Cysteine Pairs in the Third Intracellular Loop of the Muscarinic M₁ Acetylcholine Receptor Play a Role in Agonist-Induced Internalization

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ABSTRACT

We determined the functional role of a small domain in the third intracellular loop of the human muscarinic M₁ (hM₁) receptor. Using site-directed mutagenesis, several mutant hM₁ receptors were made possessing either a deletion or point mutations within the third intracellular loop domain252PETPPGRCCRC263. Wild-type and mutant hM₁ receptors were transiently expressed in Chinese hamster ovary cells, and the effects of each mutation on radioligand binding, agonist-mediated phosphoinositide hydrolysis, and agonist-induced internalization were determined. The mutant receptors exhibited a modest reduction in affinity for [³H]N-methylscopolamine (pKᵦ = −9.0) and a moderately increased binding capacity relative to the wild-type receptor. This moderate increase in binding capacity was associated with small increases in the maximal response and potency of carbachol for eliciting phosphoinositide hydrolysis through the mutant receptors (pEC₅₀ = −5.5) relative to wild-type (pEC₅₀ = 5.35 ± 0.05). In contrast, carbachol-induced internalization of mutant hM₁ receptors possessing either C259A/C260A or C262A/C263A or both double point mutations was significantly reduced compared to the wild-type hM₁ receptor. Of the hM₁ receptor mutants tested, those possessing a C262D/C263D double point mutation had the least carbachol-induced internalization. The desensitization and down-regulation of receptors possessing either Cys/Ala or Cys/Asp double point mutations were similar to those observed for the wild-type hM₁ receptor. Collectively, these observations suggest that Cys pairs Cys259/Cys260 and Cys262/Cys263 play an important role in the agonist-induced internalization of hM₁ receptors.

Muscarinic acetylcholine receptors are members of the GPCR superfamily, and five subtypes (M₁–M₅) have been cloned thus far. Muscarinic M₁ receptors couple with Gₛ to mediate phosphoinositide hydrolysis, and like other GPCRs, M₁ receptors begin to desensitize and internalize within minutes of agonist exposure (Maeda et al., 1990; Berstein et al., 1992; Lameh et al., 1992; Moro et al., 1994; Waugh et al., 1999; Willets et al., 2005). To date, several M₁ receptor domains have been determined to play a role in the agonist-dependent regulation of M₁ receptor activity, and a majority of these are putative phosphorylation sites (see review, van Koppen and Kaiser, 2003). Of the sites identified, the most critical for regulating agonist-dependent M₁ receptor internalization appears to be a Ser- and Thr-rich (S/T-rich) domain in the third intracellular (i₃) loop (284SMESLTSE292) of the receptor. Deletion of and mutagenesis within this S/T-rich domain significantly reduced agonist-induced internalization of human M₁ (hM₁) receptors in U293 cells (Lameh et al., 1992; Moro et al., 1993).

The S/T-rich domain present in the i₃ loop of hM₁ receptors is probably phosphorylated by G protein-coupled receptor kinase 2 (GRK2) in an agonist- and Gβγ-dependent manner (Haga et al., 1996; Willets et al., 2005). Based on previous observations, this activity of GRK2 may lead to desensitization and arrestin-dependent internalization of the hM₁ receptor (see review, Pierce et al., 2002). Consistent with this expectation, the suppression of GRK2 expression in rat hippocampal neurons using an antisense strategy significantly reduced M₁ receptor phosphorylation and desensitization (Willets et al., 2005). However, the effect of depleting GRK2 on agonist-induced internalization of the M₁ receptor was not investigated.

ABBREVIATIONS: GPCR, G protein-coupled receptor; S/T, Ser- and Thr-rich; CHO, Chinese hamster ovary; GRK, G protein-coupled receptor kinase; hM₁, human muscarinic M₁; i₃, third intracellular; [³H]NMS, [³H]N-methylscopolamine; [³H]QNB, [³H]quinuclidinyl benzilate; ANOVA, analysis of variance; PBS, phosphate-buffered saline; SH3, Src homology 3.
Considering the numbers of sequence and structural motifs identified in GPCRs thus far, it is expected that other yet to be described receptor domains contribute to the agonist-dependent regulation of M₁ receptor signaling and plasma membrane expression (for review, see Bockaert et al., 2004). For example, binding sites for Gβγ and β-arrestin probably exist on the M₁ receptor, because the presence of Gβγ increases GRK2-mediated phosphorylation of affinity-purified M₁ receptors and M₁ receptors colocalize with β-arrestin (Haga et al., 1996; Tolbert and Lameh, 1996; Santini et al., 2000). To date, the binding sites for Gβγ and β-arrestin have not been identified for the M₁ receptor, unlike the binding sites for both Gβγ and β-arrestin on the M₄ receptor (Wu et al., 1997, 2000). It should also be noted that the functional role of the S/T-rich domain present in the i3 loop of the M₁ receptor may be to mediate down-regulation instead of internalization. Shockley et al. (1999) demonstrated that mutations within the S/T-rich domain decreased agonist-induced hM₁ receptor down-regulation and did not affect internalization in CHO cells. Although this finding could be a consequence of the cell type used (i.e., CHO cells versus HEK293 cells), it underscores the fact that our knowledge of the receptor domains that contribute to the agonist-dependent regulation of the hM₁ receptor is incomplete.

In this investigation, we determined the functional role of a novel domain in the i3 loop of the M₁ receptor with a SH-3 binding motif, a putative proline-dependent Ser/Thr kinase site, and two pairs of Cys residues with basic Arg residues on either side (i.e., putative palmitoylation sites). A part of this domain (254TPPRCCCR261) was determined to mediate an interaction between “bait” and “prey” constructs made with the i3 loop sequence of the hM₁ receptor in yeast two-hybrid assays (G. W. Sawyer, unpublished observations). Deletion of 254-261TPPPRCR261 from the i3 loop of hM₁ receptors caused a significant decrease in the carbachol-induced internalization of the receptor. This decrease in internalization could not be attributed to the loss of either the putative SH3 binding motif or the Thr phosphorylation site but was rather caused by the deletion of Cys pair Cys259/Cys260. We also found that mutating Cys pair Cys262/Cys263 to an Asp pair almost completely inhibited carbachol-induced hM₁ receptor internalization. Thus, Cys pairs Cys259/Cys260 and Cys262/Cys263 appear to play an important role(s) in the agonist-dependent regulation of the hM₁ receptor activity.

**Materials and Methods**

**Site-Directed Mutagenesis.** The hM₁ receptor cDNA, cloned into a modified Okayma-Berg expression vector (pCD), was a generous gift from Dr. Tom I. Bonner at the National Institute of Mental Health (Bethesda, MD). Mutant hM₁ receptors were made by either deleting sequences or introducing point mutations into the hM₁ receptor cDNA of pCD-hM₁ using the QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and various mutagenesis primers. A T254A point mutation was made in the hM₁ receptor sequence (pCD-hM₁/T/A) using primers 5'-GAGGGCTCACAGAG-GCTCTCCAGGCGCCTGC-3' (forward) and 5'-GGAGGCGGTCG-AGGAGCTCCTCAGGCGCCTGC-3' (reverse), pCD-hM₁/P/A, a hM₁ receptor sequence with a P252A point mutation, was made using 5'-GCTCGAGGCTCACAGAG-GCTCTCCAGGCGCCTGC-3' (forward) and 5'-GAGAGCTCCTGCTCAGGCGCCTGC-3' (reverse), 5'-GACT-CCTCCAGGCGCCTGC-3' (forward) and 5'-GGCCGCCGACAGGCGACGCGGCGCTGGAGATT-3' (reverse) mutagenesis primers were used to make the C259A/C260A double point mutant (pCD-hM₁/C259A/C260A), a double point mutant (pCD-hM₁/C252A) was made using 5'-GGCCGCCGACAGGCGACGCGGCGCTGGAGATT-3' (reverse), 5'-GGCCGCCGACAGGCGACGCGGCGCTGGAGATT-3' (reverse), and 5'-GGCCGCCGACAGGCGACGCGGCGCTGGAGATT-3' (reverse). The C262D/C263D double point mutant (pCD-hM₁/C262D/C263D) was made using 5'-GGCCGCCGACAGGCGACGCGGCGCTGGAGATT-3' (reverse), 5'-GGCCGCCGACAGGCGACGCGGCGCTGGAGATT-3' (reverse), and 5'-GGCCGCCGACAGGCGACGCGGCGCTGGAGATT-3' (reverse). Mutant hM₁ receptors were sequenced on the Oklahoma State University core DNA sequencing facility to verify the presence of the planned mutation and to ensure that no other mutations were acquired during PCR.

**Cell Culture and Transient Transfections.** CHO cells were maintained in growth medium (F-12K supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin) in a humidified incubator at 37°C in an atmosphere of 5% CO₂-95% air and were subcultured every 2 to 3 days. For transient transfections, cells were trypsinized and plated in a 24-well plate format at 1.65 × 10⁵ cells/well in 500 μl of transfection medium (F-12K supplemented with 10% fetal bovine serum). Cells were transfected with the following day using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the product protocol. Briefly, 19.2 μg of plasmid DNA/plasmid was incubated in 1200 μl of Opti-MEM I (Invitrogen) for 5 min at room temperature. Likewise, Lipofectamine 2000 (48 μl/plate) was incubated in 1200 μl of Opti-MEM I for 5 min at room temperature in a separate tube. The DNA and lipid mixtures were combined into a single tube and mixed gently. Lipid complexes were allowed to form during a 20-min incubation at room temperature. Complexes (100 μl/well) were added to each well of the 24-well plate, and the plate was placed into a humidified incubator. After a 6-h incubation, medium was replaced in each well with fresh transfection medium (500 μl). Cells were then incubated for an additional 18 h (24 h total) in a humidified incubator before experiments were conducted.

**Receptor Internalization Assay.** CHO cells transiently expressing either wild-type or mutant hM₁ receptors were washed with F-12K (three 500-μl washes) to remove serum and then incubated with the muscarinic receptor agonist carbachol (1 mM) in F-12K (500 μl) for up to 4 h (six wells for each time point) in a humidified incubator at 37°C in an atmosphere of 5% CO₂-95% air. We chose this time period because we found that the internalization process was nearly complete in cells expressing the wild-type receptor over this time interval. Cells were washed extensively on ice with ice-cold PBS (three 500-μl washes) to remove carbachol and to prevent further receptor trafficking. Intact cell binding assays were then performed using a single concentration of the membrane-impermeable radioligand [3H]N-methylisopropylacetylmannoside ([3H]NIMA, PerkinElmer Life and Analytical Sciences, Boston, MA) as described below under Receptor Binding Assays. This assay was used to determine the amount of wild-type and mutant hM₁ receptor expressed on the plasma membrane of CHO cells before and during incubation with carbachol.

**Receptor Down-Regulation Assay.** CHO cells transiently expressing either wild-type or mutant hM₁ receptors were washed...
(three 500-μl washes) with F-12K to remove serum and then incubated with carbachol (1 mM) in F-12K (500 μl) for 24 h in a humidified incubator at 37°C and in an atmosphere of 5% CO2:95% air. We chose this time interval because Shockley et al. (1997) showed that down-regulation is a slow process, requiring approximately 24 h for a substantial loss of receptor. Cells were washed extensively on ice with ice-cold PBS (three 500-μl washes) to remove carbachol. Intact cell binding assays were then performed using either a single concentration of [3H]NMS or the membrane-permeable radioligand [3H]quinuclidinyl benzylate ([3H]QNB; PerkinElmer Life and Analytical Sciences) as described below under Receptor Binding Assays. This assay was used to determine the effect of long incubations with carbachol on the expression of wild-type and mutant hM1 receptors.

Receptor Binding Assays. Intact cell binding assays were performed using a single concentration of [3H]NMS (1.6 nM) or [3H]QNB (1.2 nM) to determine the amount of plasma membrane-expressed receptor or total receptor expressed, respectively. We chose these concentrations of the two radioligands because these conditions should yield receptor occupancies at the wild-type receptor of 86 and 87%, respectively. Washed CHO cells were incubated with either [3H]NMS or [3H]QNB in the absence (three wells for each time point or condition; total binding) and presence (three wells for each time point or condition; nonspecific binding) of atropine (1 μM) in 500 μl of binding buffer (25 mM HEPES, 113 mM NaCl, 6 mM dextrose, 3 mM CaCl2, 3 mM KCl, 2 mM MgSO4, and 1 mM Na2HPO4, pH 7.4) for either 1 h ([3H]NMS) or 18 h ([3H]QNB) at 4°C. After incubation, cells were rapidly and gently washed (three 1-ml washes) with ice-cold PBS to remove unbound [3H]NMS or [3H]QNB. Bound [3H]NMS or [3H]QNB was recovered as described previously (Griffin et al., 2003), and radioactivity was counted using a Beckman LS6500 scintillation counter. Saturation binding assays were also performed on washed, intact CHO cells transiently expressing either wild-type or mutant hM1 receptors. Cells were incubated for 2 h at 4°C with geometrically spaced (0.33 log units) concentrations of [3H]NMS (six wells of a 24-well plate for each concentration) in the absence (three wells; total binding) and presence (three wells; nonspecific binding) of atropine (1 μM) in binding buffer. Cells were rapidly and gently washed (three 1-ml washes) with ice-cold PBS to remove unbound [3H]NMS, and bound [3H]NMS was recovered as described previously (Griffin et al., 2003).

The average amount of protein expressed in CHO cells was determined for each radioligand binding assay performed. In brief, three wells of a 24-well plate were plated and transfected for each receptor construct and treatment condition as described above under Cell Culture and Transfections at the same time as experimental plates. The cells were washed two times with 500 μl of mannitol wash buffer (0.29 M mannitol, 0.01 M Tris, and 0.5 mM Ca(NO3)2, pH 7.4) to remove serum. The protein concentration was determined for each well using the bicinchoninic acid protocol as described previously (Goldschmidt and Kimelberg, 1989).

Phosphoinositide Hydrolysis and Desensitization Assays. Phosphoinositide hydrolysis assays were conducted on CHO cells transiently expressing wild-type and mutant hM1 receptors as described previously (Sawyer et al., 2006). In some experiments, the method was modified to determine the effect of acetylcholine pre-treatment on the phosphoinositide response elicited to carbachol (used as a measure of receptor desensitization). In brief, CHO cells transiently expressing wild-type hM1, hM1CC262, or hM1DP262 receptors were washed (three 500-μl washes) with F-12K to remove serum and then incubated with myo-[3H]inositol (PerkinElmer Life and Analytical Sciences) in F-12K (500 μl) for 18 h in a humidified incubator. The cells were washed the following day with F-12K (three 500-μl washes) to remove unincorporated inositol, with a 10-min incubation in a humidified incubator between each wash. Cells were then incubated for 10 min in the absence or presence of acetylcholine (1 mM) in F-12K Acetylcholinesterase (2 U/well; Sigma-Aldrich, St. Louis, MO) was added to all wells (regardless of whether they had been incubated with acetylcholine or not), and the cells were incubated for 2 min in a humidified incubator. F-12K supplemented with LiCl (10 mM) was added to each well after washing (one 500-μl wash) with F-12K, and the cells were incubated for 10 min in a humidified incubator. Cells were then incubated with geometrically spaced concentrations of carbachol (0.5 log unit) for 30 min in a humidified incubator. Total [3H]inositol phosphates were extracted from cells using perchloric acid as described previously (Sawyer et al., 2006). After extraction of [3H]inositol phosphates, 1.11 ml of chloroform/methanol/HCl (100:200:1, v/v) was added to each well of the plates, and the plates were allowed to stand for 15 min at room temperature. The solution was pipetted from each well into individual 15 × 100-mm polypropylene tubes, and 0.37 ml of water and 0.37 ml of chloroform were added to separate aqueous and organic phases. The tubes were capped, shaken, and then centrifuged at 2000g for 2 min. An aliquot of the organic phase (lower phase; 200 μl) was placed into scintillation vials, and the chloroform was allowed to evaporate. Scintillation cocktail (Scintiverse, 5 ml) was added to each vial after the chloroform had evaporated, and the radioactivity was measured with a Beckman LS6500 scintillation counter to determine the amount of [3H]phosphoinositides. The [3H]inositol phosphates are expressed relative to the total amount of [3H]inositol phosphates plus [3H]inositol phosphates to correct for variation between acetylcholine-treated and untreated cells.

Data Analysis. The Hill slope, the maximal response (E_max), and the concentration of carbachol eliciting half-maximal response (EC50) were estimated from phosphoinositide hydrolysis data using nonlinear regression analysis according to a logistic equation as described by Bowen and Jerman (1995). The same equation was used to estimate the Hill slope, maximal binding capacity (B_max), and the concentration of [3H]inositol eliciting half-maximal receptor occupancy (K_d) from saturation binding data (Bowen and Jerman, 1995). Significant values (P values) were calculated using either one- or two-way ANOVAs (GraphPad Prism, version 4.03; GraphPad Software Inc., San Diego, CA). One-way ANOVAs were performed with a Dunnett’s post-hoc test on the E_max, B_max, K_d, and EC50 values estimated from the binding and phosphoinositide hydrolysis data obtained for mutant and wild-type hM1 receptors. Two-way ANOVAs were performed with a Bonferroni post-hoc test on the data obtained for the carbachol-induced internalization of mutant and wild-type hM1 receptors. Estimates of the rate constants for wild-type and mutant hM1 receptor internalization were made by fitting data using a single-phase exponential decay equation (GraphPad Prism, version 4.03).

Results

Effects of Deleting 254TPPGRCCR261 from the i3 Loop of hM1 Receptors. We determined that a small domain in the i3 loop of the hM1 receptor, 254TPPGRCCR261, interacts with itself in yeast two-hybrid assays (G. W. Sawyer, unpublished observations). In an effort to determine the functional role of this domain, we deleted it from the i3 loop of the hM1 receptor and characterized the resulting mutant receptor, hM1del, in CHO cells.

To determine whether deletion of 254TPPGRCCR261 affects the expression of the hM1 receptor, we measured the binding of [3H]NMS to intact CHO cells transiently expressing the deletion mutant or wild-type receptor (Fig. 1A). [3H]NMS is a quaternary ammonium muscarinic ligand that does not cross the plasma membrane, and, consequently, it can be used in the intact cell to measure the expression of muscarinic receptors at the cell surface. Deletion of 254TPPGRCCR261 from the hM1 receptor caused a moderate 1.6-fold increase in both the dissociation constant (K_d) and binding capacity (B_max) of [3H]NMS relative to those measured for the wild-type receptor (Table 1). These data suggest a small enhancing effect of...
To investigate the signaling properties of the deletion mutant, we measured agonist-mediated phosphoinositide hydrolysis in CHO cells transiently expressing the hM1 del or wild-type receptor (Fig. 1B). The potency of carbachol for eliciting phosphoinositide hydrolysis increased 1.4-fold in CHO cells expressing the deletion mutant compared with that measured for the wild-type receptor. There was little difference in the maximal effect of carbachol \( (E_{\text{max}}) \) in cells expressing either hM1 or hM1 del (Table 2). The small increase in the potency of carbachol at hM1 del relative to wild-type is consistent with the greater expression of this deletion mutant at the plasma membrane (see Fig. 1A).

We next investigated the effect of deleting 254TPPRCCR261 on agonist-mediated internalization of the hM1 receptor. CHO cells transiently expressing either hM1 del or wild-type hM1 were incubated with the agonist carbachol (1 mM) for various times up to 4 h, and receptor binding at the cell surface was measured using a single concentration of \([3H]NMS\) (1.6 nM). As shown in Fig. 2A, internalization of the wild-type hM1 receptor was consistent with a first-order decay process having a half-time of 65.8 min \( (k_1 = 0.011 \text{ min}^{-1}) \) and a plateau at 34.2% of control. In the case of the hM1 del receptor, the plateau for internalization did not occur during the 4-h period of incubation. However, we estimated a half-time for internalization \( (t_{1/2}) \) of 257.3 min \( (k_1 \text{estimate} = 0.0027 \text{ min}^{-1}) \) for hM1 del, assuming a plateau the same as that of the wild-type receptor (i.e., 34.2% of control). Thus, the rate of carbachol-induced internalization of hM1 del was only 24% of wild-type receptors, corresponding to a 4.2-fold longer estimated half-time. Whereas the internalization of the hM1 del receptor is not inconsistent with a first-order decay process having a single component, it is possible that more than one component contributes to the process. Overall, these data imply that the domain 254TPPRCCR261 in the i3 loop of the hM1 receptor plays a role in agonist-induced internalization.

To determine whether the deletion of 254TPPRCCR261 affects the potency of carbachol-induced hM1 receptor internalization, we incubated CHO cells transiently expressing either wild-type hM1 or hM1 del receptors with various concentrations of carbachol for 2 h and subsequently measured specific \([3H]NMS\) binding to these intact cells. Carbachol caused a concentration-dependent decrease in specific \([3H]NMS\) binding to cells expressing the wild-type hM1 receptor as well as those expressing the hM1 del receptor (see Table 2).

### Table 1

The effects of deleting or mutating amino acid residues in the i3 loop of hM1 receptors on \([3H]NMS\) binding

<table>
<thead>
<tr>
<th>Receptor Construct (^a)</th>
<th>(pK_{i3})</th>
<th>(B_{\text{max}}) (fmol/mg protein)</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type hM1 (3) (^b)</td>
<td>9.57 ± 0.07</td>
<td>644 ± 70</td>
<td>1.48 ± 0.21</td>
</tr>
<tr>
<td>hM1del (3) (^b)</td>
<td>9.36 ± 0.04</td>
<td>1000 ± 122 (^c)</td>
<td>1.48 ± 0.21</td>
</tr>
<tr>
<td>hM1P/A (3)</td>
<td>9.23 ± 0.04</td>
<td>916 ± 34</td>
<td>1.77 ± 0.11</td>
</tr>
<tr>
<td>hM1T/A (3)</td>
<td>9.15 ± 0.04</td>
<td>686 ± 19</td>
<td>1.52 ± 0.05</td>
</tr>
<tr>
<td>hM1CC259 (3)</td>
<td>9.03 ± 0.02</td>
<td>1512 ± 17 (^d)</td>
<td>1.90 ± 0.14</td>
</tr>
<tr>
<td>hM1CC262 (3)</td>
<td>9.35 ± 0.07</td>
<td>1390 ± 99 (^d)</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>hM12CC (3)</td>
<td>9.13 ± 0.09</td>
<td>1457 ± 117 (^d)</td>
<td>1.70 ± 0.18</td>
</tr>
<tr>
<td>hM12DD (3)</td>
<td>8.95 ± 0.01 (^e)</td>
<td>1600 ± 102 (^e)</td>
<td>1.77 ± 0.06</td>
</tr>
<tr>
<td>hM12DD (5)</td>
<td>8.95 ± 0.02 (^e)</td>
<td>1949 ± 162 (^e)</td>
<td>1.47 ± 0.06</td>
</tr>
<tr>
<td>hM1C259 (3)</td>
<td>9.25 ± 0.01</td>
<td>914 ± 25</td>
<td>1.59 ± 0.01</td>
</tr>
</tbody>
</table>

\(^a\) The number of experiments conducted is denoted in parentheses. Each experiment was performed in triplicate.

\(^b\) Estimates for the \(K_{i3}\), \(B_{\text{max}}\), and Hill slope were calculated from the data shown in Fig. 1.

\(^c\) Significantly different from the \(B_{\text{max}}\) of CHO cells expressing wild-type hM1 receptors, \(P < 0.05\).

\(^d\) Significantly different from the \(B_{\text{max}}\) of CHO cells expressing wild-type hM1 receptors, \(P < 0.01\).

\(^e\) Significantly different from the \(K_{i3}\) obtained for wild-type hM1 receptors, \(P < 0.01\).

### Table 2

Effect of deleting or mutating amino acid residues in the i3 loop of hM1 receptors on carbachol-stimulated phosphoinositide hydrolysis

<table>
<thead>
<tr>
<th>Receptor Construct (^a)</th>
<th>(pEC_{50})</th>
<th>(E_{\text{max}}) (Relative to Basal)</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type hM1 (3) (^b)</td>
<td>5.36 ± 0.06</td>
<td>5.81 ± 0.19</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>hM1del (3) (^b)</td>
<td>5.49 ± 0.13</td>
<td>6.52 ± 0.38</td>
<td>0.77 ± 0.19</td>
</tr>
<tr>
<td>hM1P/A (3)</td>
<td>5.45 ± 0.01</td>
<td>9.86 ± 0.41 (^d)</td>
<td>0.78 ± 0.02</td>
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<tr>
<td>hM1T/A (3)</td>
<td>5.46 ± 0.02</td>
<td>9.59 ± 0.56 (^d)</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>hM1CC259 (3)</td>
<td>5.62 ± 0.04</td>
<td>7.82 ± 0.16</td>
<td>0.79 ± 0.05</td>
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<tr>
<td>hM1CC262 (3)</td>
<td>5.70 ± 0.11</td>
<td>6.91 ± 0.23</td>
<td>0.86 ± 0.18</td>
</tr>
<tr>
<td>hM12CC (3)</td>
<td>5.71 ± 0.09 (^e)</td>
<td>7.57 ± 0.26 (^e)</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>hM12DD (3)</td>
<td>5.61 ± 0.03 (^e)</td>
<td>9.87 ± 0.49 (^e)</td>
<td>0.87 ± 0.06</td>
</tr>
<tr>
<td>hM12DD (5)</td>
<td>5.68 ± 0.06 (^e)</td>
<td>9.35 ± 0.51 (^d)</td>
<td>0.85 ± 0.09</td>
</tr>
<tr>
<td>hM1C259 (3)</td>
<td>5.45 ± 0.05</td>
<td>7.80 ± 0.31</td>
<td>0.77 ± 0.08</td>
</tr>
</tbody>
</table>

\(^a\) The number of experiments conducted is denoted in parentheses.

\(^b\) Estimates were calculated from the data shown in Fig. 2.

\(^c\) Significantly different from the \(E_{\text{max}}\) value obtained for phosphoinositide hydrolysis elicited to carbachol by wild-type hM1 receptors, \(P < 0.05\).

\(^d\) Significantly different from the \(E_{\text{max}}\) for the wild-type hM1 receptor response, \(P < 0.01\).
The effect of deleting 254TPPRCCR261 from the i3 loop of hM₁ receptors on carbachol-induced internalization. A, CHO cells transiently expressing either wild-type hM₁ or hM₁del receptors were incubated with the agonist carbachol (1 mM) for up to 4 h. The amount of plasma membrane expressed receptor was determined at various times during the incubation by conducting intact cell [³H]NMS binding assays. Each data point represents the mean ± S.E.M. of three experiments performed in triplicate. *, significantly different from the carbachol-induced internalization of wild-type hM₁ receptors,  P < 0.01. **, significantly different from the carbachol-induced internalization of wild-type hM₁ receptors,  P < 0.001. B, CHO cells transiently expressing either wild-type hM₁ or hM₁del receptors were incubated with increasing concentrations of carbachol for 2 h. Cells were then washed and used in intact cell [³H]NMS binding assays. Each data point represents the mean ± S.E.M. of four experiments conducted in triplicate.

Fig. 2B. As described above, the maximal decrease was significantly greater for the wild-type hM₁ receptor (36.3 ± 3.8%) compared with that observed for the hM₁del receptor (14.9 ± 5.8%) (F₁,₃₀ = 24.3;  P < 0.01). The EC₅₀ value for carbachol-induced internalization of the hM₁del receptor was 1.5-fold greater than that estimated for the wild-type receptor (pEC₅₀ = 4.44 ± 0.02), although this estimate is not significantly different from wild type because of the difficulty in obtaining an accurate estimate of EC₅₀ for such a small response.

The effect of deleting 254TPPRCCR261 on carbachol-induced down-regulation of the hM₁ receptor was also investigated. CHO cells transiently expressing either wild-type hM₁ or hM₁del receptors were incubated with carbachol (1 mM) for 24 h before intact cell binding assays were conducted with a single concentration of [³H]NMS (1.6 nM) or [³H]QNB (1.2 nM). [³H]QNB crosses the plasma membrane, and, consequently, it binds to both intramembrane and extracellular receptors. A reduction in [³H]QNB binding is indicative of a reduction in the total number of receptors, consistent with receptor down-regulation. In CHO cells transiently expressing the wild-type hM₁ receptor, specific [³H]QNB binding decreased by 58.6 ± 5.0% after a 24-h incubation with carbachol (Fig. 3). A similar decrease was also observed for the hM₁del receptor after 24-h carbachol treatment (65.6 ± 4.6%) (Fig. 3). Under the same conditions, specific [³H]NMS binding decreased by 80.3 ± 2.1% in CHO cells expressing the wild-type hM₁ receptor and by 71.4 ± 3.9% in cells expressing the hM₁del receptor (Fig. 3). The intracellular pool of receptor (determined by subtracting specific [³H]NMS binding from specific [³H]QNB binding) in CHO cells expressing wild-type hM₁ receptors decreased by 27% from a control value of 0.22 pmol/mg protein after carbachol treatment. In contrast, the intracellular pool of hM₁del receptor decreased by 52% from a control value of 0.48 pmol/mg protein after carbachol treatment. These data suggest that the i3 loop domain 254TPPRCCR261 of the hM₁ receptor does not play a role in agonist-induced down-regulation.

Effect of Mutating an Adjacent Putative SH3 Binding Motif and a Pro-Dependent Ser/Thr Kinase Site. We examined whether known functional motifs were in close proximity to 254TPPRCCR261 using Scansite 2.0 (http://scansite.mit.edu). A medium stringency screen conducted on the full-length hM₁ receptor sequence identified two motifs that overlap with the deleted i3 loop domain: a putative SH3 binding motif, 252PETPP256, and a putative Pro-dependent Ser/Thr kinase site, 254TPP256 (see Fig. 4A). A high stringency screen only identified the Pro-dependent kinase site. To determine whether the defect in agonist-mediated internalization of hM₁del was caused by the loss of either of these motifs, we made point mutants to disrupt the putative SH3 binding domain (P252A) and the putative Pro-dependent Ser/Thr kinase site (T254A) and tested the mutants for agonist-mediated internalization (Fig. 4B). The extent of carbachol-induced internalization of the hM₁P/A and the hM₁T/A receptors in CHO cells was not significantly different from that observed for the wild-type receptor (see Fig. 4B). There was also little difference in carbachol-stimulated phosphoinositide hydrolysis and the binding properties of [³H]NMS in intact cells expressing either the wild-type or the two point mutants of the hM₁ receptor (see Tables 1 and 2). Therefore, the defect in carbachol-induced internalization of hM₁del...
cannot be attributed to disruption of either the putative SH3 binding motif or the putative Pro-dependent Ser/Thr kinase site.

Effect of Cys to Ala Point Mutations. We investigated Cys pairs within and adjacent to the i3 loop domain PETPPGRCCR261 to determine their potential role in receptor internalization. We made three hM1 receptor mutants, the first containing a C259A/C260A double point mutation (hM1CC259), the second containing a C262A/C263A double point mutation (hM1CC262), and the third having both of these double point mutations (hM12CC) (Fig. 5 A). The results of [3H]NMS binding experiments on intact CHO cells transiently expressing these mutants showed that the mutations caused a modest reduction in the affinity (1.7–3.5-fold increase in KD) of [3H]NMS and a substantial 2.2 to 2.4-fold increase in the binding capacity of [3H]NMS when expressed relative to the binding parameters of the wild-type receptor (Table 1). The largest effects were observed in cells expressing the hM1CC262 receptor mutant. These increases in binding capacity were associated with corresponding 1.8 to 2.2-fold increases in potency and 1.2 to 1.3-fold increases in the E\textsubscript{max} of carbachol-stimulated phosphoinositide hydrolysis relative to that for the wild type (Table 2).

Carbachol-induced internalization of these mutant receptors was substantially impaired. Whereas the wild-type receptor exhibited a 63 ± 8.7% decrease in [3H]NMS binding after a 4-h treatment with carbachol, only 39 ± 1.6, 28 ± 2.5, and 36 ± 1.7% decreases in [3H]NMS binding were noted for the hM1CC259, hM1CC262, and hM12CC mutants, respectively (Fig. 5 B). The behavior of the hM1CC259 and hM12CC receptor mutants were similar to that of the hM1del mutant and exhibited estimated half-times (t\textsubscript{1/2-estimate} 215 and 239 min, respectively) and estimated rate constants (k\textsubscript{1-estimate}, 0.0032 and 0.0029 min\textsuperscript{-1}, respectively) for loss of [3H]NMS binding that were 3.3- and 3.6-fold greater, respectively, than wild-type, assuming the same plateau as that for the wild type. In contrast, hM1CC262 exhibited even slower internalization kinetics.
with an estimated half-time ($t_{1/2}$-estimate = 440 min) and estimated rate constant ($k_{1}$-estimate = 0.0016 min$^{-1}$) that corresponded to a 6.7-fold slower process relative to wild type, assuming the same plateau value as that for the wild type.

To investigate the possibility that Cys residues in Cys pair Cys262/Cys263 function in an all-or-none manner, we made an hM1 mutant (hM1C263) possessing a single Ala point mutation in this pair (C623A) and characterized its behavior (Fig. 5A). When transiently expressed in CHO cells, the hM1C263 mutant bound $[^3H]$NMS in the intact cell with moderately reduced affinity (2.1-fold increase in $K_D$) and a slightly increased binding capacity (1.3-fold increase in $B_{max}$) (Table 1). This modest increase in expression relative to wild type was associated with increases in the potency (1.2-fold increase in $E_{max}$) and maximal response (1.3-fold increase in $E_{max}$) of carbachol-stimulated phosphoinositide hydrolysis (Table 2).

Carbachol-mediated internalization of hM1C263 was slightly attenuated compared with that for the wild-type receptor, although this difference was not significant (Fig. 5B). The data were consistent with a first-order decay model, having a 1.36-fold greater rate constant ($k_1 = 0.015$ min$^{-1}$) but a higher plateau value (51.6%) relative to that for the wild-type receptor. Overall, these data suggest that the presence of one Cys residue in Cys pair Cys262/Cys263 at least partially, or perhaps fully, rescues carbachol-induced internalization of the hM1 receptor.

**Effect of Cys to Asp Point Mutations.** We also investigated the effect of mutating Cys residues to Asp on carbachol-induced internalization of the hM1 receptor. We made two hM1 receptor mutants, one possessing a C262D/C263D double point mutation (hM1DD262) and one possessing a C259D/C260D and C262D/C263D quadruple point mutation (hM12DD) (Fig. 5A). In $[^3H]$NMS binding assays performed on intact CHO cells transiently expressing either receptor mutant, a 4.2-fold reduction in affinity and a substantial increase in binding capacity (2.5-3.0-fold) was observed (Table 1). These increases in binding capacity were associated with corresponding increases in potency (1.8-2.1-fold) and $E_{max}$ (1.6-1.7-fold) of carbachol-stimulated phosphoinositide hydrolysis relative to those for the wild type (Table 2).

Carbachol-induced internalization of hM1DD262 and hM12DD receptors showed the greatest impairment of all of the mutants tested. $[^3H]$NMS binding decreased only $18 \pm 0.8$ and $16 \pm 3.5\%$ for hM1DD262 and hM12DD, respectively, after 4 h of carbachol treatment (Fig. 5C). These decreases in binding exhibited half-times ($t_{1/2}$-estimate, 588 and 590 min, respectively) and rate constants ($k_{1}$-estimate 0.0012 and 0.002 min$^{-1}$, respectively) for hM1DD262 and hM12DD receptors that were 9-fold greater than those relative to wild-type, assuming the same plateau value (i.e., 34.2% of control).

**Effect of the C262A/C263A and C262D/C263D Double Point Mutations on hM1 Receptor Desensitization and Down-Regulation.** Desensitization of GPCRs is thought to precede internalization and is probably initiated by the agonist-dependent phosphorylation of the receptor. To investigate whether internalization of the hM1 receptor is necessary for desensitization to occur, we compared the desensitization of hM1CC262 and hM1DD262 receptors with that of the wild-type hM1 receptor. These receptor mutants were chosen for this experiment because they had the largest reduction in carbachol-induced internalization (see Fig. 5, B and C), with the fewest amino acid residues mutated (see Fig. 5A).

CHO cells transiently expressing wild-type hM1, hM1CC262, or hM1DD262 receptors were incubated in the absence or presence of acetylcholine (1 mM) for 10 min before measuring carbachol-stimulated phosphoinositide hydrolysis. Acetylcholine was used to induce receptor desensitization because its action can be rapidly terminated by the addition of purified acetylcholinesterase (see Materials and Methods). In cells expressing the wild-type hM1 receptor, the EC$_50$ value of carbachol for stimulating phosphoinositide hydrolysis was 1.6-fold greater in cells pretreated with acetylcholine than that measured in untreated cells (pEC$_{50}$ = 5.44 ± 0.05; Fig. 6A). Acetylcholine treatment also caused an 18.4% reduction in the $E_{max}$ of the response mediated by the wild-type hM1 receptor (Fig. 6A). Similar results were obtained for the mutant receptors. In cells expressing the hM1CC262 receptor, acetylcholine treatment caused a 1.9-fold increase in the EC$_{50}$ value of carbachol (control pEC$_{50}$ = 5.59 ± 0.09) and a 15.1% reduction in $E_{max}$ relative to untreated cells (Fig. 6B). In cells expressing the hM1DD262 receptor, acetylcholine treatment caused a 2.0-fold increase in EC$_{50}$ (control pEC$_{50}$ = 5.41 ± 0.13) and a 37.6% reduction in $E_{max}$ relative to untreated cells. These data indicate that hM1CC262 and hM1DD262 receptors desensitize to an extent similar to that observed for the wild-type hM1 receptor.

We also investigated receptor down-regulation by incubating cells expressing hM1CC262 and hM1DD262 receptors with carbachol (1 mM) for 24 h and then conducting binding assays with a single concentration of $[^3H]$NMS or $[^3H]$QNB. After carbachol treatment, specific $[^3H]$QNB binding in CHO cells transiently expressing either hM1CC262 or hM1DD262 receptors decreased by 54.2 ± 1.7 and 52.6 ± 2.4%, respectively (Fig. 3). In these experiments, the intracellular pool of hM1CC262 and hM1DD262 receptors decreased 1.7- and 1.9-fold, respectively, compared with control (489.4 and 430.2 fmol/mg protein, respectively). These changes in hM1CC262 and hM1DD262 receptor expression were similar to the changes observed for the wild-type hM1 receptor, suggesting that Cys pair Cys262/Cys263 does not play a role in carbachol-induced down-regulation (see Fig. 3).

**Discussion**

The i3 loop of muscarinic receptors is quite large relative to other GPCRs, and it is 158 amino acid residues long in the hM1 sequence (Tyr208/Thr366). Deletion of most of the i3 loop (Gly232/Tyr358) was shown to inhibit carbachol-induced internalization of the hM1 receptor in U293 cells without inhibiting phosphoinositide hydrolysis (Maeda et al., 1990). Lameth et al. (1992) found that agonist-mediated internalization of the hM1 receptor is complex and probably involves the contribution of multiple i3 loop sequences.

In this investigation, we characterized the functional role of a novel domain (i.e., $^{252}$PETPGRCCRCC$^{263}$) in the i3 loop of the hM1 receptor. Deletion of most of this domain ($^{254}$PETPGRCCRCC$^{261}$) decreased carbachol-induced internalization to a rate only one-fourth of that of the wild-type receptor (see Fig. 2A). This effect could not be attributed to disruption of a putative SH3 binding motif or a putative Pro-dependent Thr kinase site within this domain (see Fig. 4). In contrast, alanine mutagenesis of Cys pair Cys259/Cys260 could account for the defect in internalization of the hM1del mutant.
It seems that attenuated receptor internalization should cause an increase in receptor expression and function. This reasoning can explain why the receptor mutants with attenuated internalization exhibited a moderate increase in \[^{[3]H}\]NMS binding and agonist-mediated phosphoinositide hydrolysis relative to wild type when CHO cells were transiently transfected. It has been shown previously that the potency and $E_{\text{max}}$ for carbachol-stimulated phosphoinositide hydrolysis are proportional to hM\(_1\) receptor density in murine fibroblasts (Mei et al., 1989), illustrating that there is a receptor reserve for the phosphoinositide response. In contrast, we expect that internalization would be proportional to receptor occupancy. Accordingly, we found that the pEC\(_{50}\) value of carbachol (4.5) for eliciting internalization is approximately equal to its binding affinity (pK\(_D\)) for M\(_1\) receptors expressed in CHO K1 cells (Savarese et al., 1992) and murine fibroblasts (Mei et al., 1989).

It seems unlikely that receptor internalization is rate-limiting and that the increased expression of hM\(_1\)del and hM\(_1\)CC259 receptors, per se, causes a decrease in receptor internalization. Three lines of evidence argue against this hypothesis. First, the increases in the plasma membrane expression of hM\(_1\)del and hM\(_1\)P/A receptors were similar (i.e., 1.6- and 1.4-fold, respectively), yet the former showed a marked decrease in internalization, whereas the latter behaved like the wild-type receptor (see Table 1; Fig. 6). Second, Lameh et al. (1992) demonstrated that carbachol-induced internalization of the hM\(_1\) receptor was independent of receptor density over at least a 10-fold range in U293 cells; the maximal plasma membrane expression of the mutant receptors tested in this study fell well within this range (see Table 1). Third, Shockely et al. (1999) measured a rate of internalization of the hM\(_1\) receptor in stably transfected CHO cells similar to that reported here, yet the expression of the hM\(_1\) receptor in their study was approximately 3-fold greater than that used here. Therefore, we believe it is unlikely that the increased expression of the mutants investigated in this study (i.e., hM\(_1\)del, hM\(_1\)CC259, hM\(_1\)CC262, hM2CC, hM2DD, and hM2DD) is the cause of their decreased agonist-induced internalization.

Mutagenesis of Cys pair Cys262/Cys263 (hM1CC262) yielded a receptor with an internalization rate only half of that of the hM1del, hM1CC259, and hM12CC receptors (see Fig. 5B). This result suggests that the loss of internalization function cannot be attributed to the loss of a disulfide bond between Cys pairs Cys259/Cys260 and Cys262/Cys263 because one would expect that mutagenesis of either pair would result in equivalent defects in carbachol-induced internalization. It was anticipated that mutation of both Cys pairs Cys259/Cys260 and Cys262/Cys263 (i.e., hM12CC) would create a mutant that had internalization kinetics similar to or slower than hM1CC262 receptors. Instead, hM12CC receptors had internalization kinetics that were more consistent with hM1del and hM1CC259 receptors. Changing Cys pair Cys262/Cys263 to Asp pairs (i.e., hM1DD262 and hM12DD, respectively) further inhibited the carbachol-induced internalization of hM1 receptors (9-fold slower than wild type). Overall, our data indicate that both Cys pairs in the i3 loop of hM1 receptors are surrounded by basic Arg residues (i.e., 258RCCRCCR264) and, thus, may be palmitoylated to anchor this region of the receptor to the membrane (for review, see el-Husseini and Bredt, 2002). To date, several GPCRs (including the M2 receptor) are known to be palmitoylated on Cys residues in the C-terminal tail, and palmitoylation has been shown to affect GPCR activity, phosphorylation, and/or trafficking (for reviews, see el-Husseini and Bredt, 2002;
Torrecilla and Tobin, 2006). However, we found that 2-bromopalmitate (50 \(\mu\)M; 18-h treatment) did not inhibit carbachol-mediated internalization of wild-type, hM1CC259, and hM1CC262 receptors. 2-Bromopalmitate is known to inhibit palmitoylation of substrates in intact cells. Instead, we found that 2-bromopalmitate caused a 50% inhibition of the expression of all of the receptors tested (G. W. Sawyer, unpublished observations). Thus, palmitoylation does not appear to play a role in the agonist-dependent internalization of hM1 receptors but instead plays a role in the plasma membrane delivery and expression of the receptor. This observation is consistent with that of Sadeghi et al. (1997), who found that mutation of C-terminal Cys residues Cys341/Cys342 of the vasopressin V2 receptor to serines caused a decrease in the plasma membrane expression of the receptor. The latter Cys residues are known to be palmitoylated.

Our observations in yeast two-hybrid assays suggest that Cys pair Cys259/Cys260 may promote hM1 receptor dimerization/oligomerization. In these assays, bait and prey fusion proteins possessing the full-length i3 loop sequence of hM1 receptors interacted with one another, and this interaction was lost when 554TPPPGCCR563 was deleted from the prey i3 loop fusion construct (G. W. Sawyer, unpublished observations). Dimerization/oligomerization of muscarinic M2 and M3 receptors has been described previously, although the functional consequence of such interactions is unclear but may affect receptor signaling and/or ligand affinity (Maggio et al., 1996; Zeng et al., 1999; Zeng and Wess, 2000; Park et al., 2001). In a more recent investigation using bioluminescence resonance energy transfer, M1, M2, and M4 receptors were observed to form homodimers/oligomers in HEK 293 cells (Goin and Nathanson, 2006). The functional consequence of M1 receptor dimerization/oligomerization was not determined; however, the formation of M1/M1 heterodimers enhanced carbachol-induced M1 receptor down-regulation (Goin and Nathanson, 2006). Dimerization/oligomerization of hM1 receptors may result in the efficient delivery of receptor to clathrin-coated pits; a mechanism involved in hM1 receptor internalization (tolbert and lamesh, 1996).

Alternatively, one or both Cys pairs may act as a conformational switch, controlling the accessibility of other critical i3 loop domains to the internalization mechanism. Disulfide bonds are known to form between the side chains of adjacent Cys residues, forming an eight-membered cysteinyl-cysteine ring (Creighton et al., 2001; Carugo et al., 2003). The peptide bond between these adjacent residues can assume either a cis- or trans-conformation depending upon whether the Cys residues are oxidized or reduced, respectively (Carugo et al., 2003). This change in the conformation of the peptide bond is known to alter the conformation of peptides containing them, and thus, the conformation of the i3 loop of hM1 receptors may change in a redox-dependent manner (Creighton et al., 2001; Carugo et al., 2003). Given the close proximity of Cys pairs Cys259/Cys260 and Cys262/Cys263 to the S/T-rich domain (i.e., 20 amino acid residues away) described by Lameh et al. (1992), either one or both of the Cys pairs may fold the i3 loop of hM1 receptors to hide this critical internalization domain in an agonist-reversible manner. It is interesting to note that the presence of one Cys residue in Cys pair Cys262/Cys263 (i.e., hM1CC262; see Fig. 5A) almost completely rescued carbachol-induced internalization of hM1 receptors (see Fig. 5B). Perhaps this Cys residue is forming a disulfide linkage with either Cys residue in Cys pair Cys259/Cys260, forming the conformational switch necessary to control the exposure of other critical i3 loops domains.

A role for Cys residues in GPCR internalization has been demonstrated previously, although the residues were in the C-terminal tail of the receptor. Preisser et al. (1999) showed that mutating Cys pair Cys371/Cys372 in the C terminus of the vasopressin V1a receptor to an Ala pair significantly inhibited agonist-induced internalization. Moreover, the mutagenesis of the C-terminal sequence 336CNC337 of thyrotropin-releasing hormone receptor to either 336SGC337 or 336NGC337 also inhibited agonist-induced internalization (Nussenzveig et al., 1993). In the case of the V1a receptor, the Cys pair was eight amino acids downstream of a dileucine motif; which when mutated also significantly inhibited the agonist-induced internalization (Preisser et al., 1999). A dileucine motif is also implicated to mediate the internalization of b2-adrenergic receptors, and it is adjacent to a Cys residue that is palmitoylated (Gabilondo et al., 1997). Interestingly, there is a dileucine motif four amino acids downstream of Cys pair Cys262/Cys263 in the i3 loop of the hM1 receptor. It is possible that either or both Cys pairs are controlling the accessibility of this motif.

After 24 h of carbachol treatment, the hM1 receptor mutants hM1del, hM1CC262, and hM1DD262 were observed to down-regulate in a manner consistent with wild-type hM1 receptors, even though both of these mutants have greatly reduced internalization (see Fig. 3). This is consistent with the observations of Shockley et al. (1999), who showed that the domain(s) involved in the agonist-induced internalization of hM1 receptors appear to be separate from the domain(s) necessary for hM1 receptor down-regulation.

References


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