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

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ABSTRACT

We investigated the functional role of a conserved motif, F(x)₆LL, in the membrane proximal C-tail of the human muscarinic M₁ (hM₁) receptor. By use of site-directed mutagenesis, several different point mutations were introduced into the C-tail sequence ⁴²³FRDTFRLL⁴³¹. Wild-type and mutant hM₁ receptors were transiently expressed in Chinese hamster ovary cells, and the amount of plasma membrane-expressed receptor was determined by use of intact, whole-cell [³H]N-methylscopolamine binding assays. The plasma membrane expression of hM₁ receptors possessing either L430A or L431A or both point mutations was significantly reduced compared with the wild type. The hM₁ receptor possessing a L430A/L431A double-point mutation was retained in the endoplasmic reticulum (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 L⁴³⁰ and L⁴³¹ play a role in folding hM₁ 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), V⁴⁶ and L⁴⁷, that, when mutated, reduce the plasma membrane expression of hM₁ 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 hM₁ receptors to achieve a transport-competent state.

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 α -helical structure adjacent to the plasma membrane in the β_2 -adrenoceptor structure. This motif is necessary for the ER export of 5-HT_{1A}, 5-HT_{1B}, α_{1B} - and α_{2B} -adrenergic, angiotensin II type IA, and β_2 -adrenergic receptors (Duvernay et al., 2004, 2009; Carrel et al., 2006). Mutation of either the F or L residues in the motif

prevented the plasma membrane expression of these receptors by causing their ER retention (Duvernay et al., 2004, 2009; Carrel et al., 2006). The spacing between the F and LL residues seems to be critical, because the addition or removal of amino acid residues between them caused the ER retention of the α_{2B} -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 α_{2B} -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., 2004, 2005), or it may be required for the proper folding of some GPCRs (Duvernay et al., 2009).

The F(x)₆LL motifs of α_{1B} - and α_{2B} -adrenergic, β_2 -adrenergic, angiotensin II type IA, 5-HT_{1A}, and 5-HT_{1B} 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

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ABBREVIATIONS: GPCR, G protein-coupled receptor; ER, endoplasmic reticulum; 5-HT, 5-hydroxytryptamine; PCR, polymerase chain reaction; GFP, ANOVA, analysis of variance; GFP, green fluorescent protein; EGFP, enhanced green fluorescence protein; hM₁, human muscarinic M₁ acetylcholine receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; [³H]NMS, [³H]N-methylscopolamine; TM1, transmembrane spanning domain 1; [³H]QNB, [³H]3-quinuclidinyl benzilate; SR121463B, satavapatan.

(³³²VSELRSLL³⁴⁰) (Krause et al., 2000). Mutation of either V³³², I³³⁶, L³³⁹, or I³⁴⁰ 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)₆LL motif. In the M₁ sequence, this motif, ⁴²³FRDTFRLLL⁴³¹, is analogous to the h(x)₃h(x)₂hh (⁴²³FRDTFRLLL⁴³¹) motif described by Krause and coworkers (2000). Using site-directed mutagenesis, we investigated the role of the F(x)₆LL motif in the human M₁ muscarinic receptor (hM₁). We found that mutation of the F and L residues in the motif caused retention of the mutant hM₁ 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)₆LL motif are necessary for the ER export of the M₁ receptor.

Materials and Methods

Receptor Mutagenesis and Constructs. The hM₁ receptor cDNA, cloned into a modified Okayma-Berg expression vector (pCD), was provided by Dr. Tom I. Bonner at the National Institute of Mental Health. Mutant hM₁ receptors were made by introducing point mutations into the hM₁ receptor cDNA of pCD-hM₁ by use of 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_{1AA430-431}, and hM_{1AA46-47} receptor constructs, the sequences encoding wild-type (Bonner et al., 1988), hM_{1AA46-47}, and hM_{1AA430-431} receptors were amplified by use of the 20-cycle PCR reaction and primers with 5' EcoRI (5'-GCAGAGGAATCCAACACTTCAGCCCCAC-3'; forward) and BamHI (5'-GCAGAGGGATCCTCAGCATTGGCGGGAG-3'; reverse) restriction sites. The PCR product was purified and digested with EcoRI and BamHI and ligated into pEGFP-C2 (Clontech, Mountain View, CA) 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 hM₁ receptors as N-terminal fusion proteins to GFP.

Cell Culture and Transient Transfections. CHO cells were subcultured every 2 to 3 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% CO₂/95% air. In preparation for transient transfections, CHO cells were trypsinized and plated in a 24-well plate format at 1.65×10^5 cells per well in 500 μ l of transfection medium (F-12K supplemented with 10% fetal bovine serum). On the next day, cells were transfected by use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the product protocol. In brief, 19.2 μ g of plasmid DNA/plate was incubated in 1200 μ l of Opti-MEM I (Invitrogen) for 5 min at room temper-

ature. In a separate tube, Lipofectamine 2000 (48 μ l/plate) was incubated in 1200 μ l of Opti-MEM I for 5 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 μ l/well) were added to each well of a 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 μ l). 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 $\times$ 500 μ l) with PBS to remove serum and then used in intact, whole cell [³H]NMS or [³H]QNB binding assays as described 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 μ M) 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 μ l) 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 hM_{1AA430-431} receptors. In brief, CHO cells were transiently transfected with wild-type hM₁ or hM_{1AA430-431} receptor constructs as described under *Cell Culture and Transient Transfections*. Cells were washed three times with F-12K (500 μ l) to remove serum and then incubated with atropine (0.1 μ M) for various periods of time for up to 7 h. To remove atropine, CHO cells were washed three times with F-12K (500 μ l), 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, hM_{1AA46-47}, hM_{1AA430-431}, or hM_{1AA46,431} were incubated in the absence and presence of *N*-methylscopolamine (0.2 μ M) or scopolamine (0.5 μ M) 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 hM_{1AA430-431} receptors were stably expressed on the plasma membrane after atropine-treatment, CHO cells were incubated with atropine (0.1 μ M) for 18 h beginning 6 h after transfection with wild-type and hM_{1AA430-431} receptors. Atropine-treated cells were washed three times with F-12K (500 μ l), 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 by use of a single concentration of either [³H]QNB (1.6 nM) or [³H]NMS (1.7 nM), respectively. In brief, 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 (10 μ M) in 500 μ l of 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. After incubation, cells were rapidly and gently washed (2 $\times$ 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 by use of 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 under *Cell Culture and Transient Transfections*. Six hours 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 (nonspecific binding) of atropine (10 μM) for 24 h at 4°C. After 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 by use of 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. In brief, three wells of a 24-well plate were plated and transfected for each receptor construct and assay condition as described 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, 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 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 brief, CHO cells transiently expressing wild-type or M_{1AA430-431} receptors were incubated in a humidified incubator set at 37°C and 5% CO₂/95% air with *myo*-[³H]inositol (0.2 μM; PerkinElmer Life and Analytical Sciences, Waltham, MA) 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]Inositol phosphates were extracted from cells by adding 200 μl of perchloric acid (5%) to each well and incubating the plate for 15 min on ice. The extracts were neutralized by adding 187 μl of 0.525 M 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 3000g 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 × 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 with a 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 *myo*-[³H]inositol (0.2 μM, PerkinElmer Life and Analytical 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.

Epifluorescence Images. CHO cells were plated at 0.25 × 10⁵ 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% CO₂/95% air. On the next day, cells were cotransfected with pEGFP-C2-hM₁ (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_{1A46,A431} and pDsRed2-ER (0.2 μg/well) (Clon-

tech) with use of Lipofectamine 2000 as described under *Cell Culture and Transient Transfections*. Images of CHO cells transiently coexpressing 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 with use of an Olympus IX 71 epifluorescence microscope fitted with fluorescein isothiocyanate (Chroma Technology Corp., Brattleboro, VT) and CY3 filters (Chroma Technology Corp.), and a CoolSNAP Monochrome digital camera (Media Cybernetics, Bethesda, MD). The images captured by use of the fluorescein isothiocyanate or CY3 filters were either green or red, respectively, and overlaid by use of Metamorph 5.0r7 imaging software (Universal Imaging Corp., Downingtown, PA).

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 by use of 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 by use of nonlinear regression analysis:

$$y = \frac{X^n M_{\text{sys}}}{X^n + \left(\frac{X + K_{\text{obs}}}{\tau} \right)^n} \quad (1)$$

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 τ is proportional to receptor expression (R_T) and the intrinsic efficacy of the agonist (ϵ) and inversely proportional to the sensitivity of the signaling cascade (K_E):

$$\tau = \frac{\epsilon R_T}{K_E} \quad (2)$$

The data for agonist-stimulated phosphoinositide hydrolysis by wild-type and mutant M_{1AA430-431} receptors were fitted simultaneously to eq. 1, sharing the estimates of M_{sys} , K_{obs} , and n between the curves and obtaining unique estimates of τ (GraphPad Prism, version 4.03; GraphPad Software Inc., 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 τ for the mutant receptor (τ_{mut}), expressed relative to that of the wild-type receptor (τ_{WT}) is equivalent to the corresponding ratio of receptor densities on the plasma membrane:

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

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

Results

Epifluorescence Microscopy. Because the F(x)₆LL motif is known to be involved in the ER export of GPCRs (Duvornay et al., 2004, 2005, 2009), we examined how mutagenesis of the motif affected the cellular distribution of the hM₁

receptor relative to the ER marker DsRed-ER by use of epifluorescence microscopy. As shown in Fig. 1A, GFP-tagged wild-type hM₁ receptors did not colocalize with DsRed-ER and exhibited a distribution consistent 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 hM_{1AA430–431}) colocalized with DsRed-ER (Fig. 1C). Treatment with atropine (0.1 μ M) for 18 h had little effect on the distribution of the wild-type receptor, but converted the distribution of the hM_{1AA430–431} mutant into that of the wild-type receptor (Fig. 1, B and D).

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 (Spalding et al., 1994; Page et al., 1995). 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 hM_{1AA430–431} mutant (GFP-tagged hM_{1N105AA430–431}) affected the ability of atropine to rescue receptor expression on the plasma membrane. The D105N mutation of the wild-type receptor (GFP-tagged hM_{1N105}) had no detectable effect on cellular localization in the absence or presence of atropine (Fig. 2, A and B). Although the GFP-tagged hM_{1N105AA430–431} mutant had a distribution similar to that of hM_{1AA430–431}, its distribution was unaffected by 18-h atropine (0.1 μ M) treatment (Fig. 2, C and D). Our results show that the hM_{1AA430–431} 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.

Fig. 1. The effect of atropine on the cellular localization of wild-type hM₁ and hM_{1AA430–431} receptors. CHO cells were transiently cotransfected with pEGFP-C2-hM₁ (A and B) or pEGFP-C2-hM_{1AA430–431} (C and D) and DsRed-ER. Six hours after transfection, cells were washed and incubated for 18 h in the absence (A and C) or presence of atropine (0.1 μ M) (B and D). GFP and DsRed fluorescence was captured by use of an epifluorescence microscope, and photomicrographs were colored green (GFP) or red (DsRed). Yellow in overlaid photomicrographs indicates colocalization. Data shown are representative of multiple cells from at least four experiments. Original magnification, 60 $\times$; scale bar, 10 μ M.

Fig. 2. Effect of a D105N point mutation on the cellular localization of wild-type and mutant hM₁ receptors. CHO cells were transiently cotransfected with pEGFP-C2-hM_{1N105} (A and B), pEGFP-C2-hM_{1N105AA430–431} (C and D), pEGFP-C2-hM_{1AA46–47} (E and F), or pEGFP-C2-hM_{1N105AA46–47} (G and H), and DsRed-ER. Six hours after transfection, cells were washed and incubated for 18 h in the absence (A, C, E, and G) or presence of atropine (0.1 μ M) (B, D, F, and H). GFP and DsRed fluorescence was captured by use of an epifluorescence microscope, and photomicrographs were colored green (GFP) or red (DsRed). Yellow in overlaid photomicrographs indicates colocalization. Data shown are representative of multiple cells from at least four experiments. Original magnification, 60 $\times$; scale bar, 10 μ M.

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). The C-terminal leucines of the F(x)₆LL motif (L³³⁹ and L³⁴⁰) of V2 receptors may interact with residues at the base of TM1 (Thielen et al., 2005). We were interested in determining, therefore, whether hM₁ receptors containing mutations at the base of TM1 (hM_{1AA46–47}) associate with the ER in a manner similar to that of hM_{1AA430–431}. Figure 2 shows that the GFP-tagged hM_{1AA46–47} mutant is distributed within the ER and that incubation with atropine (0.1 μ M) for 18 h rescues its expression on the plasma membrane (Fig. 2, E and F). This effect of atropine is abolished in the corresponding D105N mutant (GFP-tagged hM_{1N105AA46–47}) (Fig. 2, G and H).

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

Figure 3 shows the specific binding of [³H]NMS to intact CHO cells expressing the wild-type hM₁ receptor (A) and receptors containing two-point mutations at the base of TM1 (hM_{1AA46-47}) (B), two-point mutations in the F(x)₆LL motif (hM_{1AA430-431}) (C), and single-point mutations in both the F(x)₆LL motif and the base of TM1 (hM_{1AA46,A431}) (D). The negative log dissociation constant of [³H]NMS (pK_D) varied modestly across the various receptors (9.2 to 9.6). In contrast, the binding capacities of the hM_{1AA430-431} (162 ± 13 fmol/mg protein), hM_{1AA46-47} (38 ± 2 fmol/mg protein), and hM_{1AA46,A431} (153 ± 7 fmol/mg protein) receptor mutants were greatly reduced relative to wild type (1700 ± 20 fmol/mg protein). Atropine treatment (0.1 μ M, 18 h) 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)₆LL motif or of residues V⁴⁶ and L⁴⁷ at the base of TM₁ or a combination of both causes a marked reduction in the expression of the hM₁ receptor at the plasma membrane and that expression of the mutants can be rescued by atropine treatment.

Because our binding experiments showed that the receptor mutants exhibited an altered binding capacity compared with wild type with little change in the dissociation constant

TABLE 1

Effect of atropine-treatment on [³H]NMS binding in CHO cells expressing wild-type hM₁, hM_{1AA46-47}, hM_{1AA430-431}, and hM_{1AA46,A431} receptors

Receptor Construct ^a	pK_D ^b	B_{max} ^b	Hill Slope ^b
<i>fmol/mg protein</i>			
Wild-type hM ₁			
Untreated (2)	9.21 ± 0.02	1700 ± 20	1.5 ± 0.1
Atropine-treated (2)	9.06 ± 0.02	2239 ± 27	1.5 ± 0.1
hM _{1AA46-47}			
Untreated (2)	9.62 ± 0.06	38 ± 2	1.6 ± 0.4
Atropine-treated (2)	9.21 ± 0.43	1178 ± 23	1.4 ± 0.1
hM _{1AA430-431}			
Untreated (2)	9.37 ± 0.19	162 ± 13	0.8 ± 0.3
Atropine-treated (2)	9.17 ± 0.03	1294 ± 25	1.4 ± 0.1
hM _{1AA46,A431}			
Untreated (2)	9.62 ± 0.18	153 ± 7	0.9 ± 0.3
Atropine-treated (2)	9.20 ± 0.02	1504 ± 20	1.4 ± 0.1

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

^b Estimates for the pK_D , B_{max} , and Hill slope were calculated from the data shown in Fig. 3.

for [³H]NMS, subsequent experiments were carried out with use of 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 hM_{1AA430-431}. To determine the potency of atropine for rescuing [³H]NMS binding to hM_{1AA430-431}, we incubated CHO cells expressing hM_{1AA430-431} with various concentrations of atropine for 18 h and then measured [³H]NMS binding (Fig. 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 (pEC_{50}) of 7.97 ± 0.11 .

Twenty-four hours after transfection, CHO cells tran-

Fig. 3. [³H]NMS binding to cells transiently expressing wild-type and mutant hM₁ receptors. CHO cells transiently expressing wild-type or mutant hM₁ receptors were incubated for 18 h in the absence (●) or presence (○) of atropine (0.1 μ M) beginning 6 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), hM_{1AA46-47} (B), hM_{1AA430-431} (C), and hM_{1AA46,A431} (D) receptors are shown. Each data point represents the mean $\pm$ S.E.M. of two experiments performed in triplicate.

Fig. 4. Effect of atropine and time on the plasma membrane expression of hM_{1AA430-431} receptors. A, CHO cells transiently expressing hM_{1AA430-431} 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 hM_{1AA430-431} receptors and incubated for 24 h. Cells were then incubated with atropine (0.1 μ M) 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 hM_{1AA430-431} receptors were incubated for 18 h with atropine (0.1 μ M), 6 h 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 $\pm$ S.E.M. of three experiments performed in triplicate.

siently expressing the hM_{1AA430-431} receptor were incubated with atropine (0.1 μ M) for various times up to 7 h and then assayed for [³H]NMS binding (Fig. 4B). Binding increased 3.3-fold after 7 h of treatment with atropine. The initial rate of delivery of the hM_{1AA430-431} receptor to the plasma membrane was estimated to be 115 ± 6 fmol/mg protein per hour from the slope of the plot of binding versus time. In contrast, atropine (0.1 μ M) only caused a 1.1-fold increase in binding to the wild-type receptor over the 7-h period (data not shown).

We also measured the decay of binding to the hM_{1AA430-431} receptor after removal of atropine (0.1 μ M) after an 18-h incubation with it (Fig. 4C). Binding appeared stable for 1.5 h (709 ± 57 fmol/mg protein), but then gradually declined 36% 8 h 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) 8 h after atropine washout (878 ± 126 fmol/mg protein). We fitted an exponential decay equation to the data in Fig. 4C, assuming that the equilibrium level (i.e., plateau level) of receptor expression is equivalent to that measured in Fig. 3B (i.e., 162 ± 13 fmol/mg protein). The estimate of the rate constant for loss of hM_{1AA430-431} 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. After a 7-h incubation with scopolamine (0.5 μ M), [³H]NMS binding to hM_{1AA46-47},

hM_{1AA430-431}, and hM_{1A46,A431} increased 4.7- to 14.4-fold relative to untreated controls (Fig. 5 and Table 2). In cells treated with NMS for 7 h, [³H]NMS binding also increased, but to a much lesser extent than that observed with scopolamine (Fig. 5 and Table 2). The binding of [³H]NMS to the wild-type hM₁ receptor was unaffected by a prior 7-h 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

Fig. 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 ($\square$) or presence of NMS (0.2 μ M) ($\boxtimes$) or scopolamine (0.5 μ M) ($\blacksquare$) for 7 h. Cells were washed extensively and used in intact, whole-cell [³H]NMS binding assays. Each bar represents the mean $\pm$ S.E.M. of two experiments performed in triplicate.

TABLE 2

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

Receptor Construct ^a	Specifically Bound [³ H]NMS		
	Untreated Cells ^b	Scopolamine-Treated Cells (0.5 μM) ^c	NMS-Treated Cells (0.2 μM) ^d
	<i>fmol/mg protein</i>		
Wild-type hM ₁ (2)	1862 ± 116	2179 ± 182 ^{ns}	1804 ± 100 ^{ns}
hM _{1AA46-47} (2)	58 ± 7	842 ± 102 [*]	175 ± 22 ^{ns}
hM _{1AA430-431} (2)	263 ± 21	1240 ± 186 [*]	440 ± 70 ^{ns}
hM _{1A46,A431} (2)	197 ± 5	1182 ± 17 [*]	347 ± 17 ^{ns}

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

^{*}, significantly different from untreated cells expressing the same receptor construct ($P < 0.001$).

^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 Fig. 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 by use of a two-way ANOVA with Bonferroni's 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 by use of a two-way ANOVA with Bonferroni's post hoc test.

receptor expressed in intact CHO cells (Fig. 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 receptor 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 (Fig. 6). The binding estimates of [³H]QNB to the hM_{1AA46-47}, hM_{1AA430-431}, and hM_{1A46,A431} receptor mutants were only

Fig. 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 (□) or presence (■) of atropine (0.1 μM) beginning 6 h after transfection. Cells were washed extensively and used in intact, whole-cell [³H]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 with use of a paired Student's t test (two-tailed). ns, not significant; *, $P < 0.001$.

4.6, 11.8, and 9.8% of wild type, respectively (Fig. 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 (Fig. 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 with Use of Mutagenesis. The M₁ F(x)₆LL motif (⁴²³FRDTRFLL⁴³¹) also conforms to the hydrophobic folding motif h(x)₃h(x)₂hh (⁴²³FRDTRFLL⁴³¹) described by Krause and co-workers (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, hM_{1AA430-431}, hM_{1VV430-431}, 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 hM_{1AA430-431} was greatly reduced relative to wild type (8.2%), whereas mutation of the same terminal leucines to hydrophobic valines (hM_{1VV430-431}) partially restored binding to 65.9% of wild type (Fig. 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 (Fig. 7A and Table 3).

We also further investigated the plasma membrane expression of receptors containing mutations in residues at the base of TM1 (Fig. 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 (Fig. 7B and Table 3). Binding to the double mutant, hM_{1AA46-47}, was greatly reduced relative to wild type (2.1%) as described above (Fig. 3C). Atropine treatment (0.1 μM, 18 h) restored binding to all of the mutants (Fig. 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 hM_{1AA46-47} or hM_{1AA430-431} mutants could explain the large loss of atropine-rescuable receptor expression on the plasma membrane. We investigated, therefore, whether the *trans*-mutations (hM_{1A46,A431} and hM_{1A47,A430}) caused atropine-restorable deficits of receptor expression on the plasma membrane comparable with those of the *cis*-mutations (hM_{1AA46-47} and hM_{1AA430-431}). As shown in Fig. 7C (see also Fig. 3D for hM_{1A46,A431}), the [³H]NMS binding values for hM_{1A46,A431} and hM_{1A47,A430} were greatly reduced relative to wild type (7.1 and 1.2%, respectively) and comparable with those of hM_{1AA46-47} (2.1%) and hM_{1AA430-431} (8.2%). In contrast, specific binding to the double mutant hM_{1A48,A431} was similar to that of the single mutant hM_{1A431}, 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. After atropine treatment (0.1 μM, 18 h), [³H]NMS binding to

Fig. 7. The plasma membrane expression of wild-type hM₁ and mutant hM₁ receptors after an incubation in the absence and presence of atropine (0.1 μM). A, the plasma membrane expression of wild-type and hM₁ receptors possessing mutations in the F(X)₆LL motif. CHO cells were transiently transfected with the indicated receptor constructs and 6 h after transfection, cells were incubated for 18 h in the absence (□) and presence (■) of atropine (0.1 μM). After washing to remove atropine, intact, whole-cell [³H]NMS binding assays were performed. B, the plasma membrane expression of hM₁ receptors possessing mutations at the base of TM1. CHO cells were transiently transfected with the indicated receptor constructs. Six hours after transfection, cells were incubated in the absence (□) and presence (■) of atropine (0.1 μM) for 18 h. Afterward, intact, whole-cell [³H]NMS binding assays were performed. C, the plasma membrane expression of hM₁ receptors possessing mutations at the base of TM1 and the F(X)₆LL motif. CHO cells were transiently transfected with the indicated receptor constructs and incubated for 6 h. Cells were then incubated an additional 18 h in the absence (□) or presence (■) of atropine (0.1 μM). Intact, whole-cell [³H]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 by use of a paired Student's *t* test (two-tailed). *, *P* ≤ 0.01; **, *P* ≤ 0.001.

hM₁A46,A431, hM₁A47,A430 and hM₁A48,A431 was greatly restored (Fig. 7C).

Agonist-Stimulated Phosphoinositide Hydrolysis. We measured carbachol-stimulated phosphoinositide hydrolysis in CHO cells expressing hM₁AA430-431 to determine whether 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 *E*_{max} values of carbachol were both less for the hM₁AA430-431 mutant (4.70 ± 0.06 and 6.9-fold above basal, respectively) compared with 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 *τ* for each curve. This parameter is proportional to the density of functional receptors. The estimate of *τ* ± S.E.M. for the data obtained with the hM₁AA430-431 mutant was 10.2 ± 1.2% of wild type, which agrees with the *B*_{max} 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 *E*_{max}). Analysis with the operational model gave an estimate of *τ* that was 43% of wild type, which is similar to the estimate of the *B*_{max} of [³H]NMS relative to wild type (63%).

TABLE 3

The effects of mutating amino acid residues in the F(x)₆LL motif and at the base of TM1 on the plasma membrane expression of hM₁ receptors as determined by intact, whole-cell [³H]NMS binding

Receptor Construct ^a	Specifically Bound [³ H]NMS in Untreated Cells ^b	Specifically Bound [³ H]NMS in Atropine-Treated Cells ^c	Fold Increase ^d
	<i>fmol/mg protein</i>		
Wild-type hM ₁ (4)	1547 ± 93	1807 ± 99	1.2 ± 0.03
hM _{1AA44} (3)	1261 ± 35*	1634 ± 21 ^{ns}	1.3 ± 0.04
hM _{1AA45} (3)	954 ± 21***	1731 ± 73 ^{ns}	1.8 ± 0.04
hM _{1AA46} (3)	670 ± 47***	1642 ± 62 ^{ns}	2.5 ± 0.09
hM _{1AA47} (3)	667 ± 28***	1531 ± 60 ^{ns}	2.3 ± 0.08
hM _{1AA48} (3)	1204 ± 28***	1665 ± 64 ^{ns}	1.4 ± 0.02
hM _{1AA46-47} (3)	33 ± 4***	995 ± 91***	27.3 ± 0.45
hM _{1AA423} (4)	808 ± 47***	1480 ± 111 ^{ns}	1.8 ± 0.04
hM _{1AA427} (4)	957 ± 121***	1259 ± 128**	1.3 ± 0.04
hM _{1AA430} (3)	405 ± 87***	858 ± 183***	2.1 ± 0.01
hM _{1AA431} (3)	788 ± 45***	1319 ± 88*	1.7 ± 0.03
hM _{1V430-431} (3)	1019 ± 56***	1433 ± 75 ^{ns}	1.4 ± 0.01
hM _{1AA430-431} (3)	127 ± 14***	971 ± 106***	7.6 ± 0.33
hM _{1AA46,AA431} (3)	110 ± 13***	1107 ± 137***	10.1 ± 0.19
hM _{1AA47,AA430} (3)	19 ± 4***	678 ± 139***	36.7 ± 1.9
hM _{1AA48,AA431} (3)	450 ± 24***	1140 ± 121***	2.5 ± 0.17

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

*, $P < 0.05$, when compared with wild-type hM₁ receptors; **, $P < 0.01$, when compared with wild-type hM₁ receptors; ***, $P < 0.001$ when compared with wild-type hM₁ receptors.

^a The number of experiments is denoted in parentheses. Each experiment was performed in triplicate. Specifically bound [³H]NMS and fold increase in specific [³H]NMS binding was calculated from the data shown in Fig. 7.

^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. P values were calculated by use of a one-way ANOVA with Dunnett's post hoc test.

^c The mean ± S.E.M. of data obtained from intact, whole-cell [³H]NMS binding assays performed on atropine-treated CHO cells (0.1 μM, 18 h) transiently expressing the indicated receptor constructs. P values were calculated by use of a one-way ANOVA with Dunnett's post hoc test.

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

Fig. 8. Carbachol-mediated phosphoinositide hydrolysis in CHO cells expressing wild-type hM₁ or hM_{1AA430-431} receptors. CHO cells transiently expressing wild-type hM₁ (■) or hM_{1AA430-431} (●, ○) receptors were incubated with *myo*-[³H]inositol for 18 h in the absence (■, ●) or presence (○) of atropine (0.1 μM). Cells were washed extensively to remove unincorporated [³H]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.

TABLE 4

Effect of atropine treatment on carbachol-stimulated phosphoinositide hydrolysis elicited by wild-type hM₁ and hM_{1AA430-431} receptors

Receptor Construct ^a	pEC ₅₀ ^b	E _{max} ^b	Hill Slope
Wild-type hM ₁ (4) ^c	5.62 ± 0.11	8.6 ± 0.4	0.96 ± 0.22
hM _{1AA430-431} Untreated (3) ^c	4.70 ± 0.06***	6.9 ± 0.3*	1.05 ± 0.13
Atropine-treated (3) ^c	5.16 ± 0.10*	8.7 ± 0.5 ^{ns}	0.82 ± 0.18

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

*, $P < 0.05$, when compared with wild-type hM₁ receptors; ***, $P < 0.001$, when compared with wild-type hM₁ receptors.

^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 pEC₅₀ and E_{max} values, relative to basal, and the Hill slope were calculated from the data shown in Fig. 8.

The estimate of the negative log dissociation constant of carbachol was 4.07 ± 0.29 .

There was no difference in basal [³H]inositol phosphate accumulation between CHO cells expressing wild-type hM₁ and hM_{1AA430-431} receptors (3041 ± 154 dpm and 2573 ± 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 μM, 18 h) (3386 ± 134 dpm and 2733 ± 112 dpm, respectively). In addition, there was no difference between the labeling of [³H]phosphoinositides with [³H]inositol in CHO cells expressing wild-type ($49,623 \pm 4061$ dpm) and hM_{1AA430-431} receptors ($48,316 \pm 3441$ dpm). Atropine treatment (0.1 μM, 18 h) did not affect labeling ($48,761 \pm 3474$ dpm and $48,442 \pm 2996$ dpm, respectively) either. In addition, [³H]inositol phosphate accumulation elicited to a maximally effective concentration of carbachol (1 mM) by wild-type hM₁ receptors in untreated cells (9.5 ± 1.3 -fold over basal) was not significantly different from that obtained in atropine-treated cells (0.1 μM, 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 EC₅₀ 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 EC₅₀ 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 hM₁ receptor with a dissociation constant of 1 nM, which represents 10-fold higher affinity than the EC₅₀ 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 per hour (see Fig. 4). Dividing this rate by the maximal receptor expression after 18 h of atropine treatment yields a rate constant of 0.12 h^{-1} . Using RPD regulated secretion/aggregation technology at low hM₁ receptor expression, we previously estimated an initial rate of delivery of the wild-type hM₁ receptor to the plasma membrane of 18 fmol/mg protein per hour (Sawyer et al., 2006). When normalized relative to receptor expression at 18 h, 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 hM_{1AA430-431} and enabling ER export and not by preventing receptor degradation.

It might also be argued that atropine stabilizes hM_{1AA430-431} 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 hM_{1AA430-431} receptor after atropine washout (0.09 h^{-1} ; Fig. 4), was similar to that (0.07 h^{-1}) estimated for the wild-type hM₁ receptor by use of RPD regulated secretion/aggregation technology (Sawyer et al., 2006). The similarity between the two estimates shows that the atropine-rescued hM_{1AA430-431} receptor exhibits stability comparable with that of the wild-type hM₁ receptor, and that the effect of atropine cannot be attributed to prevention of receptor internalization and degradation. The hM_{1AA430-431} 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 (Dowling et al., 2006; Nelson et al., 2006).

Our results showed that alanine mutagenesis of single residues in the consensus sequence (h(x)₃h(x)₂hh) within the C terminus of hM₁ caused a moderate reduction in plasma membrane expression (approximately 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 β_2 -adrenoceptor structure (Cherezov et al., 2007; Rosenbaum et al., 2007; Duvernay et al., 2009) and the homology model of Thielen and co-workers (2005), the F(x)₆LL motif forms an α -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 co-workers (2005) showed that the membrane-permeable vasopressin V₂ receptor antagonist SR121463B rescues the plasma membrane expression of a V₂ receptor mutant possessing mutations similar to that of hM_{1AA430-431} receptors (i.e., V₂ receptor, L339T/L340T). They postulated, based on the observations of Wüller and co-workers (2004), that the SR121463B 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 ($1 \mu\text{M}$) 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 hM_{1AA430-431} mutant after an 18-h 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 hM_{1AA430-431} 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 pre-existing pool of rescuable receptor in the ER as described above.

Once the hM_{1AA430-431} mutant reaches the plasma membrane, it seems 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 τ -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 (eq. 2). Because the estimate of τ for the hM_{1AA430-431} mutant expressed relative to that of the wild-type receptor was approximately the same as the corresponding relative B_{max} values for [³H]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 observed by use of fluorescence microscopy (with a GFP-tagged receptor) or [³H]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 with use of thermoreversible folding mutants (Hirschberg et

al., 1998) and RPD 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.

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