A Conserved Motif in the Membrane Proximal C-Terminal Tail of Human Muscarinic M1 Acetylcholine Receptors Affects Plasma Membrane Expression

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Running title page

a) A Conserved Motif Affects M_1 Receptor Plasma Membrane Expression

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d) Nonstandard abbreviations: EGFP, enhanced green fluorescence protein; hM_1, human muscarinic M_1 acetylcholine receptor; [^3H]NMS, [^3H]-N-methylscopolamine; TM1, transmembrane spanning domain 1; [^3H]QNB, [^3H]-3-quinuclidinyl benzilate

e) Cellular and Molecular
Abstract

We investigated the functional role of a conserved motif, F(x)_6LL, in the membrane proximal C-tail of the human muscarinic M_1 (hM_1) receptor. Using site-directed mutagenesis, several different point mutations were introduced into the C-tail sequence \(^{423}\text{FRDTFRLLL}^{431}\). Wild-type and mutant hM_1 receptors were transiently expressed in CHO cells and the amount of plasma membrane expressed receptor was determined using intact, whole cell \(^3\text{H}\)NMS binding assays. The plasma membrane expression of hM_1 receptors possessing either L430A or L431A or both point mutations was significantly reduced when compared to the wild type. The hM_1 receptor possessing a L430A/L431A double point mutation was retained in the ER, and atropine treatment caused the redistribution of the mutant receptor from the ER to the plasma membrane. Atropine treatment also caused an increase in the maximal response and potency of carbachol-stimulated phosphoinositide hydrolysis elicited by the L430A/L431A mutant. The effect of atropine on the L430A/L431A receptor mutant suggests that L\(^{430}\) and L\(^{431}\) play a role in folding hM_1 receptors, which is necessary for exit from the ER. Using site-directed mutagenesis, we also identified amino acid residues at the base of transmembrane spanning domain 1 (TM1), V\(^{46}\) and L\(^{47}\), that when mutated reduce the plasma membrane expression of hM_1 receptors in an atropine-reversible manner. Overall, these mutagenesis data show that amino acid residues in the membrane proximal C-tail and base of TM1 are necessary for hM_1 receptors to achieve a transport-competent state.
Introduction

Many GPCRs contain a conserved motif F(x)6LL (x indicates any amino acid residue, and L indicates either Leu or Iso) (Duvernay et al., 2004) in their c-terminal tails that is contained within an alpha helical structure adjacent to the plasma membrane in the β2-adrenoceptor structure. This motif is necessary for the ER export of 5-HT1A, 5-HT1B, α1B- and α2B-adrenergic, angiotensin II type IA, and β2-adrenergic receptors (Carrel et al., 2006; Duvernay et al., 2009; Duvernay et al., 2004). Mutation of either the F or L residues in the motif prevented the plasma membrane expression of these receptors by causing their ER retention (Carrel et al., 2006; Duvernay et al., 2009; Duvernay et al., 2004). The spacing between the F and LL residues appears to be critical, as the addition or removal of amino acid residues between them caused the ER retention of the α2B receptor (Duvernay et al., 2004). Other hydrophobic amino acids could not fully substitute for the F or L residues of the F(x)6LL motif in the α2B-adrenoceptor, suggesting that they have unique properties necessary for ER export (Duvernay et al., 2009). The mechanism by which the F(x)6LL motif influences ER export is unclear, but it may be an independent ER-exit motif (Duvernay et al., 2005; Duvernay et al., 2004) or be required for the proper folding of some GPCRs (Duvernay et al., 2009).

The F(x)6LL motifs of α1B- and α2B-adrenergic, β2-adrenergic, angiotensin II type IA, 5-HT1A, and 5-HT1B receptors are structurally analogous to the membrane-proximal hydrophobic folding motif (i.e., h(x)3h(x)2hh, where h is a hydrophobic amino acid) of the vasopressin V2 receptor (332VSELRSLL340) (Krause et al., 2000). Mutation of either V332, L336, L339, or L340 in this motif caused ER retention of the receptor (Krause et al., 2000; Thielen et al., 2005). The membrane-permeable V2 receptor antagonist SR121463B rescued the plasma membrane expression (Thielen et al., 2005), suggesting that the mutant V2 receptor could not achieve a transport-competent conformation (Krause et al., 2000; Thielen et al., 2005). Perhaps the F(x)6LL motif is another type of folding motif that mediates transport-competent folding necessary for many GPCRs to exit the ER and enter the secretory pathway.
All five subtypes of the muscarinic receptor also possess an F(x)_{6}LL motif. In the M_{1} sequence, this motif, 423FRDTFRLLL^{431}, is analogous to the h(x)_{3}h(x)_{2}hh (423FRDTFRLLL^{431}) motif described by Krause and coworkers (2000). Using site-directed mutagenesis, we investigated the role of the F(x)_{6}LL motif in the human M_{1} muscarinic receptor (hM_{1}). We found that mutation of the F and L residues in the motif caused retention of the mutant hM_{1} receptor in the ER and a consequent loss of plasma membrane expression. The membrane permeable muscarinic antagonist atropine, but not the quaternary antagonist NMS, restored the plasma membrane expression and signaling of the mutant receptor. Our data indicate that amino acids in the F(x)_{6}LL motif are necessary for the ER export of the M_{1} receptor.
Methods

Receptor Mutagenesis and Constructs. The hM<sub>1</sub> receptor cDNA, cloned into a modified Okayma-Berg expression vector (pCD), was provided by Dr. Tom I. Bonner at the NIMH. Mutant hM<sub>1</sub> receptors were made by introducing point mutations into the hM<sub>1</sub> receptor cDNA of pCD-hM<sub>1</sub> using the QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and mutagenesis primers. All receptor mutants were sequenced at 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.

To make GFP-tagged wild-type, hM<sub>1AA430-431</sub>, and hM<sub>1AA46-47</sub> receptor constructs, the sequences encoding wild-type (Bonner et al., 1988), hM<sub>1AA46-47</sub>, and hM<sub>1AA430-431</sub> receptors were amplified using 20-cycle PCR reaction and primers with 5’ EcoRI (5’-GCAGAGGAATCCAACTTCAGCCCCAC-3’; forward) and BamHI (5’-GCAGAGGGATCTCAGCATTGGCGGGAG-3’; reverse) restriction sites. The PCR product was purified and digested with EcoRI and BamHI and ligated into pEGFP-C2 (Clontech) digested with the same restriction enzymes. To ensure the translation of the receptor constructs in the ER, the pEGFP-C2 construct was modified to include a human growth hormone secretion signal sequence (amino acids -26 to -1 of human growth hormone) 5’ of the sequence encoding GFP (Volchuk et. al., 2000). This construct was used to express wild-type and mutant hM<sub>1</sub> receptors as N-terminal fusion proteins to GFP.

Cell Culture and Transient Transfections. CHO cells were subcultured every two to three days and 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 set at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air. In preparation for transient transfections, CHO cells were trypsinized and plated in a 24-well plate format at 1.65 x 10<sup>5</sup> cells per well in 500 μl of transfection medium (F-12K supplemented with 10% fetal bovine serum). On the following day,
cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the product protocol. Briefly, 19.2 μg of plasmid DNA/plate was incubated in 1,200 μl of Optimem I (Invitrogen, Carlsbad, CA) for five min at room temperature. In a separate tube, Lipofectamine 2000 (48 μl/plate) was incubated in 1,200 μl of Optimem I for five min at room temperature. The DNA and lipid mixtures were combined into a single tube and mixed gently. The lipid complexes were allowed to form during a 20 min incubation at room temperature. Complexes (100 μl/well) were added to each well of a 24-well plate and the plate was placed into a humidified incubator. After a six-hr incubation, medium was replaced in each well with fresh transfection medium (500 μl). Cells were incubated for an additional 18 h (24 h total) in a humidified incubator before conducting experiments.

**Characterization of Wild-type and Mutant hM1 Receptor Plasma Membrane Expression.** CHO cells were transiently transfected with wild-type and mutant hM1 receptor constructs as described under “Cell Culture and Transient Transfections”. Cells were washed (3 x 500 μl) with PBS to remove serum and then used in intact, whole cell [3H]NMS or [3H]QNB binding assays as described below under “Receptor Binding Assays.”

To determine the effect of atropine on the plasma membrane expression of wild-type and mutant receptors, CHO cells were incubated with either a single concentration of atropine (0.1 μM) or increasing concentrations of atropine (0.5 log-unit) in transfection medium for 18 h beginning 6 h after transfecting cells with either wild-type or mutant hM1 receptor constructs. To remove atropine, cells were washed three times with F-12K (500 μl) with a 20 min incubation between each wash in a humidified incubator set at 37°C in an atmosphere of 5% CO₂/95% air. Cells were then used in intact, whole cell [3H]NMS or [3H]QNB binding assays as described under “Receptor Binding Assays.”

We also determined the effect of short-term atropine treatment on the plasma membrane expression of wild-type and hM1AA430-431 receptors. Briefly, CHO cells were transiently transfected with wild-type hM1 or hM1AA430-431 receptor constructs as described under “Cell
Culture and Transient Transfections.” Cells were washed three times with F-12K (500 μl) to remove serum and then incubated with atropine (0.1 μM) for various periods of time for up to 7 h. To remove atropine, CHO cells were washed three times with F-12K (500 μl), with a 20 min incubation between each wash in a humidified incubator set at 37°C in an atmosphere of 5% CO₂/95% air. Cells were then used in intact, whole cell [³H]NMS binding assays as described under “Receptor Binding Assays.” In some experiments, CHO cells expressing either wild-type hM₁, hM₁AA46-47, hM₁AA430-431, and hM₁A46,A431 were incubated in the absence and presence of N-methylscopolamine (0.2 μM) or scopolamine (0.5 μM) for various periods of time for up to 7 h. Cells were washed as described above and used in intact, whole cell [³H]NMS binding assays as described under “Receptor Binding Assays”.

To determine whether hM₁AA430-431 receptors were stably expressed on the plasma membrane after atropine-treatment, CHO cells were incubated with atropine (0.1 μM) for 18 h beginning 6 h after transfection with wild-type and hM₁AA430-431 receptors. Atropine-treated cells were washed three times with F-12K (500 μl), with a 20 min incubation between each wash in a humidified incubator. After the third and final wash, cells were incubated for various periods of time for up to 8 h in a humidified incubator set at 37°C in an atmosphere of 5% CO₂/95% air, then used in intact, whole cell [³H]NMS binding assays as described under “Receptor Binding Assays.”

Receptor Binding Assays. To determine total receptor expression and the amount of receptor expressed on the plasma membrane of CHO cells, intact, whole cell binding assays were performed using a single concentration of either [³H]QNB (1.6 nM) or [³H]NMS (1.7 nM), respectively. Briefly, 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 binding buffer (25 mM HEPES, 113 mM NaCl, 6 mM dextrose, 3 mM CaCl₂, 3 mM KCl, 2 mM MgSO₄, 1 mM NaH₂PO₄, pH 7.4) for 24 h at 4°C. Following incubation, cells were rapidly and gently
washed (2 x 1 ml) 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 LS 6500 scintillation counter.

To determine the affinity of [3H]NMS for wild-type and mutant hM1 receptors, CHO cells were transiently transfected as described above in “Cell Culture and Transient Transfections”. Six h after transfection, cells were incubated in transfection medium with or without atropine (0.1 μM) for 18 h. Cells were washed three times with F12K (500 μl) with a 20 min. incubation between each wash. After washing, cells were incubated with equally-spaced concentrations of [3H]NMS in binding buffer (0.33-log unit) in the absence (total binding) or presence (non-specific binding) of atropine (10 μM) for 24 h at 4°C. Following incubation, cells were washed with ice-cold PBS (1 ml) two times on ice. Bound [3H]NMS was recovered as described previously (Griffin et al., 2003) and radioactivity was counted using a Beckman LS6500 scintillation counter.

The average amount of protein expressed in CHO cells was determined for each radioligand binding assay performed and specific [3H]NMS or [3H]QNB binding was normalized to the amount of protein measured. Briefly, three wells of a 24-well plate were plated and transfected for each receptor construct and assay condition as described above in “Cell Culture and Transfections” at the same time as experimental plates. The cells were washed two times with 500 μl mannitol wash buffer (0.29 M mannitol, 0.01 M Tris, 0.5 mM Ca(NO3)2, pH 7.4) to remove serum. The protein concentration was determined for each well using the bicinchoninic acid (BCA) protocol as described previously (Goldschmidt and Kimelberg, 1989).

Phosphoinositide Hydrolysis Assays. Phosphoinositide hydrolysis assays were conducted on CHO cells transiently expressing wild-type and mutant hM1 receptors as described previously (Sawyer et al., 2006). Briefly, CHO cells transiently expressing wild-type or M1AA430-431 receptors were incubated in a humidified incubator set at 37°C and 5% CO2/95% air with [3H]myo-inositol (0.2 μM, NEN Life Sciences) for 18 h in F-12K media. Cells were washed
extensively to remove unincorporated $[^3H]$inositol, then incubated for 30 min with equally spaced concentrations (0.5 log units) of the muscarinic agonist carbachol in the presence of lithium (10 mM). $[^3H]$inositolphosphates were extracted from cells by adding 200 µl PCA (5%) to each well and incubating the plate for 15 min on ice. The extracts were neutralized by adding 187 µl of 0.525M KOH containing 10 mM Tris to each well of the plate, followed by a 15 min incubation on ice. Neutralized extracts were pipetted into individual 1.5-ml tubes and wells were washed with 400 µl of 25 mM Tris, pH 7.4, which was added to the appropriate tube. Tubes were centrifuged at 3000 x g for 10 min and the supernatant was transferred into glass tubes containing 2 ml of 25 mM Tris, pH 7.4. The solution was applied to a 1 ml column of Dowex AG 1-X8 (formate form, 100-200 mesh). Columns were washed with water (4 x 4 ml) and eluted into 25-ml scintillation vials with 2.5 ml of 1 M ammonium formate, 0.1 M formic acid. Scintiverse (20 ml) was added and the radioactivity was counted using Beckman LS 6500 scintillation counter.

In some experiments, CHO cells were incubated with atropine (0.1 µM) for 18 h beginning 6 h after transfection with wild-type or hM$_{1AA430-431}$ receptor constructs. The atropine containing medium was replaced with F-12K medium (500 µl) containing atropine (0.1 µM) and $[^3H]$myo-inositol (0.2 µM, NEN Life Sciences). Cells were incubated for 18 h in a humidified incubator, then washed three times with F-12K (500 µl) with a 20 min incubation between each wash in a humidified incubator. Cells were then used in phosphoinositide hydrolysis assays as described above.

**Epi-fluorescence Images.** CHO cells were plated at 0.25 x $10^5$ cells/well on poly-D-lysine treated plates in transfection medium (500 µl) and placed into a humidified incubator at 37°C in an atmosphere of 5% CO$_2$/95% air. On the following day, cells were co-transfected with pEGFP-C2-hM$_1$ (0.2 µg/well), pEGFP-C2-hM$_{1AA46-47}$ (0.2 µg/well), pEGFP-C2-hM$_{1AA430-431}$ (0.2 µg/well), or pEGFP-C2-hM$_{1AA46,A431}$ and pDsRed2-ER (0.2 µg/well) (Clontech) using Lipofectamine 2000 as described under “Cell Culture and Transient Transfections.” Images of
CHO cells transiently co-expressing GFP-tagged wild-type hM₁ or mutant receptor constructs with DsRed2-ER were captured after a 24 h incubation in a humidified incubator set at 37°C in an atmosphere of 5% CO₂/95% air using an Olympus IX 71 epi-fluorescence microscope fitted with FITC (Chroma Technology Corp.) and CY3 filters (Chroma Technology Corp.), and a Coolsnap Monchrome digital camera. The images captured using the FITC or CY3 filters were colored either green or red, respectively, and overlaid using Metamorph 5.0r7 imaging software.

Data Analysis. The Hill slope, maximal response (E_{max}), and the concentration of carbachol eliciting half-maximal response (EC_{50}), 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, E_{max}, and EC_{50} of atropine-mediated plasma membrane expression of hM_{1AA430-431} receptors (Bowen and Jerman, 1995). The operational model (Black et al., 1985) was used to analyze agonist-induced phosphoinositide hydrolysis to obtain a relative estimate of the plasma membrane expression of mutant M₁ receptors. The following equation was fitted to the concentration-response curves using nonlinear regression analysis:

\[ y = \frac{X^n M_{sys}}{X^n + \left(\frac{X + K_{obs}}{\tau}\right)^n} \]  

In this equation, \( y \) denotes the response, \( X \) denotes the concentration of agonist, \( M_{sys} \) represents the maximum response of the system, \( n \) represents the transducer slope factor, and \( K_{obs} \) denotes the observed dissociation constant of the agonist. The parameter \( \tau \) is proportional to receptor expression (R_T) and the intrinsic efficacy of the agonist (\( \varepsilon \)) and inversely proportional to the sensitivity of the signaling cascade (K_E):

\[ \tau = \frac{\varepsilon R_T}{K_E} \]  

The data for agonist-stimulated phosphoinositide hydrolysis by wild type and mutant M_{1AA430-431} receptors were fitted simultaneously to equation 1 sharing the estimates of \( M_{sys} \), \( K_{obs} \) and \( n \)
between the curves and obtaining unique estimates of $\tau$ (GraphPad Prism, ver. 4.03; La Jolla, CA) for each receptor. If the intrinsic efficacy of the agonist is the same at both wild type and mutant receptors, then the estimate of $\tau$ for the mutant receptor ($\tau_{\text{mut}}$), expressed relative to that of the wild type receptor ($\tau_{\text{WT}}$) is equivalent to the corresponding ratio of receptor densities on the plasma membrane:

$$\frac{\tau_{\text{mut}}}{\tau_{\text{WT}}} = \frac{eR_{T-mut}}{K_E} = \frac{eR_{T-WT}}{K_E} = \frac{R_{T-mut}}{R_{T-WT}}$$ (3)

The significance of differences between sets of data were calculated using either Student’s t-tests (two-tailed) or an one-way ANOVA with Dunnett’s post-hoc test (GraphPad Prism).
Results

Epifluorescence microscopy: Since the F(x)_6LL motif is known to be involved in the ER export of GPCRs (Duvernay et al., 2009; Duvernay et al., 2005; Duvernay et al., 2004), we examined how mutagenesis of the motif affected the cellular distribution of the hM1 receptor relative to the ER marker DsRed-ER using epifluorescence microscopy. As shown in Figure 1A, GFP-tagged wild-type hM1 receptors did not co-localize with DsRed-ER and exhibited a distribution not inconsistent with expression on the plasma membrane. In contrast, GFP-tagged receptors containing two L to A point mutations in the adjacent, C-terminal leucines of the F(x)_6LL motif (GFP-tagged hM1AA430-431) colocalized with DsRed-ER (Figure 1C). Treatment with atropine (0.1 µM) for 18 hours had little effect on the distribution of the wild-type receptor, but converted the distribution of the hM1AA430-431 mutant into that of the wild-type receptor (Figure 1B and 1D).

Receptors for amine neurotransmitters within the rhodopsin class contain a highly conserved aspartic acid in TM3 (D105 in the M1 sequence) that is thought to form a counter ion for the amine moiety of the neurotransmitter (Page et al., 1995; Spalding et al., 1994). The D105N point mutant of the hM1 receptor has greatly reduced affinity for acetylcholine, [3H]NMS and other orthosteric ligands (Page et al., 1995). We examined how introducing the D105N mutation into the GFP-tagged hM1AA430-431 mutant (GFP-tagged hM1N105AA430-431) affected the ability of atropine to rescue receptor expression on the plasma membrane. The D105N mutation of the wild-type receptor (GFP-tagged hM1N105) had no detectable effect on cellular localization in the absence or presence of atropine (Figure 2A and 2B). While the GFP-tagged hM1N105AA430-431 mutant had a distribution similar to that of hM1AA430-431, its distribution was unaffected by 18-hour atropine (0.1 µM) treatment (Figure 2C and 2D). Our results show that the hM1AA430-431 mutant is probably trapped in the ER and that the binding of atropine to the orthosteric binding site can rescue its expression on the plasma membrane.

In the vasopressin V2 receptor, the F(x)_6LL motif exists in an amphipathic α-helix referred to as helix 8 (Thielen et al., 2005).
and L\textsuperscript{340}) of V2 receptors may interact with residues at the base of TM1 (Thielen et al., 2005). We were interested in determining, therefore, whether hM\textsubscript{1} receptors containing mutations at the base of TM1 (hM\textsubscript{1AA46-47}) associate with the ER in a manner similar to that of hM\textsubscript{1AA430-431}.

Figure 2 shows that the GFP-tagged hM\textsubscript{1AA46-47} mutant is distributed within the ER and that incubation with atropine (0.1 µM) for 18 hours rescues its expression on the plasma membrane (Figure 2E and 2F). This effect of atropine is abolished in the corresponding D105N mutant (GFP-tagged hM\textsubscript{1N105AA46-47}) (Figure 2G and 2H).

[\textsuperscript{3}H]NMS binding: To quantify receptor expression on the plasma membrane, we measured the binding of [\textsuperscript{3}H]NMS to intact CHO cells. [\textsuperscript{3}H]NMS is a quaternary ammonium muscarinic antagonist that does not penetrate the plasma membrane. Differences in [\textsuperscript{3}H]NMS binding across receptor mutants, therefore, should reflect differences in their expression on the plasma membrane.

Figure 3 shows the specific binding of [\textsuperscript{3}H]NMS to intact CHO cells expressing the wild-type hM\textsubscript{1} receptor (panel A) and receptors containing two point mutations at the base of TM1 (hM\textsubscript{1AA46-47}) (panel B), two point mutations in the F(x)\textsubscript{6}LL motif (hM\textsubscript{1AA430-431}) (panel C), and single point mutations in both the F(x)\textsubscript{6}LL motif and the base of TM1 (hM\textsubscript{1A46,A431}) (panel D). The negative log dissociation constant of [\textsuperscript{3}H]NMS (pK\textsubscript{D}) varied modestly across the various receptors (9.2 – 9.6). In contrast, the binding capacities of the hM\textsubscript{1AA430-431} (162 ± 13 fmol/mg protein), hM\textsubscript{1AA46-47} (38 ± 2 fmol/mg protein) and hM\textsubscript{1A46,A431} (153 ± 7 fmol/mg protein) receptor mutants were greatly reduced relative to wild-type (1700 ± 20 fmol/mg protein). Atropine treatment (0.1 µM, 18 hour) caused a modest increase (1.3-fold) in the binding capacity of the wild-type receptor, but much greater increases (23-, 7.8- and 9.8-fold, respectively) in those of the receptor mutants. These data are summarized in Table 1. Our results show that alanine mutagenesis of the terminal leucines of the F(x)\textsubscript{6}LL motif or of residues V\textsuperscript{46} and L\textsuperscript{47} at the base of TM\textsubscript{1} or a combination of both causes a marked reduction in the expression of the hM\textsubscript{1} receptor at the plasma membrane and that expression of the mutants can be rescued by atropine treatment.
Since our binding experiments showed that the receptor mutants exhibited an altered binding capacity compared to wild type with little change in the dissociation constant for [3H]NMS, subsequent experiments were carried out using a single concentration of [3H]NMS to monitor changes in receptor expression on the cell membrane.

Characterization of the effect of atropine on the plasma membrane expression of hM1AA430-431:

To determine the potency of atropine for rescuing [3H]NMS binding to hM1AA430-431, we incubated CHO cells expressing hM1AA430-431 with various concentrations of atropine for 18 hours and then measured [3H]NMS binding (Figure 4A). Atropine caused a concentration-dependent increase in binding. The maximal effect was an 8.6-fold increase in binding, and the half-maximal effect occurred at a negative log atropine concentration (pEC50) of 7.97 ± 0.11.

Twenty-four hours after transfection, CHO cells transiently expressing the hM1AA430-431 receptor were incubated with atropine (0.1 µM) for various times up to seven hours and then assayed for [3H]NMS binding (Figure 4B). Binding increased 3.3-fold after seven-hour treatment with atropine. The initial rate of delivery of the hM1AA430-431 receptor to the plasma membrane was estimated to be 115 ± 6 fmol/mg protein h⁻¹ from the slope of the plot of binding vs. time. In contrast, atropine (0.1 µM) only caused a 1.1-fold increase in binding to the wild type receptor over the seven-hour time period (data not shown).

We also measured the decay of binding to the hM1AA430-431 receptor following removal of atropine (0.1 µM) after an 18-hour incubation with it (Figure 4C). Binding appeared stable for 1.5 hours (709 ± 57 fmol/mg protein), but then gradually declined 36% eight hours after removal of atropine (455 ± 28 fmol/mg protein). In CHO cells expressing the wild-type hM1 receptor, [3H]NMS binding was not significantly different from control (932 ± 130 fmol/mg protein) eight hours after atropine washout (878 ± 126 fmol/mg protein). We fitted an exponential decay equation to the data in Figure 4C assuming that the equilibrium level (i.e., plateau level) of receptor expression is equivalent to that measured in Figure 3B (i.e., 162 ± 13 fmol/mg protein). The estimate of the rate constant for loss of hM1AA430-431 was 0.09 ± 0.0002 h⁻¹.
To investigate whether atropine is binding to receptors inside the cell (presumably in the ER) to rescue expression on the plasma membrane, we compared the effects of atropine with those of two close structural analogs, scopolamine and NMS. Like atropine, scopolamine readily penetrates the plasma membrane, whereas its N-methyl derivative (NMS) does not. Following a seven-hour incubation with scopolamine (0.5 µM), [3H]NMS binding to hM1AA46-47, hM1AA430-431 and hM1AA46,A431 increased 4.7- to 14.4-fold relative to untreated controls (Figure 5 and Table 2). In cells treated with NMS for seven hours, [3H]NMS binding also increased, but to a much lesser extent than that observed with scopolamine (Figure 5 and Table 2). The binding of [3H]NMS to the wild-type hM1 receptor was unaffected by a prior seven-hour incubation with either scopolamine or NMS (Table 2).

[3H]QNB binding to receptor mutants: We also used [3H]QNB to measure the amount of wild-type and mutant receptor expressed in intact CHO cells (Figure 6). [3H]QNB is a membrane permeable muscarinic antagonist that should penetrate to intracellular compartments including the ER, Golgi and endosomes. When used at a nearly receptor-saturating concentration of 1.6 nM, [3H]QNB labeled 2.48 ± 0.08 pmol/mg protein of wild-type hM1 receptors. This value was similar to the binding capacity of [3H]NMS for the wild-type receptor in intact CHO cells suggesting that the majority of receptors is expressed on the plasma membrane (see Table 1). Atropine treatment (0.1 µM, 18 h) caused a small 1.1-fold increase in [3H]QNB binding to the wild-type receptor (Figure 6). The binding estimates of [3H]QNB to the hM1AA46-47, hM1AA430-431 and hM1AA46,A431 receptor mutants were only 4.6, 11.8 and 9.8% of wild-type, respectively (Figure 6). Treatment with atropine (0.1 µM) for 18 h caused 12.4-, 5.7- and 6.6-fold increases, respectively in [3H]QNB binding to the receptor mutants (Figure 6). Our results with [3H]QNB are similar to those with [3H]NMS and suggest, therefore, that at a concentration of 1.6 nM, [3H]QNB binds to few misfolded receptor mutants in the ER or that the latter are degraded or a combination of both.
Further characterization of the F(x)_6LL motif using mutagenesis: The M1 F(x)_6LL motif (423FRDTRFLLL431) also conforms to the hydrophobic folding motif h(x)_3h(x)_2hh (423FRDTRFLLL431) described by Krause and coworkers (2000). To explore the role of this hM1 motif further, we made point mutations in the F and L residues of the consensus sequences and determined the consequences on receptor expression at the plasma membrane. Figure 7A shows the binding of [³H]NMS to intact CHO cells transiently expressing wild-type hM1, hM1AA430-431, hM1VV430-431, and receptors containing single point mutations within the F(x)_6LL motif. The [³H]NMS binding values for the single point mutants (F423A, F427A, L430A and L431A) were 52.2, 61.8, 26.2 and 50.9% that of wild type, respectively (Table 3). As described above, [³H]NMS binding to hM1AA430-431 was greatly reduced relative to wild type (8.2%), whereas mutation of the same terminal leucines to hydrophobic valines (hM1VV430-431) partially restored binding to 65.9% of wild type (Figure 7A and Table 3). Atropine treatment (0.1 µM, 18 h) caused a 1.2-fold increase in [³H]NMS binding to the wild-type hM1 receptor and a large rescue of binding to the other mutants (Figure 7A and Table 3).

We also further investigated the plasma membrane expression of receptors containing mutations in residues at the base of TM1 (Figure 7B). The specific binding values of [³H]NMS in intact CHO cells expressing the point mutants L44A, L45A, V46A, L47A and I48A were 81.5, 61.7, 43.3, 43.1 and 77.8% of wild type, respectively (Figure 7B and Table 3). Binding to the double mutant, hM1AA46-47, was greatly reduced relative to wild type (2.1%) as described above (Figure 3C). Atropine treatment (0.1 µM, 18 h) restored binding to all of the mutants (Figure 7B and Table 3).

In the homology model of the V2 vasopressin receptor, the terminal leucines (430LL431) of the F(x)_6LL motif may interact with adjacent hydrophobic residues at the base of TM1 (Thielen et al., 2005). This interaction is thought to be necessary for the transport competent folding of the receptor. Disruption of this interaction in the hM1AA46-47 or hM1AA430-431 mutants could explain the large loss of atropine-rescuable receptor expression on the plasma membrane. We investigated, therefore, whether the trans-mutations (hM1A46,A431 and hM1A47,A430) caused
atropine-restorable deficits of receptor expression on the plasma membrane comparable to those of the cis-mutations (hM1AA46-47 and hM1AA430-431). As shown in Figure 7C (see also Figure 3D for hM1A46,A431), the [3H]NMS binding values for hM1A46,A431 and hM1A47,A430 were greatly reduced relative to wild type (7.1 and 1.2%, respectively) and comparable to those of hM1AA46-47 (2.1%) and hM1AA430-431 (8.2%). In contrast, specific binding to the double mutant hM1A48,A431 was similar to that of the single mutant hM1A431, suggesting that 48I is not involved in an interaction with the F(x)6LL motif and that the interaction is specific to 46VL47 at the base of TM1. Following atropine treatment (0.1 µM, 18 h), [3H]NMS binding to hM1A46,A431, hM1A47,A430 and hM1A48,A431 was greatly restored (Figure 7C).

**Agonist-stimulated phosphoinositide hydrolysis:** We measured carbachol-stimulated phosphoinositide hydrolysis in CHO cells expressing hM1AA430-431 to determine if the loss of function was similar to the loss of the receptor expression on the plasma membrane. Figure 8 shows carbachol-stimulated phosphoinositide hydrolysis in CHO cells expressing the wild-type hM1 receptor and hM1AA430-431. The pEC50 and Emax values of carbachol were both less for the hM1AA430-431 mutant (4.70 ± 0.06 and 6.9-fold above basal, respectively) compared to wild type (5.62 ± 0.11 and 8.6-fold above basal, respectively) (Table 4). We fitted the operational model (Black et al., 1985) to the data sharing the estimate of the dissociation constant of carbachol between the curves and estimating values of tau for each curve. This parameter is proportional to the density of functional receptors. The estimate of tau ± SEM for the data obtained with the hM1AA430-431 mutant was 10.2 ± 1.2% of wild type, which agrees with the Bmax value of [3H]NMS in this mutant relative to wild type (8.2%). Atropine treatment (0.1 µM, 18 h), partially prevented the loss in function in the hM1AA430-431 mutant relative to wild type (one third the potency of wild type, no change in Emax). Analysis with the operational model gave an estimate of tau that was 43% of wild type, which is similar to the estimate of the Bmax of [3H]NMS relative to wild type (63%). The estimate of the negative log dissociation constant of carbachol was 4.07 ± 0.29.
The was no difference in basal $[^3\text{H}]$inositolphosphate accumulation between CHO cells expressing wild-type hM$_1$ and hM$_{1AA430-431}$ receptors ($3041 \pm 154$ dpm and $2573 \pm 31$ dpm, respectively), indicating no evidence of altered constitutive activity of the mutant relative to wild type. Similar results were observed in atropine treated cells (0.1 $\mu$M, 18 h) ($3386 \pm 134$ dpm and $2733 \pm 112$ dpm, respectively). Also, there was no difference between the labeling of $[^3\text{H}]$phosphoinositides with $[^3\text{H}]$inositol in CHO cells expressing wild-type (49,623 ± 4,061 dpm) and hM$_{1AA430-431}$ receptors (48,316 ± 3,441 dpm). Atropine treatment (0.1 $\mu$M, 18 h) did not effect labeling (48,761 ± 3,474 dpm and 48,442 ± 2,996 dpm, respectively) either. Additionally, $[^3\text{H}]$inositolphosphate accumulation elicited to a maximally effective concentration of carbachol (1 mM) by wild-type hM$_1$ receptors in untreated cells (9.5 ± 1.3-fold over basal) was not significantly different from that obtained in atropine treated cells (0.1 mM, 18 h) (8.9 ± 1.5-fold over basal).
Discussion

The effect of atropine on the expression of hM_{1AA430-431} and the other mutants is caused by its binding to the orthosteric site of the receptor because its EC_{50} value for affecting expression exhibits high potency (i.e., 10 nM) and is within the range of that expected for a specific muscarinic effect. In addition, atropine’s effect was prevented by the mutation D105N in the receptor, which is known to disrupt the binding of muscarinic agonists and antagonists. It might be expected that the rescuing effect of atropine on receptor expression should be proportional to receptor occupancy by atropine, in which case the EC_{50} value for rescuing expression should be similar to the dissociation constant of atropine for hM_{1AA430-431} receptors in the ER. It is known that atropine binds to the wild-type hM_{1} receptor with a dissociation constant of 1 nM, which represents 10-fold higher affinity than the EC_{50} value for rescuing expression of hM_{1AA430-431}. We speculate that in the ER the hM_{1AA430-431} mutant exists primarily in a conformation exhibiting low affinity for atropine and that atropine selects for the high-affinity wild type conformation that is required for ER export.

It is known that high affinity ligands often stabilize their respective binding proteins and inhibit their degradation. If it is assumed that hM_{1AA430-431} and the other mutants are rapidly degraded within the ER and that atropine inhibits this degradation, then this mechanism could explain the atropine-induced increase in receptor expression on the plasma membrane. However, upon atropine treatment, we found that hM_{1AA430-431} receptors appeared on the plasma membrane at an initial rate of 115 ± 6 fmol/mg protein h^{-1} (see Figure 4). Dividing this rate by the maximal receptor expression after 18 hours of atropine treatment yields a rate constant of 0.12 h^{-1}. Using RPD^{TM} Regulated Secretion/Aggregation technology at low hM_{1} receptor expression, we previously estimated an initial rate of delivery of the wild-type hM_{1} receptor to the plasma membrane of 18 fmol/mg protein h^{-1} (Sawyer et al., 2006). When normalized relative to receptor expression at 18 hr, the rate constant for ER export of the wild-type receptor (0.10 h^{-1}) is approximately the same as that of hM_{1AA430-431}. If the hM_{1AA430-431} receptor were rapidly...
degraded in the ER, then its initial atropine-induced rate of expression should be much less than the rate of delivery of the wild-type receptor to the plasma membrane. The similarity in the rates suggests that atropine acts by binding to a substantial pool of hM1AA430-431 and enabling ER export and not by preventing receptor degradation.

It might also be argued that atropine stabilizes hM1AA430-431 on the plasma membrane and inhibits its endocytosis and ultimate degradation, leading to an increase in expression on the plasma membrane. We found, however, that the rate constant for the internalization of the hM1AA430-431 receptor after atropine washout (0.09 h⁻¹, Figure 4), was similar to that (0.07 h⁻¹) estimated for the wild-type hM1 receptor using RPD™ Regulated Secretion/Aggregation technology (Sawyer et al., 2006). The similarity between the two estimates shows that the atropine-rescued hM1AA430-431 receptor exhibits stability comparable to that of the wild-type hM1 receptor and that the effect of atropine cannot be attributed to prevention of receptor internalization and degradation. The hM1AA430-431 mutant behaves differently from M2 and M3 muscarinic receptors bearing a N6.58Y mutation that causes internalization and constitutive activation in an atropine-reversible manner (Nelson et al., 2006; Dowling et al., 2006).

Our results showed that alanine mutagenesis of single residues in the consensus sequence (h(x)₂h(x)₂hh) within the C terminus of hM1 caused a moderate reduction in plasma membrane expression (about 50%), whereas the double point mutations cause a substantial decline (>90%) in expression. The additive effect of the point mutations suggests that the motif consists of a concatenation of residues, each contributing to the stabilization of the transport-competent conformation of the receptor.

We also found that alanine mutagenesis of critical residues at the base of TM1 had consequences on receptor expression analogous to those of mutations within the F(x)₆LL motif. In the β2 adrenoceptor structure (Cherezov et al., 2007; Duvernay et al., 2009; Rosenbaum et al., 2007) and the homology model of Thielen and coworkers (2005), the F(x)₆LL motif forms an alpha helical structure that interacts with residues at the base of TM1. It is possible that the proper folding of the hM1 receptor requires the interaction of the F(x)₆LL motif with critical
hydrophobic residues at the base of TM1. Our results suggest that alanine mutagenesis of either locus disrupts this interaction and prevents the transport-competent folding of the receptor. We cannot rule out the possibility, however, that the two motifs act independently to promote receptor folding.

We are not the only group to observe a ligand-induced rescue in the plasma membrane expression of a GPCR with a folding mutation. Thielen and coworkers (2005), showed that the membrane-permeable vasopressin V2 receptor antagonist SR121463 rescues the plasma membrane expression of a V2 receptor mutant possessing mutations similar to that of hM1AA430-431 receptors (i.e., V2 receptor, L339T/L340T). They postulated, based on the observations of Wuller and coworkers (2004), that the SR121463 rescues the plasma membrane expression of the mutant V2 receptor by binding to it, causing the mutant receptor to fold adequately to exit the ER (Thielen et al., 2005). In addition, alanine mutagenesis of some highly conserved residues in TM domains 2, 3, 4, 6 and 7 of the hM1 receptor greatly reduces receptor expression, and treatment with atropine (1 µM) for 24 h has been shown to rescue expression (Hulme et al., 2003). These residues are thought to be involved in intermolecular interactions that stabilize the receptor.

Our binding experiments with the membrane permeable muscarinic antagonist radioligand [3H]QNB (1.6 nM) showed little binding to the hM1AA430-431 mutant after an 18-hour incubation at a temperature (4°C) that prevents receptor trafficking. A simple explanation is that at 4°C [3H]QNB occupies few misfolded receptors at a concentration of 1.6 nM because of its low affinity for the misfolded receptor and possibly because isomerization of the unfolded receptor to the folded state may be inhibited at this low temperature. It is unlikely that the hM1AA430-431 mutant is rapidly degraded in the ER because of the substantial fluorescence of the fusion protein associated with the ER. In addition, the substantial rate of delivery of the fusion protein after atropine treatment implies a large preexisting pool of rescuable receptor in the ER as described above.
Once the hM$_{1AA430-431}$ mutant reaches the plasma membrane, it appears to function as well as the wild-type receptor in terms of agonist stimulation of phosphoinositide hydrolysis. Our analysis of the concentration-response curves of carbachol for eliciting phosphoinositide hydrolysis in CHO cells transfected with the wild-type receptor or hM$_{1AA430-431}$ showed that the data could be attributed entirely to changes in the $\tau$ parameter of the operational model. The value of this parameter is proportional to receptor expression and intrinsic efficacy and inversely proportional to the sensitivity of the signaling cascade (equation 2). Since the estimate of $\tau$ for the hM$_{1AA430-431}$ mutant expressed relative to that of the wild type receptor was approximately the same as the corresponding relative $B_{\text{max}}$ values for $[^3\text{H}]$NMS, our data show that the loss of function displayed by the mutant can be attributed to decreased expression on the plasma membrane and not to a loss in affinity or efficacy.

The stability of hM$_{1AA430-431}$ on the plasma membrane suggests that it might be a useful construct for investigating receptor trafficking. In cells expressing the mutant, a pulse of properly folded receptor can be synchronously released from the ER with a period of atropine treatment. The fate of these receptors can be followed using fluorescence microscopy (with a GFP-tagged receptor) or $[^3\text{H}]$NMS binding (plasma membrane expression) to investigate receptor trafficking (i.e., plasma membrane delivery, internalization, recycling and degradation) without interference from constitutively expressed receptor. Similar approaches have been described using thermo-reversible folding mutants (Hirschberg et al., 1998) and RPD$^\text{TM}$ Regulated Secretion/Aggregation technology (Sawyer et al., 2006).

Naturally occurring mutations in certain GPCRs are implicated in causing the ER retention of these receptors and, thus, causing human disease (Duvernay et al., 2005). The effect of atropine on the expression of hM$_{1AA430-431}$ receptors suggests that membrane-permeable ligands may be useful in rescuing the plasma membrane expression of certain misfolded GPCRs that are retained in the ER. Our results with the hM$_{1AA430-431}$ mutant show that atropine treatment rescues expression and that the rescued receptor has a stability and functional activity equivalent to the wild-type receptor. The identification of novel membrane-permeable muscarinic receptor
ligands that stabilize the receptor by binding to allosteric sites that do not interfere with orthosteric ligand signaling may represent a useful therapeutic strategy for rescuing misfolded mutants.
References


Footnotes

a) This work was supported by an Oklahoma Health Research award [project number HR03-1072] from the Oklahoma Center for the Advancement of Science and Technology, an intramural grant [CHS-0803] from Oklahoma State University, Center for Health Sciences, and a grant from National Institute of Neurological Disorders and Stroke [1R15NS057742].

b) Parts of this work were presented in an abstract entitled “A small domain in the C-terminal tail of muscarinic M₁ and M₄ receptors is necessary for expression on the plasma membrane” at Neuroscience, San Diego, CA., 2007.

c) Gregory W. Sawyer, Oklahoma State University, Center for Health Sciences, Department of Biochemistry and Microbiology, 1111 W. 17th Street, Tulsa, OK 74107-1898.
Legends for Figures

Figure 1. The effect of atropine on the cellular localization of wild-type hM1 and hM1AA430-431 receptors. CHO cells were transiently co-transfected with pEGFP-C2-hM1 (A and B) or pEGFP-C2-hM1AA430-431 (C and D) and DsRed-ER. Six h post-transfection, cells were washed and incubated for 18 h in the absence (A and C) or presence of atropine (0.1 μM) (B and D). GFP and DsRed fluorescence was captured using an epi-fluorescence microscope and photomicrographs were colored green (GFP) or red (DsRed). Yellow in overlayed photomicrographs indicates colocalization. Data shown is representative of multiple cells from at least four experiments. Original magnification = 60x; scale bar = 10 μM.

Figure 2. Effect of a D105N point mutation on the cellular localization of wild-type and mutant hM1 receptors. CHO cells were transiently co-transfected with pEGFP-C2-hM1N105 (A and B), pEGFP-C2-hM1N105,AA430-431 (C and D), pEGFP-C2-hM1AA46-47 (E and F), or pEGFP-C2-hM1N105,AA46-47 (H and G) and DsRed-ER. Six h post-transfection, cells were washed and incubated for 18 h in the absence (A, C, E, and H) or presence of atropine (0.1 μM) (B, D, F, and G). GFP and DsRed fluorescence was captured using an epi-fluorescence microscope and photomicrographs were colored green (GFP) or red (DsRed). Yellow in overlayed photomicrographs indicates colocalization. Data shown is representative of multiple cells from at least four experiments. Original magnification = 60x; scale bar = 10 μM.

Figure 3. [3H]NMS binding to cells transiently expression wild-type and mutant hM1 receptors. CHO cells transiently expressing wild-type or mutant hM1 receptors were incubated for 18 h in the absence (closed circles) or presence (open circles) of atropine (0.1 μM) beginning six h after transfection. Cells were washed extensively and incubated with equally spaced concentrations (0.33 log-unit) of [3H]NMS for 24 h at 4°C. Binding data for wild-type hM1 (A), hM1AA46-47 (B),
hM1AA430-431 (C), and hM1A46,A431 (D) receptors are shown. Each data point represents the mean ± S.E.M. of two experiments performed in triplicate.

Figure 4. Effect of atropine and time on the plasma membrane expression of hM1AA430-431 receptors. A, CHO cells transiently expressing hM1AA430-431 receptors were incubated with equally spaced concentrations (0.5-log unit) of atropine for 18 h beginning 6 h after transfection. Cells were washed extensively to remove atropine and used in intact, whole cell [3H]NMS binding assays. B, CHO cells were transiently transfected with hM1AA430-431 receptors and incubated for 24 h. Cells were then incubated with atropine (0.1 μM) for various periods of time for up to 7 h, washed extensively to remove atropine, and used in intact, whole cell [3H]NMS binding assays. C, CHO cells transiently expressing hM1AA430-431 receptors were incubated for 18 h with atropine (0.1 μM), 6 after transfection. Cells were washed extensively to remove atropine and then incubated for various periods of time for up to 8 h. After incubation, cells were used in intact, whole cell [3H]NMS binding assays. Each data point represents the mean ± S.E.M. of three experiments performed in triplicate.

Figure 5. The effect of NMS and scopolamine on the plasma membrane expression of wild-type and mutant hM1 receptors in CHO cells. CHO cells were transiently transfected with the indicated receptor constructs and incubated for 24 h. Cells were then washed and incubated in the absence (open bars) or presence of NMS (0.2 μM) (checked bars) or scopolamine (0.2 μM) (closed bars) for seven h. Cells were washed extensively and used in intact, whole cell [3H]NMS binding assays. Each bar represents the mean ± SEM of two experiments performed in triplicate.

Figure 6. The effect of atropine treatment on intact, whole cell [3H]QNB binding in CHO cells transiently expressing wild-type and mutant hM1 receptors. CHO cells transiently expressing the indicated receptor constructs were incubated for 18 h in the absence (open bars) or presence (closed bars) of atropine (0.1 μM) beginning six h after transfection. Cells were washed
extensively and used in intact, whole cell $[^3]$H]QNB binding assays. Each bar represents the mean ± S.E.M. of three experiments performed in triplicate. $P$ values were calculated for receptor plasma membrane expression in untreated versus atropine treated cells using a paired Student’s t-test (two-tailed). ns = not significant, * $P < 0.001$.

Figure 7. The plasma membrane expression of wild-type hM$_1$ and mutant hM$_1$ receptors following an incubation in the absence and presence of atropine (0.1 μM). A, the plasma membrane expression of wild-type and hM$_1$ receptors possessing mutations in the F(X)$_6$LL motif. CHO cells were transiently transfected with the indicated receptor constructs and six h after transfection, cells were incubated for 18 h in the absence (open bars) and presence (closed bars) of atropine (0.1 μM). After washing to remove atropine, intact, whole cell $[^3]$H]NMS binding assays were performed. B, the plasma membrane expression of hM$_1$ receptors possessing mutations at the base of TM1. CHO cells were transiently transfected with the indicated receptor constructs. Six h after transfection, cells were incubated in the absence (open bars) and presence (closed bars) of atropine (0.1 μM) for 18 h. Afterwards, intact, whole cell $[^3]$H]NMS binding assays were performed. C, the plasma membrane expression of hM$_1$ receptors possessing mutations at the base of TM1 and the F(X)$_6$LL motif. CHO were transiently transfected with the indicated receptor constructs and incubated for six h. Cells were then incubated an additional 18 h in the absence (open bars) or presence (closed bars) of atropine (0.1 μM). Intact, whole cell $[^3]$H]NMS binding assays were then performed. Each bar represents the mean ± the S.E.M. of three to four experiments performed in triplicate. $P$ values were calculated for receptor plasma membrane expression in untreated versus atropine treated cells using a paired Student’s t-test (two-tailed). * $P \leq 0.01$; ** $P \leq 0.001$.

Figure 8. Carbachol-mediated phosphoinositide hydrolysis in CHO cells expressing wild-type hM$_1$ or hM$_{1AA430-431}$ receptors. CHO cells transiently expressing wild-type hM$_1$ (■) or hM$_{1AA430-431}$ (●, ○) receptors were incubated with $[^3]$H]myo-inositol for 18 h in the absence (■, ●) or
presence (O) of atropine (0.1 μM). Cells were washed extensively to remove unincorporated 
[^3]H]inositol (and atropine in treated cells) and then used to measure carbachol-mediated 
phosphoinositide hydrolysis. Each data point represents the mean ± S.E.M. of three experiments 
conducted in triplicate.
Tables

TABLE 1

Effect of atropine-treatment on [³H]NMS binding in CHO cells expressing wild-type hM₁, hM₁AA46-47, hM₁AA430-431 and hM₁A46,A431 receptors.

<table>
<thead>
<tr>
<th>Receptor Construct&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pK&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (fmol/mg protein)</th>
<th>Hill Slope&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Wild-type hM₁</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (2)</td>
<td>9.21 ± 0.02</td>
<td>1700 ± 20</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Atropine-treated (2)</td>
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<td>2239 ± 27</td>
<td>1.5 ± 0.1</td>
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<tr>
<td>hM₁AA46-47</td>
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<tr>
<td>Untreated (2)</td>
<td>9.62 ± 0.06</td>
<td>38 ± 2</td>
<td>1.6 ± 0.4</td>
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<tr>
<td>Atropine-treated (2)</td>
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<td>1178 ± 23</td>
<td>1.4 ± 0.1</td>
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<tr>
<td>hM₁AA430-431</td>
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<td>Untreated (2)</td>
<td>9.37 ± 0.19</td>
<td>162 ± 13</td>
<td>0.8 ± 0.3</td>
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<td>Atropine-treated (2)</td>
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<td>Untreated (2)</td>
<td>9.62 ± 0.18</td>
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<td>Atropine-treated (2)</td>
<td>9.20 ± 0.02</td>
<td>1504 ± 20</td>
<td>1.4 ± 0.1</td>
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</tbody>
</table>

<sup>a</sup> The number of experiments conducted is denoted in parentheses.

<sup>b</sup> Estimates for the pK<sub>D</sub>, B<sub>max</sub>, and Hill slope were calculated from the data shown in Figure 3.
TABLE 2

The effect of NMS and scopolamine on the plasma membrane expression of wild-type and mutant hM₁ receptors.

<table>
<thead>
<tr>
<th>Receptor Constructᵃ</th>
<th>Specifically Bound [³H]NMS (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td></td>
<td>Cellsᵇ</td>
</tr>
<tr>
<td>Wild-type hM₁ (2)</td>
<td>1862 ± 116</td>
</tr>
<tr>
<td>hM₁AA46-47 (2)</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>hM₁AA430-431 (2)</td>
<td>263 ± 21</td>
</tr>
<tr>
<td>hM₁A46,A431 (2)</td>
<td>197 ± 5</td>
</tr>
</tbody>
</table>

ᵃ The number of experiments is denoted in parentheses. Each experiment was performed in triplicate. Specifically bound [³H]NMS was calculated from the data shown in Figure 5.

ᵇ The mean ± S.E.M. of data obtained from intact, whole cell [³H]NMS binding assays performed on untreated CHO cells transiently expressing the indicated receptor constructs.

ᶜ The mean ± S.E.M. of data obtained from intact, whole cell [³H]NMS binding assays performed on scopolamine treated CHO cells transiently expressing the indicated receptor constructs. *P* values were calculated using an two-way ANOVA with Bonferroni post-hoc test.

ᵈ The mean ± S.E.M. of data obtained from intact, whole cell [³H]NMS binding assays performed on NMS treated CHO cells transiently expressing the indicated receptor constructs. *P* values were calculated using an two-way ANOVA with Bonferroni post-hoc test.

ⁿˢ not significantly different from untreated cells expressing the same receptor construct.

* significantly different from untreated cells expressing the same receptor construct (*P* < 0.001).
TABLE 3

The effects of mutating amino acid residues in the F(x)\textsubscript{6}LL motif and at the base of TM1 on the plasma membrane expression of hM\textsubscript{1} receptors as determined by intact, whole cell [\textsuperscript{3}H]NMS binding.

<table>
<thead>
<tr>
<th>Receptor Construct (^a)</th>
<th>Specifically Bound [\textsuperscript{3}H]NMS in untreated cells(^b) (fmol/mg protein)</th>
<th>Specifically Bound [\textsuperscript{3}H]NMS in atropine treated cells(^c) (fmol/mg protein)</th>
<th>Fold Increase(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type hM\textsubscript{1} (4)</td>
<td>1547 ± 93</td>
<td>1807 ± 99</td>
<td>1.2 ± 0.03</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{44} (3)</td>
<td>1261 ± 35 (^*)</td>
<td>1634 ± 21 (^*)</td>
<td>1.3 ± 0.04</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{45} (3)</td>
<td>954 ± 21 (^***)</td>
<td>1731 ± 73 (^*)</td>
<td>1.8 ± 0.04</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{46} (3)</td>
<td>670 ± 47 (^***)</td>
<td>1642 ± 62 (^*)</td>
<td>2.5 ± 0.09</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{47} (3)</td>
<td>667 ± 28 (^***)</td>
<td>1731 ± 60 (^*)</td>
<td>2.3 ± 0.08</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{48} (3)</td>
<td>1204 ± 28 (^***)</td>
<td>1665 ± 64 (^*)</td>
<td>1.4 ± 0.02</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{46-47} (3)</td>
<td>33 ± 4 (^***)</td>
<td>995 ± 91 (^***)</td>
<td>27.3 ± 0.45</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{423} (4)</td>
<td>808 ± 47 (^***)</td>
<td>1480 ± 111 (^*)</td>
<td>1.8 ± 0.04</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{427} (4)</td>
<td>957 ± 121 (^***)</td>
<td>1259 ± 128 (^*)</td>
<td>1.3 ± 0.04</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{430} (3)</td>
<td>405 ± 87 (^***)</td>
<td>858 ± 183 (^***)</td>
<td>2.1 ± 0.01</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{431} (3)</td>
<td>788 ± 45 (^***)</td>
<td>1319 ± 88 (^*)</td>
<td>1.7 ± 0.03</td>
</tr>
<tr>
<td>hM\textsubscript{1}V\textsubscript{430-431} (3)</td>
<td>1019 ± 56 (^***)</td>
<td>1433 ± 75 (^*)</td>
<td>1.4 ± 0.01</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{430-431} (3)</td>
<td>127 ± 14 (^***)</td>
<td>971 ± 106 (^**)</td>
<td>7.6 ± 0.33</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{46,431} (3)</td>
<td>110 ± 13 (^***)</td>
<td>1107 ± 137 (^***)</td>
<td>10.1 ± 0.19</td>
</tr>
<tr>
<td>hM\textsubscript{1}A\textsubscript{47,430} (3)</td>
<td>19 ± 4 (^***)</td>
<td>678 ± 139 (^***)</td>
<td>36.7 ± 1.9</td>
</tr>
</tbody>
</table>

\(^a\) Specific constructs.
\(^b\) Calculated as fmol/mg protein.
\(^c\) Calculated as fmol/mg protein.
\(^d\) Fold increase in atropine treated cells over untreated cells.
| hM1A48,A431 (3) | 450 ± 24*** | 1140 ± 121*** | 2.5 ± 0.17 |

* The number of experiments is denoted in parentheses. Each experiment was performed in triplicate. Specifically bound \(^{3}H\)NMS and fold-increase in specific \(^{3}H\)NMS binding was calculated from the data shown in Figure 7.

b The mean ± S.E.M. of data obtained from intact, whole cell \(^{3}H\)NMS binding assays performed on untreated CHO cells transiently expressing the indicated receptor constructs. \(P\) values were calculated using an one-way ANOVA with Dunnett’s post-hoc test.

c The mean ± S.E.M. of data obtained from intact, whole cell \(^{3}H\)NMS binding assays performed on atropine-treated CHO cells (0.1 \(\mu\)M, 18 h) transiently expressing the indicated receptor constructs. \(P\) values were calculated using an one-way ANOVA with Dunnett’s post-hoc test.

d The mean ± S.E.M. fold-increase in specifically bound \(^{3}H\)NMS as determined by dividing specific \(^{3}H\)NMS binding observed in atropine-treated cells by that observed in untreated cells for each of the indicated receptor constructs.

* \(P < 0.05\), when compared to wild-type hM1 receptors.

** \(P < 0.01\), when compared to wild-type hM1 receptors.

*** \(P < 0.001\) when compared to wild-type hM1 receptors.

ns, not significantly different from wild-type hM1 receptors.
TABLE 4

Effect of atropine-treatment on carbachol-stimulated phosphoinositide hydrolysis elicited by wild-type hM1 and hM1AA430-431 receptors.

<table>
<thead>
<tr>
<th>Receptor Construct</th>
<th>pEC$_{50}^b$</th>
<th>$E_{\text{max}}^b$</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type hM1 (4)$^c$</td>
<td>5.62 ± 0.11</td>
<td>8.6 ± 0.4</td>
<td>0.96 ± 0.22</td>
</tr>
<tr>
<td>hM1AA430-431</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (3)$^c$</td>
<td>4.70 ± 0.06***</td>
<td>6.9 ± 0.3*</td>
<td>1.05 ± 0.13</td>
</tr>
<tr>
<td>Atropine-treated (3)$^c$</td>
<td>5.16 ± 0.10*</td>
<td>8.7 ± 0.5ns</td>
<td>0.82 ± 0.18</td>
</tr>
</tbody>
</table>

$^a$ The number of experiments conducted is denoted in parentheses.

$^b$ $P$ values were calculated using an one-way ANOVA with Dunnett’s post-hoc test.

$^c$ Estimates for the pEC$_{50}$, $E_{\text{max}}$, and Hill slope were calculated from the data shown in Figure 8.

* $P < 0.05$, when compared to wild-type hM1 receptors.

*** $P < 0.001$, when compared to wild-type hM1 receptors.

ns, not significantly different from wild-type hM1 receptors.
Figure 3
Figure 5


- **Untreated**
- **Scopolamine (0.5 μM)**
- **NMS (0.2 μM)**

Data points for:
- hM1
- hM1AA46-47
- hM1AA450-431
- hM1AA46-A431
Figure 6

Specifically Bound $[^3H]QNB$ (fmol/mg protein)

- Untreated
- Atropine-treated (0.1 μM)

- hM$_1$
- hM$_1$A46-A47
- hM$_1$A430-A431
- hM$_1$A46-A431

* indicates significant difference.
Figure 7
Figure 8