with the untransfected HEK 293 cells. Data are mean ± standard error of four or five experiments.

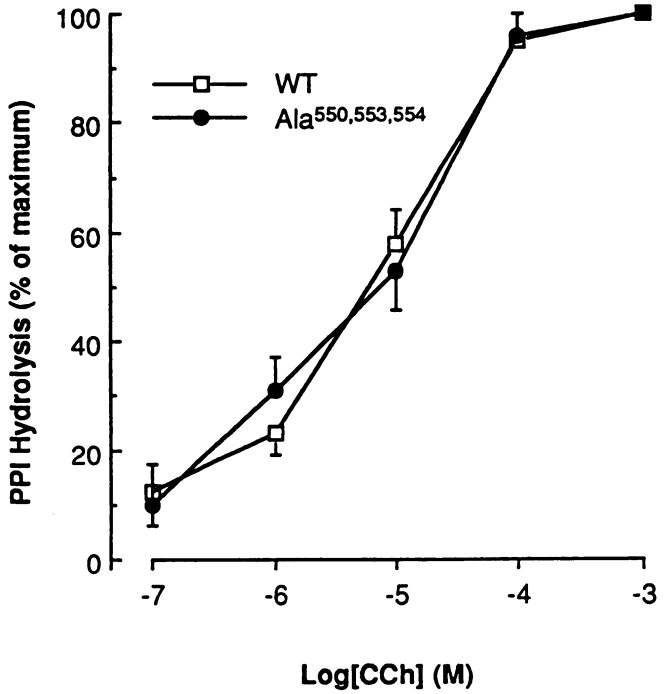

Fig. 3. Dose dependence of PPI hydrolysis for wild-type (*WT*) and Ala^{550,553,554} mAChRs. Transfected cells were stimulated with indicated concentration of CCh for 1 hr at 37° in the presence of 20 mM LiCl. The maximum increase in PPI hydrolysis in 1 mM CCh stimulation group was defined as 100%. Data are mean ± standard error of three experiments.

transduction pathways in transiently transfected HEK 293 cells were similar to the wild-type receptors.

CCh-induced wild-type and mutant m3 mAChR sequestration and down-regulation. To investigate receptor sequestration and down-regulation, transfected HEK 293 cells were pretreated with 1 mM CCh for different time periods at 37° and then cooled to 4°, and cell surface and total m3 receptors were determined. A time-dependent sequestration of receptor was observed in cells transfected with the wild-type receptor as measured by the difference in the level of binding to the hydrophilic ligand [^{3}H]NMS and lipophilic ligand [^{3}H]scopolamine (Fig. 4, *left*). Receptor sequestration was initially seen after a 30-min preincubation with CCh and reached a maximum at 1 hr. After a 1-hr pretreatment with 1 mM CCh, ~36% of surface wild-type receptors measured by hydrophilic ligand were sequestered, but the total number of the receptors as determined by the lipophilic ligands remained unchanged (Table 2). The extent of receptor sequestration was not increased by prolonged preincubation times. Sequestration of the wild-type receptor was also dependent on the CCh concentration (Fig. 5). A measurable response was found at 10^{-5} M, and a maximum effect occurred at 10^{-3} M CCh. The EC_{50} for wild-type receptor sequestration was 12 μM.

In contrast, the Ala^{550,553,554} mutant receptor did not display any agonist-induced receptor sequestration (Fig. 4, *right*). The Ala^{553,554} mutant also displayed a reduced receptor sequestration after 1 hr of CCh treatment (Table 2). The single mutants Ala^{550} and Ala^{553} and the Gly^{561,563} mutant showed levels of receptor sequestration equal to those of the wild-type receptor. These latter results suggested that m3

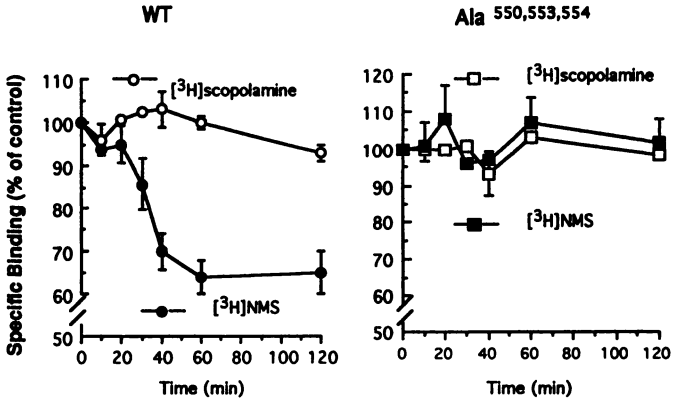

Fig. 4. Time course of CCh-induced sequestration in wild-type (*WT*) and Ala^{550,553,554} mAChRs. At 48 hr after transfection, cells were plated onto 24-well culture dishes and incubated at 37° with 1 mM CCh for the indicated times. At the end of the incubation, cells were washed three times with PBS, and radioligand binding assays were performed at 4° in the presence of 2 nM [^{3}H]NMS or [^{3}H]scopolamine for 24 hr. Then, 10 μm atropine was added to determine nonspecific binding, which was less than 10% of total binding at each time point. Data are mean ± standard error of three experiments.

receptor carboxyl-terminal cysteine residues are not involved in the receptor regulation and that the effects seen with the Ala^{550,553,554} are specific. Prolonged treatment with CCh, up to 24 hr, induced only a 19% receptor down-regulation in the HEK 293 cells, which was much less than observed results obtained with CHO cells (10). This lack of major receptor down-regulation in HEK 293 cells has been reported previously (11).

TABLE 2

CCh-induced wild-type and mutant receptor sequestration and downregulation in HEK 293 cells

At 48 hr after transfection, cells were plated onto 24-well dishes and incubated with 1 mM CCh for indicated time periods. After subsequent washing with ice-cold PBS three times, radioligand binding assays were performed with [^{3}H]NMS or [^{3}H]scopolamine at 4°. Data are mean ± standard error of three or four experiments and expressed as percentage of binding observed without CCh pretreatment.

Clone	[^{3}H]NMS		[^{3}H]Scopolamine	
	1 hr	24 hr	1 hr	24 hr
Wild-type	57 ± 5	51 ± 5	95 ± 3	81 ± 4
Ala^{550}	69 ± 8	60 ± 6	96 ± 7	76 ± 4
Ala^{553}	58 ± 5	47 ± 5	87 ± 4	69 ± 3
$Ala^{550,553,554}$	89 ± 9[a]	92 ± 6[b]	92 ± 9	86 ± 4
$Ala^{553,554}$	107 ± 6[b]	102 ± 2[b]	104 ± 1	98 ± 3
$Gly^{561,563}$	71 ± 4	56 ± 2	93 ± 5	86 ± 8

[a] $p < 0.05$,
[b] $p < 0.01$ compared with the wild-type.

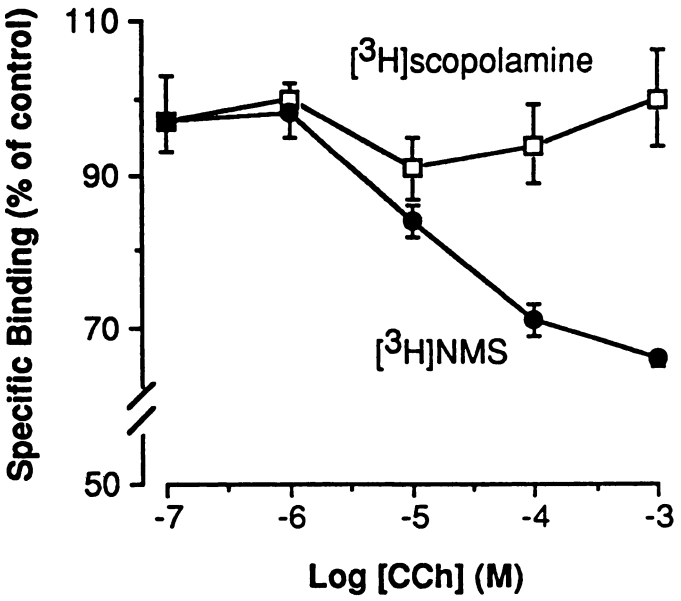

Fig. 5. Concentration dependence of CCh-induced sequestration in wild-type mAChRs. Cells were plated onto 24-well culture dishes 48 hr after transfection and then treated with the indicated concentration of CCh for 1 hr at 37°. At the end of incubation, cells were washed three times with PBS, and radioligand binding assays were performed at 4° for 24 hr in the presence of 2 nM [^{3}H]NMS or [^{3}H]scopolamine. Then, 10 μm atropine was added to determine nonspecific binding, which was less than 10% of total binding at each time point. Data are mean ± standard error of three experiments.

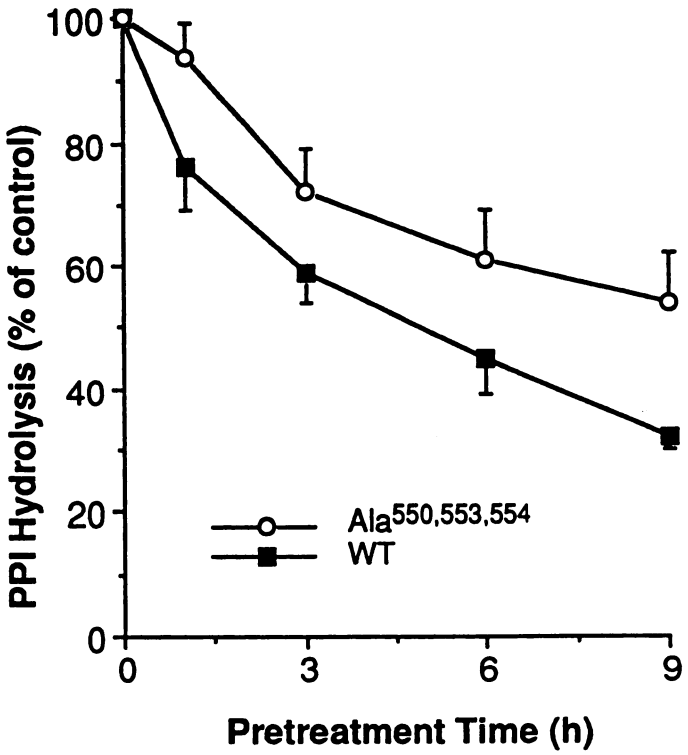

Fig. 6. Time course of CCh-induced PPI hydrolysis desensitization in wild-type (*WT*) and $Ala^{550,553,554}$ mAChRs. After 48-hr transfection, cells were plated onto 24-well culture dishes and labeled with 3 μCi/ml *myo*-[^{3}H]inositol for 24 hr. Then, 1 mM CCh was added to the plates during the labeling time period. After preincubation, cells were washed twice with PBS and once with buffer A plus LiCl and restimulated with 1 mM CCh for 1 hr at 37° in the same buffer. Inositol phosphates were extracted. The basal counts were subtracted from each time point, and the restimulated PPI hydrolysis response was compared with that of the nonpretreated group, which was defined as 100%. Basal counts did not vary significantly between wild-type and $Ala^{550,553,554}$-bearing cells (229 ± 33 cpm and 282 ± 51 cpm, $n = 5$, respectively). Data are mean ± standard error of four to eight experiments.

Desensitization of PPI hydrolysis in cells bearing wild-type and mutant receptors by CCh-induced prolonged receptor activation. Desensitization in HEK 293 cells was studied by measuring PPI hydrolysis after the cells were pretreated with CCh. The basal level of PPI hydrolysis in the transfected cells was slightly increased by the preincubation of CCh at the early time periods (1–6 hr), and it returned to control levels after a prolonged pretreatment. When wild-type receptors were exposed to 1 mM CCh for 1 hr, the subsequent ability of the agonist to maximally stimulate PPI hydrolysis was reduced by approximately 24% ($n = 7$). In contrast, the $Ala^{550,553,554}$ mutant did not show any significant PPI desensitization after being exposed to CCh for 1 hr (Fig. 6). On prolonged exposure to CCh, both wild-type and mutant receptors exhibited a decreased response to CCh restimulation (Fig. 6). However, the extent of desensitization of the mutant receptors was less than that of the wild-type receptors at all times. After 9 hr of pretreatment with CCh, the cell PPI responses to the second CCh challenge decreased by 68 ± 2% for the wild-type receptor and decreased by 46 ± 7% for the $Ala^{550,553,554}$ mutant receptor ($n = 5$).

We then investigated the concentration dependence of desensitization to a 9-hr exposure to CCh (Fig. 7). The desensitization of m3 mAChR PPI hydrolysis at the 9-hr time point was CCh concentration dependent for both wild-type and $Ala^{550,553,554}$ mutant receptors. The initial PPI desensitization could be observed at 10^{-5} M, and the maximum desensitization occurred at 10^{-3} M. $Ala^{550,553,554}$ cells showed a decreased desensitization response at any given CCh concentration compared with the wild-type receptors. These results suggested that cellular processes other than receptor sequestration and down-regulation, which required greater concentrations of agonist, were responsible for long term PPI hydrolysis desensitization of m3 receptors.

At early time points, the wild-type receptor was significantly desensitized and sequestered, whereas the $Ala^{550,553,554}$ mutant receptor was not. This suggested that sequestration may have been a component of desensitization in the wild-type receptor, especially at early times after ag-

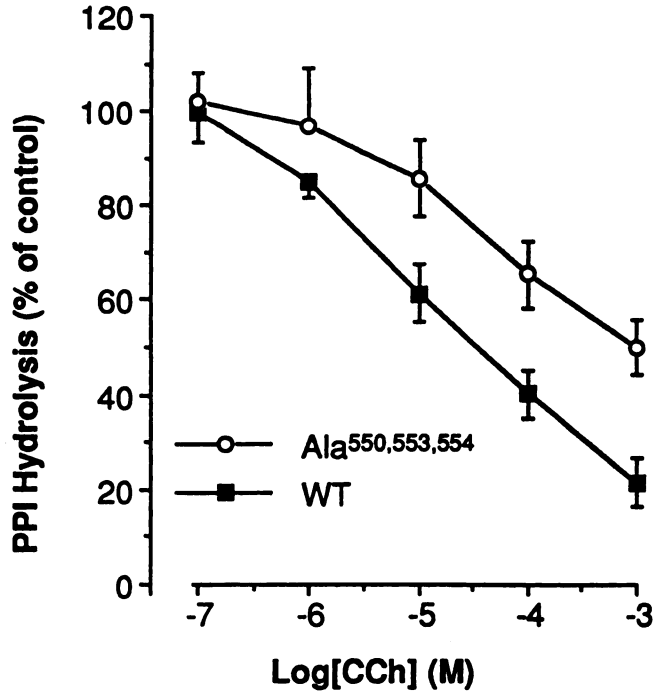

Fig. 7. Dose dependence of CCh-induced PPI hydrolysis desensitization in wild-type (*WT*) and $Ala^{550,553,554}$ mAChRs. Transfected cells were stimulated with the indicated concentration of CCh for 9 hr in the presence of 3 μCi/ml *myo*-[^{3}H]inositol. Cells were then washed with PBS and buffer A plus LiCl and restimulated with 1 mM CCh for 1 hr at 37° in the same buffer A. Inositol phosphates were extracted. The basal counts were subtracted from each time points, and the restimulated PPI hydrolysis response was compared with that of the nonpretreated group, which was defined as 100%. Data are mean ± standard error of four to seven experiments.

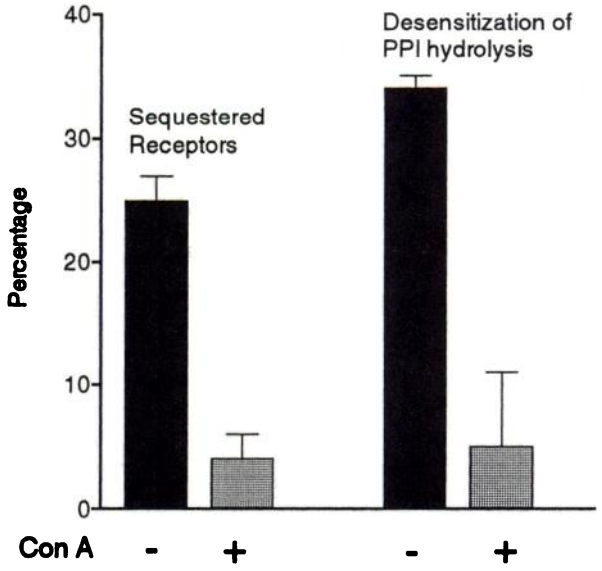

Fig. 8. Effect of Con A pretreatment on wild-type m3 receptor sequestration and PPI hydrolysis desensitization. HEK 293 cells were transiently transfected with wild-type m3 receptor cDNA. Transfected cells were washed free of culture media 48 hr after transfection and pretreated with or without 0.25 mg/ml of Con A in buffer A minus glucose at 37° for 20 min. Receptor sequestration and desensitization were induced by exposure of the cells to 1 mM CCh for 1 hr. For analysis of receptor sequestration, at the end of the incubation cells were washed three times with PBS, and radioligand binding assays were performed at 4° in the presence of 2 nM [^{3}H]NMS and [^{3}H]scopolamine for 24 hr. Data shown represent binding to sequestered receptors ([^{3}H]scopolamine binding minus [^{3}H]NMS binding) after CCh treatment, with or without Con A treatment, as a percentage of binding in untreated controls and are the mean ± standard error of four experiments. For analysis of PPI desensitization, transfected cells were labeled with 3 μCi/ml *myo*-[^{3}H]inositol for 48 hr. After preincubation with or without Con A and with or without CCh, cells were washed twice with PBS and once with buffer A plus LiCl and restimulated with 1 mM CCh for 1 hr at 37° in the same buffer. Inositol phosphates were extracted, basal counts were subtracted, and the difference in the PPI hydrolysis response between nonpretreated and CCh-pretreated cells was calculated as a percentage of the response in the control group. Data are the mean ± standard error of five experiments.

onist treatment. To further explore this issue, we used Con A treatment to block sequestration of the wild-type receptor and determined the effects on receptor desensitization. Con A blocked both the sequestration and desensitization normally observed after 1 hr of treatment of cells expressing the wild-type m3 receptor with CCh (Fig. 8).

An alternative explanation for the reduction in responsiveness of the cells after CCh pretreatment could be a decrease in the PPI precursor pool. To control for this variable, the total cellular pool of inositol lipids was assessed after various periods of CCh treatment. Total cellular contents of inositol phospholipids were unchanged after 9 hr of CCh treatment (97.5 ± 5.5% of control, $n = 4$). To further determine whether the PIP2 precursor pool was affected by CCh treatment, the inositol phospholipid pools were analyzed with chloroform-methanol extraction (Table 3). These data showed that control inositol phspholipids were distributed 91% as PI, 6% as PIP, and 3% as PIP2. More important, CCh pretreatment did not significantly decrease the PIP2 pool for up to 9 hr.

TABLE 3

Effect of CCh treatment on cellular phosphoinositol contents

HEK 293 cells stably expressing human m3 mAChRs were labeled with 3 μCi/ml *myo*-[^{3}H]inositol for 24 hr. Then, 1 mM CCh was added to the plates during the labeling time period for either 1 or 9 hr before the cells were harvested. Lipids were then extracted with chloroform-methanol and were separated by thin layer chromatography. PI, PIP, and PIP2 bands were visualized by autoradiography, scrapped, and counted. Results are expressed as a percentage of counts in cells not treated with CCh and are the mean ± standard error of 7 or 8 experiments.

CCh pretreatment	PI	PIP	PIP2
1 hr	90 ± 6	126 ± 19	94 ± 4
9 hr	96 ± 9	143 ± 28	123 ± 7

Discussion

We previously observed that when expressed in CHO-K1 cells, an m3 mAChR with three threonines at positions 550, 553, and 554 in the carboxyl terminus mutated to alanines bound ligand and coupled to phospholipase C activation and $[Ca^{2+}]_i$ release equal to wild-type receptors but did not down-regulate (10). However, due to the lack of significant sequestration of the wild-type m3 mAChR in this cell model, efforts to understand the roles of these threonine residues in sequestration were frustrated. In the present study, we transiently transfected wild-type and mutant receptors into HEK 293 cells, in which we were able to study further the roles of the mutations in receptor sequestration and desensitization. Our results showed that mutation of these threonine residues blocked m3 mAChR sequestration and significantly reduced receptor desensitization.

In the present study, we found that mutations of threonine residues in the carboxyl terminus of the m3 mAChR that had previously been shown to prevent receptor down-regulation

in CHO cells also prevented receptor sequestration in HEK 293 cells. Thus, these data support a relationship between these two phenomena. However, the nature of the relationship between receptor sequestration and down-regulation is unclear. Sequestration involves the removal of the receptors from the cell surface into a space where they are no longer accessible to hydrophilic ligands. Evidence from a variety of morphological and biochemical studies suggested that this process involves the formation of intracellular vesicles (17–20). Furthermore, lysosomal inhibitors have been shown to prevent agonist-induced receptor degradation (21, 22). These observations have led to a model in which sequestration and down-regulation involve receptor internalization into vesicles that may be targeted to different cellular compartments (20, 23). If the receptor-containing vesicles recycle to the cell surface with the receptors remaining intact, no decrease in binding is observed and no down-regulation has occurred. Alternatively, the vesicles may deliver the receptors to lysosomes, where they are degraded, resulting in a net loss in receptor number, i.e., down-regulation. Therefore, according to this model, sequestration is a prerequisite stage for down-regulation but may occur independently. That receptors can sequester independent of down-regulation is apparent from a large number of studies with different receptors. Studies on adrenergic receptors showed that β2AR with impaired coupling to G_s sequestered normally but exhibited a reduced extent of down-regulation (24). Lameh *et al.* (11) found that partial deletion of the third intracellular loop of human m1 mAChR resulted in a mutant receptor deficient in agonist-induced sequestration but not down-regulation. In the present study, we found that HEK 293 cells supported m3 mAChR sequestration without significant down-regulation.

However, there are a number of recent reports of down-regulation occurring in the absence of sequestration. We found that CHO cells downregulated m3 mAChR without an observable increase in the proportion of receptors sequestered (10). Also, mutation of the proximal portion of the carboxyl terminus of the β_2AR abolished receptor sequestration yet did not affect receptor down-regulation (25). In addition, it has been shown that mutation of tyrosine residues in the β_2AR carboxyl terminus were selectively involved in receptor down-regulation (26) or sequestration (6). These studies suggested that at least for the βAR, sequestration and down-regulation are not dependent on each other. What may reconcile these observations is the realization that the sequestration compartment is transient and dynamic. In specific cell lines or with specific mutations of receptor residues, the receptors may target directly a degradative pathway, or the rate of receptor recycling may exceed the rate of internalization. In either of these cases, no significant accumulation of intracellular receptors would be expected. If the receptors target directly and rapidly to a degradative pathway, then down-regulation in the absence of measurable sequestration would be observed experimentally. Alternatively, separate pathways for sequestration and down-regulation that are completely independent may exist. In this situation, our observation that the same mutations that block sequestration also block down-regulation would be due to effects on independent mechanisms.

A variety of receptor domains have previously been suggested as important in receptor internalization. In particular, portions of the second cytoplasmic loop (27), the third cytoplasmic loop (27–29), and the carboxyl terminus (6, 9, 10, 25, 30–34) have been found to influence internalization. Of particular relevance to the present investigation are studies on the role of the carboxyl terminus. Several early studies suggested that there is either no influence or a negative influence of the carboxyl terminus on receptor sequestration. For example, truncation of much of the distal carboxyl terminus of β_2ARs had no effect on sequestration (35). Furthermore, the avian β_2AR is not normally sequestered, but truncation of the carboxyl terminus unmasks the receptor's ability to be internalized (30). Also, truncation of the carboxyl terminus of the luteinizing hormone/chorionic gonadotropin receptor enhances internalization (36). However, several recent studies have shown that the carboxyl terminus plays an important permissive role in receptor internalization. In bombesin receptors, removal of large stretches of carboxyl-terminal residues blocks sequestration without interfering with receptor coupling (32). In the adrenergic system, it was found that mutation of a single tyrosine (Tyr^{326}) in the seventh transmembrane domain or proximal carboxyl terminus blocks β_2AR internalization (6). This residue was selected as potentially forming part of an endocytotic consensus sequence based on sequences found to be important in the cellular cycling of receptors for growth factors. It was suggested that this tyrosine may form part of an endocytotic domain with a consensus sequence of [NP $(X)_{2-3}$Y] in G protein-coupled receptors. However, more recent studies have found that this motif is present in the gastrin-releasing peptide receptor, where it is not involved in receptor sequestration and therefore is not a general "sequestration signal" (37). The present study indicates that mutation of three threonine residues within 6–10 residues of this putative consensus sequence completely blocks sequestration of the m3 AChR. Thus, the endocytotic site may extend beyond the originally proposed sequence. Alternatively, changes in the conformation of the proximal carboxyl terminus may indirectly influence this domain. However, mutation of two cysteine residues in the same vicinity had no effect on receptor sequestration. Further study will be necessary to firmly establish the existence of an endocytotic site. However, these observations suggest that the overall confirmation of the carboxyl terminus plays an important permissive role in receptor sequestration.

It has been proposed that receptor desensitization may include the following mechanisms: a rapid uncoupling of receptor from its effector, sequestration of receptor from the plasma membrane, and long term receptor degradation (2, 4). More recent studies suggested that receptor sequestration in βAR does not play a role in receptor desensitization but instead is required for receptor resensitization (5, 6). In the present study, the mutant m3 mAChR, which did not sequester, was less desensitized than the wild-type receptors. Also, treatment of the wild-type receptor-bearing cells with Con A prevented both sequestration and desensitization normally observed at early times. These observations argue against resensitization being the major role of sequestration because in that situation, greater desensitization would be expected under conditions of reduced sequestration.

Desensitization studies performed previously in mAChR system have shown that the m3 mAChR-stimulated PPI response occurs in two phases (38). The first phase of PPI hydrolysis is transient, reaching a peak after 10 sec, and is

followed by a secondary sustained phase. The decline of the initial rapid phase has been dissociated from the sequestration of the mAChR in two previous studies (38, 39). However, it may be causally related to a reduction in receptor calcium mobilization (38). The second phase of PPI hydrolysis is relatively resistant to desensitization as extended periods of agonist exposure are required for reducing receptor responsiveness (38–42). Previous evidence suggested that the later phase of desensitization is related to receptor sequestration and subsequent down-regulation (41, 42). Our present study showed that 1 hr of pretreatment with agonist decreased PPI hydrolysis induced by a second CCh challenge for wild-type receptors but had no effect on the response of mutant receptors. Because at 1 hr receptor sequestration was complete for wild-type receptors and totally abolished for the $Ala^{550,553,554}$ mutant, the PPI desensitization observed at this time in the wild-type receptor is likely due to sequestration. Furthermore, inhibition of sequestration of wild-type receptors with treatment with Con A also blocked desensitization. Thus, sequestration appears to play an important role in m3 mAChR desensitization at early times, at least in HEK 293 cells. This is consistent with other evidence suggesting that the internalized m3 mAChR is not coupled to G proteins (43).

Prolonged CCh treatment reduced the PPI hydrolysis response of mutant as well as wild-type receptors. This suggests that mechanisms other than sequestration of m3 receptor desensitization come into play at later times. The blockage of m3 mAChR sequestration or down-regulation in $Ala^{550,553,554}$ did not totally abolish receptor desensitization after prolonged agonist exposure in either HEK 293 cells or CHO-K1 cells (10). Because little down-regulation was observed in HEK 293 cells, this suggested that down-regulation of the receptor in the later stage of m3 mAChR desensitization was not very important in these cells. Although it was proposed that down-regulation contributes to βAR long term desensitization (2, 4), this mechanism may not be important for other receptors or may depend on the cell model. Studies showed that long term agonist exposure-induced desensitization of an α_2AR uncoupling-resistant mutant was not due to an overall decrease in receptor number but rather due to a reduction in G proteins (44). We investigated G protein levels in CHO-K1 cells expressing wild-type and $Ala^{550,553,554}$ mutant receptors, and our preliminary results showed that there was a similar agonist-induced decrease in G_q but not G_s α subunit protein levels for both receptors (45). Full clarification of the role of G protein down-regulation in the later stage of desensitization will require further study.

The absence of significant agonist-induced sequestration in CHO cells, which exhibited agonist-induced m3 mAChR down-regulation, and the absence of major down-regulation in HEK 293 cells, which exhibited agonist-induced m3 mAChR sequestration, suggests that these cell lines differ in the possession of important components of m3 mAChR regulatory mechanisms. Also, because β_2ARs are sequestered normally in CHO cells (6), these mechanisms appear to vary between receptors. The nature of the cellular mechanisms responsible for these differences between cell lines and receptors are currently unknown.

In summary, we have shown that threonine residues in the carboxyl terminus previously found to be required for down-regulation are also required for m3 mAChR sequestration. We also found that mutation of these residues reduced the extent of desensitization. This reduction in desensitization may be due in part to the lack of sequestration. However, that the mutant receptors were significantly desensitized over the long term indicated that regulation and desensitization of m3 mAChRs involve multiple components.

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