0026-895X/93/061158-07$03.00/0

MOLECULAR PHARMACOLOGY, 44:1158-1164

Human m3 Muscarinic Acetylcholine Receptor Carboxyl-Terminal Threonine Residues Are Required for Agonist-Induced Receptor Down-regulation

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Received July 6, 1993; Accepted September 11, 1993

SUMMARY

The mechanisms involved in agonist-induced down-regulation of the human m3 muscarinic acetylcholine receptor were investigated by site-directed mutagenesis of the receptor cytoplasmic carboxyl terminus. Threonine residues ($Thr^{550,553,554}$) were converted into alanines collectively and individually. The mutated and wild-type receptor cDNAs stably expressed in Chinese hamster ovary cells displayed similar antagonist- and agonist-binding properties. Furthermore, mutant receptors showed the same efficacy and potency for carbachol-induced activation of phosphoinositide hydrolysis as did the wild-type clone. In all cases the maximal increase in phosphoinositide hydrolysis was 8–9-fold. In contrast to normal intracellular signaling, however, the mutant receptor with all three threonines changed to alanines ($Ala^{550,553,554}$) failed to undergo normal down-regulation in response to carbachol. After a 24-hr incubation in the presence of 1 mM carbachol, subsequent *N*-[^{3}H]methylscopolamine binding was reduced by 66% for the wild-type clone but by only 12% for the mutant receptor. The $Ala^{553,554}$ mutant also showed a profound reduction in receptor down-regulation. Subsequent studies showed that a small but significant blockage of receptor down-regulation also could be produced by converting a single threonine residue (Thr^{553}) to alanine. The fact that these effects were not due to nonspecific conformational changes was suggested by the lack of effects on binding, signal transduction, and down-regulation of converting Thr^{550} to alanine or converting two cysteine residues ($Cys^{561,563}$) to glycines in an adjacent region. A similar reduction in receptor number also was observed in binding studies using the membrane-permeant ligand [^{3}H]scopolamine. These results show that threonine residues in the carboxyl-terminal domain of the human m3 muscarinic acetylcholine receptor are important in agonist-induced receptor down-regulation.

Molecular cloning studies have identified five subtypes of mammalian mAChRs, designated m1 through m5 (1–4). All mAChRs possess seven putative, hydrophobic, transmembrane domains and belong to the G protein-coupled receptor family. m1, m3, and m5 mAChRs signal primarily by activating PPI hydrolysis, whereas m2 and m4 mAChRs act mainly by inhibiting adenylate cyclase (for review, see Ref. 5). The m3 mAChR is a predominant form of the receptor expressed in glandular tissues, and it mediates the secretion of pancreatic enzymes, saliva, and gastric acid (4, 6). In addition to the stimulatory function, ACh and its analogues also can affect the numbers and responsiveness of mAChRs. In pancreatic acini, cholinergic agonists have been shown to both desensitize and down-regulate the mAChR (7–9). However, the molecular mechanism of m3 mAChR down-regulation is not clear.

The involvement of various domains and specific amino acids in receptor down-regulation has been previously studied in several members of the G protein-coupled receptor family (10–15). The results suggest that domains and individual amino acids located in the third intracellular loop and carboxyl terminus are important in receptor down-regulation. We used site-directed mutagenesis techniques to examine the role of carboxyl-terminal threonine residues in m3 mAChR regulation. Four mutant receptors were constructed in which the carboxyl-terminal threonine residues at positions 550, 553, and 554 were changed to alanines collectively and individually. Cysteine residues at positions 561 and 563 in the carboxyl-terminal domain were converted to glycines to eliminate the possibility that the receptor functional change could be due to a nonspecific effect of amino acid changes in the carboxyl-terminal region. Our data suggest that threonine residues in the m3 mAChR car-

This work was supported by National Institutes of Health Grants DK41225 and DK41350 and by the Michigan Gastrointestinal Peptide Digestive Disease Center.

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; PKC, protein kinase C; βAR, β-adrenergic receptor; CHO, Chinese hamster ovary; CCh, carbachol; PPI, phosphoinositide; NMS, *N*-methylscopolamine; PBS, phosphate-buffered saline; WT, wild-type; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACh, acetylcholine.

boxyl-terminal region are required for agonist-induced receptor down-regulation but are not necessary for receptor binding and signal transduction.

Experimental Procedures

Materials

Radiochemicals. [^{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 was 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.

Biochemicals. Atropine and CCh were obtained from Sigma Chemical Co. (St. Louis, MO). Analytical grade Dowex 1-X8 (AG1-X8, 100–200 mesh) was obtained from Bio-Rad (Rockville Center, NY). Restriction endonucleases were purchased from GIBCO (Grand Island, NY). Other enzymes were from Boehringer Mannheim (Indianapolis, IN).

Tissue culture supplies. Dulbecco's modified Eagle's medium, Dulbecco's PBS, trypsin-EDTA, and fetal bovine serum were purchased from GIBCO. Tissue culture plastic-ware (24- and six-well plates and 10- and 15-cm Petri dishes) were obtained from Costar (Cambridge, MA). CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD).

Methods

Construction of vectors expressing human m3 mAChR and mutant receptors. The cDNA encoding the human m3 mAChR (4) was obtained from Dr. E. G. Peralta (Genentech Inc., South San Francisco, CA). The 2.0-kilobase cDNA was subcloned into *Eco*RI and *Bam*HI sites of pBluescript SK(−) vector. Site-directed mutagenesis was performed according to the method of Kunkel *et al.* (16). Uracil-containing single-strand DNA was isolated from pBluescript SK(−)-transformed *Escherichia coli* strain CJ236 (*ung*$^-$, *dut*$^-$) in the presence of helper phage R408.

Oligonucleotides were synthesized by an Applied Biosystems 380B DNA synthesizer. The 22–39-mers were designed to change Thr^{550,553,554} residues (ACA, ACC, and ACT) in the carboxyl terminus to alanines (GCA, GCC, and GCT) and Cys^{561,563} residues (TGC and TGT) to glycines (GGC and GGT). The single-strand uridine-containing DNA was annealed to synthetic oligonucleotides, and *in vitro* synthesis of the DNA second strand was carried out using T_4 DNA polymerase and deoxynucleoside triphosphates. After ligation, the newly synthesized double-strand DNA was transformed into *E. coli* strain XLI-blue (*ung*$^+$, *dut*$^+$), which selectively replicates the synthetic oligonucleotide-primered DNA strand. All mutations were confirmed by dideoxy sequencing with Sequenase version 2 (United States Biochemicals, Cleveland, OH). The entire coding region of the mutated receptor gene was removed from pBluescript SK(−), and both mutant and WT receptor cDNAs were inserted into *Eco*RI and *Bam*HI sites of the mammalian expression vector pTEJ-8 (17). The vector utilizes a ubiquitin promoter to drive the inserted gene and also possesses a neomycin resistance gene for use as a selectable marker.

Cell culture and transfections. CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, in an environment of 5% CO_2. Transfections of CHO cells were carried out by the calcium phosphate precipitation method (18). Stable transfectants were selected in the presence of G418 at a concentration of 1 mg/ml and were maintained with a concentration of 0.25 mg/ml. The G418-resistant CHO cell colonies were screened for receptor binding using [^{3}H]NMS.

Radioligand binding and down-regulation assays. Receptor binding assays were carried out on cells plated at a density of 1×10^5/well in 24-well cell culture dishes. After overnight attachment, the radioactive antagonist [^{3}H]NMS or [^{3}H]scopolamine was added at 4° for 24 hr. The concentration of radioligands used for most binding studies was 2 nM. The cells were incubated in 1 ml of 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, 30 mM Na-HEPES buffer, pH 7.4). Nonspecific binding determined in the presence of 10 μM atropine was <10% of total binding. For saturation binding studies, 0.01–2 nM concentrations of radioligand were used. At 2 nM concentration, 95% of the receptor binding sites were occupied by radioligands. For agonist competition studies, 10^{-3} to 10^{-6} M concentrations of CCh were added in the presence of 2 nM [^{3}H]NMS. For the study of receptor down-regulation, cells were preincubated at 37° with the indicated concentrations of CCh for different time periods and were then washed with PBS, after which binding assays using either [^{3}H]NMS or [^{3}H]scopolamine were performed at 4°. The binding reactions were terminated by aspiration of the binding buffer, and the cells were then washed four times with 2 ml of ice-cold 0.9% NaCl solution. After removal of the cells with 0.5 ml of 0.1 M NaOH, radioactivity was determined in 5 ml of Bio-Safe II scintillation fluid (Research Products international Corp., Mount Prospect, IL). Protein concentrations were determined according to the method of Bradford (19), using a Bio-Rad protein assay kit. DNA was assayed according to the method of Cesarone *et al.* (20).

PPI hydrolysis assays. CHO cells expressing WT and mutant receptor clones were plated at a density of 1×10^5/well in 35-mm six-well culture dishes and were labeled with *myo*-[^{3}H]inositol (3 μCi/ml) in culture medium for 24 hr at 37°. For studies involving agonist pretreatment, cells were incubated with *myo*-[^{3}H]inositol with or without 1 mM CCh. Cells were then washed three times with buffer A containing 20 mM LiCl and were incubated in 1 ml of this buffer, with or without CCh, for another 1 hr. After stimulation cells were extracted with 10% trichloroacetic acid, and total inositol phosphates were isolated according to the method of Berridge *et al.* (21).

Data analysis. Specific receptor binding was calculated by subtracting nonspecific binding obtained in the presence of atropine from the amount bound in the absence of atropine. Saturation binding and agonist competition data were analyzed by a radioligand binding analysis program adapted for the IBM PC (Elsevier-BIOSOFT, Cambridge, UK). Data were fitted with one- or two-site models and the best fit was selected. All values are represented as the mean ± standard error. Student's *t* test was used for statistical analysis of the data.

Results

Ligand-binding properties and agonist-stimulated PPI hydrolysis of WT and mutant receptors. WT and mutant human m3 mAChR cDNAs were transfected into CHO-K1 cells, which lack endogenous mAChRs. G418-resistant cell colonies were screened for the expression of mAChR by using the radiolabeled antagonist [^{3}H]NMS and by Northern blot analysis. Binding studies showed that the level of receptor expression varied. To account for possible differences in receptor function caused by differences in the level of receptor expression, three WT cell clones (clones 8, 14, and 17) with different receptor numbers were studied in detail. The three WT clones showed similar mAChR antagonist binding affinities (Table 1), with WT clone 17 possessing the highest binding capacity (401,000 receptor sites/cell) and WT clone 8 having the lowest (80,500 sites/cell). WT clone 14 expressed an intermediate number of receptor binding sites (152,000 sites/cell) and was used as a standard with which the cell clones containing mutant genes were compared.

The WT clones showed a K_d of about 100 pM for antagonist NMS binding, and all of the mutant receptor clones studied had binding affinities similar to those of the WT receptor clones (Table 1). In addition, agonist competition studies indicated that CCh could distinguish high and low affinity states similarly in both WT clones and the Ala^{550,553,554} mutant (Table 1). These studies suggested that threonine and cysteine residues

TABLE 1

Antagonist and agonist binding parameters for WT and mutant human m3 mAChRs

The equilibrium dissociation constant (K_d) for the radioligand [^{3}H]NMS and the maximum receptor binding capacity (B_{max}) were determined in saturation binding experiments with intact cells, using radioligand concentrations of 0.1–2 nM. Agonist competitive displacement data were obtained using CCh concentrations from 10^{-8} to 10^{-3} M, in the presence of 2 nM [^{3}H]NMS. Incubation with [^{3}H]NMS was for 24 hr at 4°. The equilibrium dissociation constants for the high affinity binding state (K_{dH}) and the low affinity binding state (K_{dL}) and the percentage of high affinity state receptor amount, relative to the total receptor amount (R_H/R_T), were determined by the radioligand drug displacement program. Data are mean ± standard error of three to eight experiments.

Clone	[^{3}H]NMS binding		CCh competition		
	K_d	B_{max}	K_{dH}	K_{dL}	R_H/R_T
	pM	fmol/mg	μM	μM	%
WT 8	110 ± 21	447 ± 78	3.4 ± 0.8	198 ± 31	33 ± 5
WT 14	104 ± 14	846 ± 37	2.9 ± 0.6	135 ± 34	19 ± 2
WT 17	139 ± 20	2230 ± 186	2.9 ± 0.5	149 ± 33	15 ± 4
Ala^{550,553,554}	118 ± 23	759 ± 59	1.2 ± 0.2	168 ± 7	16 ± 3
Ala^{553,554}	90 ± 10	1011 ± 142	–[a]	–	–
Ala^{550}	100 ± 22	787 ± 5	–	–	–
Ala^{553}	124 ± 23	882 ± 101	–	–	–
Gly^{561,563}	107 ± 13	1047 ± 155	–	–	–

[a] –, Not determined.

in the receptor carboxyl-terminal region are not important in the regulation of antagonist and agonist binding. This is consistent with previous conclusions that the carboxyl-terminal region is not a part of the ligand-binding domain of seven-transmembrane region G protein-coupled receptors (22).

The m3 mAChR is coupled to a pertussis toxin-insensitive G protein and mediates stimulation of PPI hydrolysis upon incubation with the agonist CCh (6). To examine the physiological response to agonist stimulation of the WT and mutant m3 mAChRs in CHO cells, PPI hydrolysis assays were performed. Untransfected CHO cells displayed no increase in PPI hydrolysis upon CCh addition. WT receptor cell clones showed a concentration-dependent increase in total PPI hydrolysis, with a maximum increase of 8.7 ± 0.5-fold for WT clone 17, 8.1 ± 0.4-fold for WT clone 14, and 5.5 ± 0.5-fold for WT clone 8 (Fig. 1). These results suggest that the efficacy of activation of the signal transduction pathway is affected by the receptor density, as has been reported previously (23–25). Moreover, WT clone 17 was about 2-fold more sensitive to CCh than was WT clone 14.

The Ala^{550,553,554} clone showed a similar level of agonist-induced PPI hydrolysis as did the WT clones, with a maximum increase of 8.3 ± 0.4-fold (Fig. 2). All of the other threonine mutant clones exhibited the same level of PPI hydrolysis response to CCh as did WT clone 14 (data not shown). These studies show that alteration of threonine residues in the carboxyl terminus does not affect m3 mAChR coupling to this signal transduction pathway. The Gly^{561,563} clone in which cysteine residues were replaced also displayed a CCh concentration-dependent response similar to that of WT clone 14 (Fig. 2). This suggests that these cysteine residues are not involved in receptor binding and activation.

Agonist-induced receptor down-regulation studies in WT and mutated receptor clones. Upon pretreatment of the WT clones with CCh, time-dependent receptor down-regulation was observed, as measured by a loss of binding of the hydrophilic ligand [^{3}H]NMS (Fig. 3). Initial studies with WT clone 17 showed that this time-dependent decrease in receptor number was significant after 6 hr and complete after a 24-hr incubation with CCh (data not shown). Down-regulation of m3 mAChRs was also found to depend on the concentration of

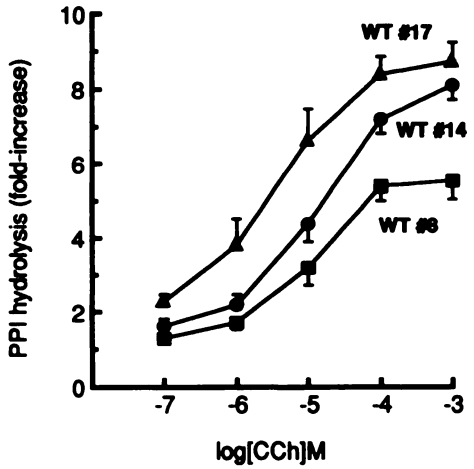

Fig. 1. CCh-induced PPI hydrolysis in clones expressing different numbers of m3 mAChRs. Cell clones (clones 8, 14, and 17) (Table 1) expressing WT human m3 mAChRs were labeled with [^{3}H]inositol for 24 hr and then treated with increasing concentrations of CCh, as indicated, for 1 hr at 37°. Inositol phosphates were extracted and compared with the amount in cells not stimulated with CCh. Data are mean ± standard error of three or four experiments.

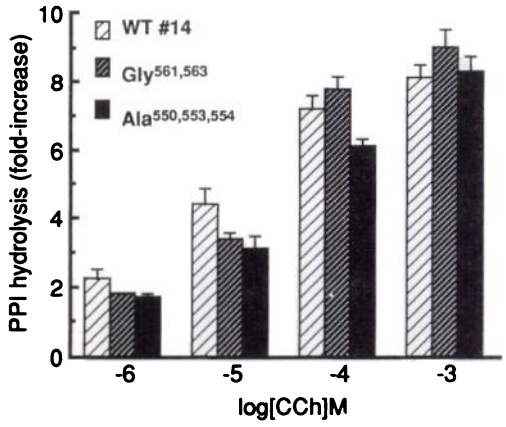

Fig. 2. CCh-induced PPI hydrolysis in CHO cells expressing WT clone 14, Gly^{561,563}, and Ala^{550,553,554} human m3 mAChRs. Cells labeled with [^{3}H]inositol were incubated with or without the indicated concentration of CCh at 37° for 1 hr, after which total inositol phosphates were extracted. Data are mean ± standard error of three experiments.

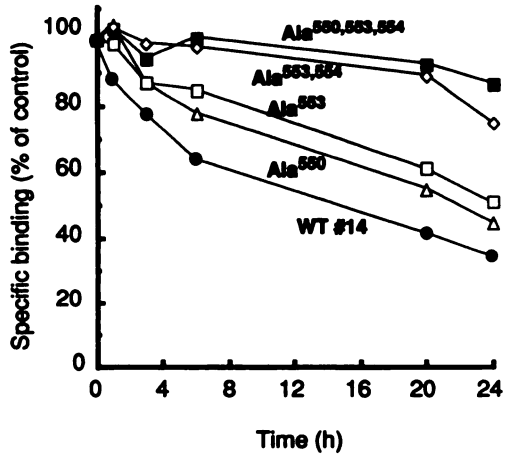

Fig. 3. Time course of CCh-induced receptor down-regulation in CHO cells expressing WT and mutated human m3 mAChRs. Cells were treated at 37° with 1 mM CCh as indicated and were washed three times with PBS, and radioligand binding assays were then performed at 4° using 2 nM [^{3}H]NMS. Atropine (10 μM) was added to determine nonspecific binding, which was <10% of total binding at each time point. Data are means of three or four experiments.

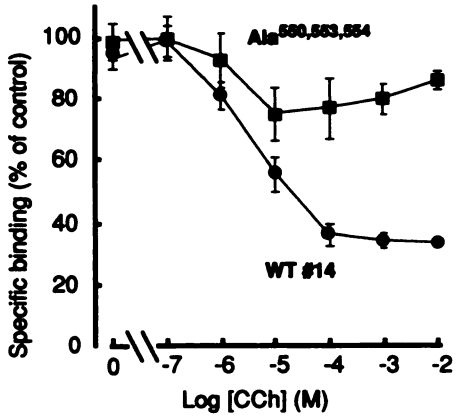

Fig. 4. Concentration dependence of CCh-induced receptor down-regulation in CHO cells expressing WT or Ala^{550,553,554} human m3 mAChRs. Cells were treated for 24 hr at 37° with the indicated concentration of CCh, and radioligand binding assays were then performed at 4° using 2 nM [^{3}H]NMS, with or without 10 μM atropine. To eliminate any effect of CCh-stimulated cell growth on receptor down-regulation, a DNA assay was performed. No influence of changes in DNA content on receptor down-regulation was observed. Data are mean ± standard error of three experiments.

CCh (Fig. 4). Measurable down-regulation was observed at 10^{-6} M, with a maximum effect at 10^{-4} M CCh. The EC_{50} for receptor down-regulation for clone 14 was 4.6 ± 1.1 μM, which was similar to the EC_{50} for PPI hydrolysis for clone 14 (8.4 ± 3.1 μM). Cells transfected with mutant receptor genes, however, did not show normal CCh-induced receptor down-regulation (Figs. 3 and 4). The blockage of receptor down-regulation was most prominently exhibited with the Ala^{550,553,554} clone. Ala^{553,554} also showed a profound reduction in down-regulation. A statistically significant effect in reducing receptor down-regulation was also observed with the Ala^{553} clone (Table 2). In contrast, the mutant Ala^{550} clone showed no difference in receptor down-regulation. Substitution of two conserved cysteine residues by glycine (Gly^{561,563}) in an adjacent region of the carboxyl terminus also did not alter the WT pattern of down-regulation (Table 2). The results described above indicate that threonine residues

TABLE 2

CCH-induced down-regulation of WT and mutant human m3 mAChRs

Receptor down-regulation was determined in cells incubated in the presence of 1 mM CCh at 37° for 24 hr. Cells were washed and incubated with 2 nM [^{3}H]NMS at 4° to measure binding. Data are mean ± standard error of four to 10 experiments (*n*).

Clone	NMS binding	*n*
	% of control	
WT 8	41 ± 2	6
WT 14	34 ± 1	6
WT 17	34 ± 2	8
Ala^{550}	41 ± 3	7
Ala^{553}	49 ± 3[a]	7
Ala^{553,554}	79 ± 3[b]	6
Ala^{550,553,554}	88 ± 4[b]	10
Gly^{561,563}	39 ± 4	4

[a] $p < 0.05$ compared with WT clone 14.
[b] $p < 0.01$.

in the carboxyl-terminal region of the m3 mAChR are involved in receptor down-regulation.

Receptor down-regulation versus redistribution. To determine whether the loss of observed [^{3}H]NMS binding, which indicates numbers of cell surface receptors, represented true receptor down-regulation, as opposed to receptor redistribution or sequestration, the binding of a lipophilic radioactive ligand, [^{3}H]scopolamine, was studied and compared with that of the hydrophilic ligand [^{3}H]NMS. Scopolamine penetrates intact cells and detects intracellular receptors as well as receptors located on the cell surface (26). Saturation binding studies performed at 4° showed that WT clone 14 had a binding affinity for [^{3}H]scopolamine of 85 ± 8 pM and a maximum binding capacity of 1027 ± 83 fmol/mg of protein (three experiments). Compared with the data from saturation studies using [^{3}H]NMS, the results indicated that 18% of total m3 receptors were present intracellularly and were inaccessible to the hydrophilic ligand.

A receptor down-regulation study was then performed with WT clone 14 and the Ala^{550,553,554} clone, using 2 nM [^{3}H]NMS or [^{3}H]scopolamine. The results showed that total receptor down-regulation, as measured by a loss of scopolamine binding, occurred in a pattern parallel to that of the decrease in cell surface receptor number for WT clone 14 (Fig. 5, *left*). Careful observation at short time intervals revealed the same results (Fig. 5, *inset*). When the mutant receptors were studied, both ligands failed to detect a significant loss of receptor sites (Fig. 5, *right*). The loss of m3 mAChRs, as assessed with NMS and scopolamine, was 472 ± 25 and 452 ± 69 fmol/mg of protein, respectively, for WT clone 14 and 33 ± 50 and 16 ± 71 fmol/mg of protein, respectively, for Ala^{550,553,554} (four experiments). The similarity of results obtained with the WT and mutant receptors for both [^{3}H]NMS and [^{3}H]scopolamine suggests that the data generated with the hydrophilic ligand represent changes in receptor number, rather than a redistribution of cellular receptors.

Receptor functioning after prolonged exposure to agonist. To study the functional response of cells after incubation with CCh for 24 hr, PPI hydrolysis assays were performed. Basal PPI hydrolysis was not affected by CCh pretreatment. However, both WT clone 14 and Ala^{550,553,554} displayed a reduction in stimulated PPI hydrolysis after exposure to CCh (Table 3). CCh decreased subsequent PPI hydrolysis for WT clone 14 to 24%, compared with the control group; CCh decreased the

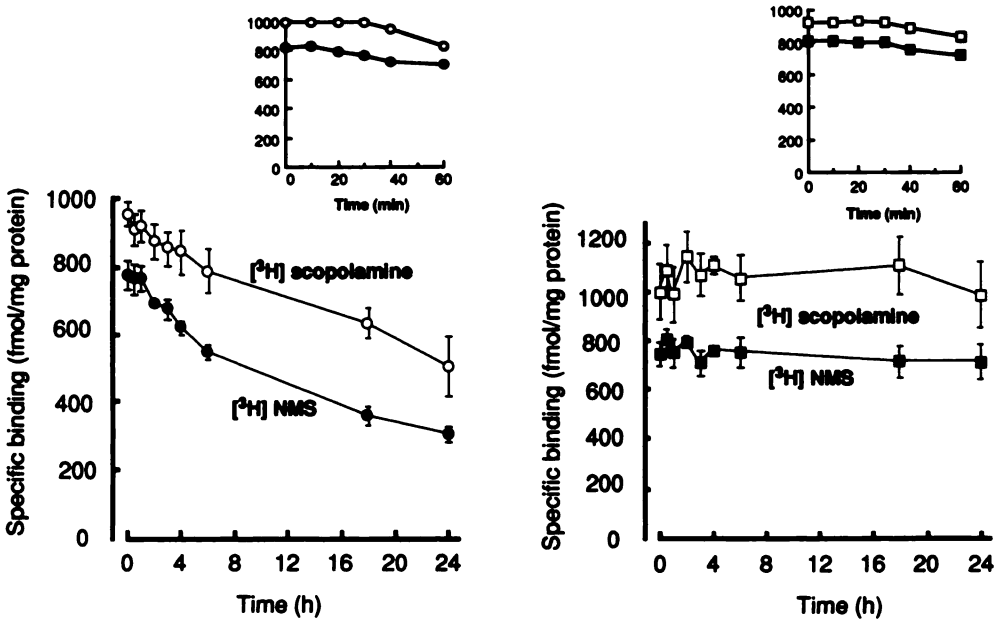

Fig. 5. Receptor down-regulation measured with [^{3}H]NMS and [^{3}H]scopolamine in WT clone 14 (*left*) and Ala^{550,553,554} (*right*). Cells were incubated with 1 mM CCh at 37° for different time periods. After the treatment the cells were washed three times with PBS and incubated for 24 hr at 4° with 2 nM radioactive antagonists, with or without 10 μM atropine. *Insets*, separate study to evaluate the early time periods. Data are mean ± standard error of four experiments. Error bars for insets are not shown, for clarity.

TABLE 3

PPI hydrolysis after 24-hr exposure to CCh

Cells expressing m3 mAChRs were labeled with [^{3}H]inositol at 37°, with or without 1 mM CCh, for 24 hr. Cells were then washed with buffer A plus 20 mM LiCl and were stimulated with 1 mM CCh in the same buffer for 1 hr at 37°, after which total inositol phosphates were extracted. Data are mean ± standard error of three experiments.

Clone	Control	24-hr exposure	
	fold increase	*fold increase*	*% of control*
WT 14	9.9 ± 1.0	2.4 ± 0.2	24 ± 1
Ala^{550,553,554}	10.3 ± 1.9	5.3 ± 1.3	51 ± 6[a]

[a] $p < 0.01$, compared with WT clone.

response for the Ala^{550,553,554} mutant to 51% of control. The difference in PPI hydrolysis reduction between cells bearing WT and mutant receptors was significant.

Discussion

We constructed a series of carboxyl-terminal threonine residue mutations in the human m3 mAChR to study the effect of these residues on m3 mAChR function. Our data showed that changing selected amino acid residues in the carboxyl-terminal region did not affect antagonist- and agonist-binding properties of the receptors, nor was receptor coupling to PPI hydrolysis affected. These data are consistent with previous findings for adrenergic receptors and mAChRs (for review, see Ref. 27). However, mutation of threonine residues in the carboxyl-terminal domain blocked the m3 mAChR down-regulation induced by CCh. The m3 mAChR is the predominant form in most glandular tissues, including pancreas (4). Studies of agonist-induced mAChR down-regulation have previously been carried out in pancreas and neuroblastoma cell lines (8, 9, 28, 29). The mechanism of this down-regulation, however, is still not known. With the development of gene cloning and sequence analysis, we are now able to study the mechanism at the gene structural level and to locate the amino acid residues involved in this function.

The results of this mutagenesis study suggest that receptor phosphorylation may be involved in CCh-induced down-regulation. The threonine residues we mutated include two possible PKC phosphorylation sites (Thr^{550,554}) and an additional site at Thr^{553}. Mutation to alanines of all three, only two, or even one (Thr^{553}) of these sites leads to a significant decrease in agonist-induced down-regulation. This suggests that PKC may be involved in this process. However, we found that the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate could not induce receptor down-regulation in CHO cells transfected with the WT m3 mAChR and staurosporine, a PKC inhibitor, could not block the CCh-induced receptor down-regulation.[2] Furthermore, a recent study showed that CCh-induced m3 mAChR phosphorylation was not related to PKC activation (30). Therefore, protein kinases other than PKC may be acting on the carboxyl-terminal threonine sites to induce receptor down-regulation. Kwatra *et al.* (31) showed that desensitization of m2 mAChRs could be induced by receptor phosphorylation via a βAR-specific kinase. Many down-regulation studies have been performed in the βAR system. Early observations obtained with a mutated β_2AR in which protein kinase A phosphorylation sites had been eliminated suggested that the rate of receptor internalization and down-regulation had been affected (32). However, other recent reports have not confirmed this relationship between down-regulation and phosphorylation (33–35).

We cannot exclude, however, the possibility that the receptor carboxyl-terminal domain identified in this study is specifically recognized by some cellular mechanisms involved in the process of receptor down-regulation. Recently, regional involvement in receptor function has been reported to be important in βAR regulation. Hausdorff *et al.* (34, 36) showed that mutations of threonine and serine residues in the distal region of the β_2AR

[2] J. Yang, C. D. Logsdon, and J. A. Williams, unpublished observations.

carboxyl terminus could overcome the effects induced by mutations in the proximal region of the tail. Furthermore, studies showed that the lack of receptor internalization and down-regulation of the avian βAR could be restored by carboxyl-terminal truncation and that addition of some carboxyl-terminal amino acids removed from the avian βAR to the carboxyl terminus of the mammalian βAR made the latter lose its ability to internalize (11, 13). Furthermore, the replacement with alanines of two tyrosine residues in the carboxyl-terminal region of the β_2AR dramatically decreased the ability of the receptor to undergo down-regulation (12). These findings suggest that certain carboxyl-terminal domains may be structurally important in interacting with cellular elements mediating receptor down-regulation.

Studies on the role of intracellular domains in m1 AChR internalization and down-regulation have suggested the involvement of specific regions enriched in serine and threonine residues in the third intracellular loop (13–15), rather than the carboxyl terminus (15). This may reflect a subtype specificity between the m1 and m3 mAChRs. Alternately, results obtained from deletion analysis studies could be different from those reported using single amino acid substitutions due to the possibility of receptor structural changes. The identification of important sites in both the third intracellular loop and the carboxyl tail may also suggest involvement of multiple regions in mAChR down-regulation.

In the present study we have also shown that the cysteine residues in the carboxyl-terminal region of the m3 mAChR are not necessary for receptor activation. This finding is in agreement with previous studies of the m1 and m2 mAChRs (24, 37). In contrast, the importance of the analogous cysteine residues in receptor activation was observed with rhodopsin (38), β_2ARs (39), and serotonin 5-hydroxytryptamine type 2 receptors (40). Thus, the function of the conserved cysteine residues in the carboxyl-terminal region of the G protein-coupled receptor family is dependent on the individual receptor.

The possibility exists that the results of this mutation study are due to nonspecific conformational changes in the carboxyl terminus of the m3 mAChR. However, this is unlikely for the following reasons. First, all of the threonine mutants showed normal ligand binding and induction of PPI hydrolysis, which excludes the possibility of gross conformational disruption. Second, replacing with glycines the cysteine residues adjacent to the threonines did not alter CCh-induced receptor down-regulation. Finally, the existence of nonspecific conformational effects is made less likely by the observation that changing one single threonine residue (Thr^{550}) had no effect on receptor down-regulation, whereas changing another (Thr^{553}) did.

After agonist binding, receptors typically undergo sequestration and down-regulation. We show that unoccupied m3 mAChRs expressed in CHO cells are present both inside the cell and on the cell surface. In the resting state about 18% of the receptors are located intracellularly. This is similar to the results obtained in SK-N-SH cells (26). However, whether sequestration occurs as a prerequisite for down-regulation is controversial. In the case of β_2ARs, sequestration was not required for receptor down-regulation (36). In our study, the losses of receptor binding sites detected with [^{3}H]NMS and [^{3}H]scopolamine were parallel in the WT receptor clones, which suggested an absence of changes in receptor sequestration in the CHO cell lines. The threonine mutations profoundly affected the receptor down-regulation process, with no effect on sequestration. However, the CHO cells used showed little sequstration, suggesting that this process may be better studied in other cell lines. Additional studies will be required to elucidate the relationship between receptor sequestration and down-regulation.

The cellular response after prolonged exposure to CCh was also examined in our study. Cells bearing WT m3 mAChRs showed a profound decrease in inositol phosphate production after a 24-hr exposure to CCh. Similar results have been found with m1 receptors (14). Interestingly, the mutant receptor $Ala^{550,553,554}$, whose down-regulation was almost totally blocked, showed a smaller but significant reduction in PPI hydrolysis. These results suggest that prolonged agonist exposure induced receptor desensitization as well as down-regulation. Furthermore, they also indicate that additional regions of the m3 mAChR, distinct from those required for down-regulation, are involved in desensitization. More detailed studies will be required, however, to fully evaluate the role of receptor down-regulation in desensitization.

In summary, our study has shown that certain threonine residues in the m3 mAChR carboxyl-terminal domain are required for receptor down-regulation. These results suggest that protein phosphorylation may play an important role in this receptor function.

Acknowledgments

We thank Dr. S. K. Fisher for helpful discussion on mAChRs throughout this work.

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