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

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Received August 25, 2009; accepted October 19, 2009

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 423FRDTFRLLL431. 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 β2-adrenoceptor structure. This motif is necessary for the ER export of 5-HT1A, 5-HT1B, e1β-adrenergic, angiotensin II type IA, and β2-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 α2H-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 α2H-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 α1β- and α2H-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.

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-quinoxidinyl benzilate; SR121463B, satavapatan.
brane-permeable V2 receptor antagonist SR121463B rescued 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, 422FDDTFRL431, is analogous to the h(x)3h(x)2hh (423FRDTFRLLL431) motif described by Krause and coworkers (2000). Using site-directed mutagenesis, we investigate 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 antagonists 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 Okayama-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, hM1AA430–431, and hM1AA46–47 receptor constructs, the sequences encoding wild-type (Bonner et al., 1988), hM1AA46–47, and hM1AA430–431 receptors were amplified by using the 20-cycle PCR reaction and primers with 5′ EcoRI (5′-GCAGAGGCATCCACACTTTGCCCAC3′; forward) and BamHI (5′-GCAGAGGCTCCTGCAGGCGGGAG3′; 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 placement 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. Cells were 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 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 hM1AA46–47 receptors. In brief, CHO cells were transiently transfected with wild-type hM1 or hM1AA46–47 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.

We also determined the effect of atropine on the plasma membrane expression of wild-type and mutant receptors. In brief, CHO cells were transiently transfected with wild-type hM1, hM1AA46–47, hM1AA430–431, or hM1AA46, hM1AA430–431, or hM1AA46, hM1AA430–431 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 hM1AA46–431 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 hM1AA46–431 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 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 μM) in 500 μl of binding buffer (25 mM HEPES, 113 mM NaCl, 6 mM dextrose, 5 mM CaCl2, 3 mM KCl, 2 mM MgSO4, 1 mM Na2HPO4, pH 7.4) for 24 h at 4°C. After incubation, cells were rapidly and gently washed (2 × 1 ml) with ice-cold PBS to remove unbound [3H]NMS or [3H]QNB.
[3H]NQB. Bound [3H]NMS or [3H]NQNB 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]NQNB 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 previously (Goldschmidt and Kimelberg, 1989). After washing, cells were incubated in transfection medium with or without atropine (0.1 μM) and [3H]NMS was added (200 μl of 0.525 M KOH containing 10 mM Tris/HCl, pH 7.4) to remove serum. The protein concentration was measured. In brief, three wells of a 24-well plate were plated and transfected for each receptor construct and assay condition as described previously (Griffin et al., 2003), and radioactivity was counted by use of a Beckman LS 65000 scintillation counter.

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., 2016). In brief, 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 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 3000 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 1-8 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 hM1AA430–431 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 g/well), pEGFP-C2-hM1AA430–431 with a 20-min incubation between each wash. After washing, cells were incubated 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 3000 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 1-8 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.

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Epifluorescence Images. CHO cells were plated at 0.25 × 10⁵ cells/well on poly(t-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 co-transfected with pEGFP-C2-hM1 (0.2 μg/well), pEGFP-C2-hM1AA430–431 (0.2 μg/well), pEGFP-C2-hM1AA430–431 (0.2 μg/well), or pEGFP-C2-hM1AA430–431 and pDsRed2-ER (0.2 μg/well) (Clontech) 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 (Emax), and the concentration of carbachol eliciting half-maximal response (EC50) 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, Emax, and EC50 of atropine-mediated plasma membrane expression of hM1AA430–431 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:

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

In this equation, y denotes the response, X denotes the concentration of agonist, Msys represents the maximum response of the system, n represents the transducer slope factor, and Kobs denotes the observed dissociation constant of the agonist. The parameter τ is proportional to receptor expression (Rτ) and the intrinsic efficacy of the agonist (ε) and inversely proportional to the sensitivity of the signaling cascade (Kε):

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

The data for agonist-stimulated phosphoinositide hydrolysis by wild-type and mutant M1AA430–431 receptors were fitted simultaneously to eq. 1, sharing the estimates of Msys, Kobs, 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 (τmut), expressed relative to that of the wild-type receptor (τWT) is equivalent to the corresponding ratio of receptor densities on the plasma membrane:

\[ \frac{\tau_{\text{mut}}}{\tau_{\text{WT}}} = \frac{K_E}{K_S} \]  

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

Results

Epifluorescence Microscopy. Because the F(3)δLL motif is known to be involved in the ER export of GPCRs (Durnernay 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 hM₁₄₃₀₋₄₃₁) colocalized with DsRed-ER (Fig. 1C). Treatment with atropine (0.1 μM) for 18 h had little effect on the distribution of the wild-type receptor, but converted the distribution of the hM₁₄₃₀₋₄₃₁ 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 hM₁₄₃₀₋₄₃₁ mutant (GFP-tagged hM₁₁₀₅₄₃₀₋₄₃₁) affected the ability of atropine to rescue receptor expression on the plasma membrane. The D105N mutation of the wild-type receptor (GFP-tagged hM₁₁₀₅₄₃₀₋₄₃₁) had no detectable effect on cellular localization in the absence or presence of atropine (Fig. 2, A and B). Although the GFP-tagged hM₁₁₀₅₄₃₀₋₄₃₁ mutant had a distribution similar to that of hM₁₄₃₀₋₄₃₁, its distribution was unaffected by 18-h atropine (0.1 μM) treatment (Fig. 2, C and D). Our results show that the hM₁₄₃₀₋₄₃₁ 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. 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-hM₁N₁₀₅ (A and B), pEGFP-C2-hM₁₄₃₀₋₄₃₁-C₁₀₅ (C and D), pEGFP-C2-hM₁₄₆₋₄₇ (E and F), or pEGFP-C2-hM₁₁₀₅₄₆₋₄₇ (H and G), and DsRed-ER. Six hours after 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 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×; scale bar, 10 μM.

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 (L¹₃₅⁹ and L¹₄₄⁰) 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 (hM₁₄₄₆₋₄₇) associate with the ER in a manner similar to that of hM₁₄₃₀₋₄₃₁. Figure 2 shows that the GFP-tagged hM₁₄₄₆₋₄₇ mutant is distributed within the ER and that incubation with atropine (0.1 μM) 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 hM₁₁₀₅₄₆₋₄₇) (Fig. 2, G and H).
To quantify receptor expression on the plasma membrane, we measured the binding of \({}^3\text{H}\)NMS to intact CHO cells. \({}^3\text{H}\)NMS is a quaternary ammonium muscarinic antagonist that does not penetrate the plasma membrane. Differences in \({}^3\text{H}\)NMS binding across receptor mutants, therefore, should reflect differences in their expression on the plasma membrane.

Figure 3 shows the specific binding of \({}^3\text{H}\)NMS to intact CHO cells expressing the wild-type hM1 receptor (A) and receptors containing two-point mutations at the base of TM1 (hM1AA46–47) (B), two-point mutations in the F(x)6LL motif (hM1AA430–431) (C), and single-point mutations in both the F(x)6LL motif and the base of TM1 (hM1A46,A431) (D). The negative log dissociation constant of \({}^3\text{H}\)NMS (p\(K_D\)) varied modestly across the various receptors (9.2 to 9.6). In contrast, the binding capacities of the hM1AA430–431 (162 ± 2 fmol/mg protein), hM1AA46–47 (38 ± 2 fmol/mg protein), and hM1A46,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 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 for \({}^3\text{H}\)NMS, subsequent experiments were carried out with use of a single concentration of \({}^3\text{H}\)NMS to monitor changes in receptor expression on the cell membrane.

**Table 1**

<table>
<thead>
<tr>
<th>Receptor Construct</th>
<th>p(K_D)^b</th>
<th>(B_{\text{max}}) (\text{fmol/mg protein})</th>
<th>Hill Slope(^b)</th>
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<tr>
<td>Wild-type hM1</td>
<td></td>
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<tr>
<td>Untreated (2)</td>
<td>9.21 ± 0.02</td>
<td>1700 ± 20</td>
<td>1.5 ± 0.1</td>
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<tr>
<td>Atropine-treated (2)</td>
<td>9.06 ± 0.02</td>
<td>2239 ± 27</td>
<td>1.5 ± 0.1</td>
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<tr>
<td>hM1AA46–47</td>
<td></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>
<td>9.21 ± 0.43</td>
<td>1178 ± 23</td>
<td>1.4 ± 0.1</td>
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<tr>
<td>hM1AA430–431</td>
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<tr>
<td>Untreated (2)</td>
<td>9.37 ± 0.19</td>
<td>162 ± 13</td>
<td>0.8 ± 0.3</td>
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<tr>
<td>Atropine-treated (2)</td>
<td>9.17 ± 0.03</td>
<td>1294 ± 25</td>
<td>1.4 ± 0.1</td>
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<tr>
<td>hM1A46,A431</td>
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<td></td>
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<tr>
<td>Untreated (2)</td>
<td>9.62 ± 0.18</td>
<td>153 ± 7</td>
<td>0.9 ± 0.3</td>
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<tr>
<td>Atropine-treated (2)</td>
<td>9.20 ± 0.20</td>
<td>1504 ± 20</td>
<td>1.4 ± 0.1</td>
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</table>

\(^a\) The number of experiments conducted is denoted in parentheses.  
\(^b\) Estimates for the \(pK_D\), \(B_{\text{max}}\), and Hill slope were calculated from the data shown in Fig. 3.
siently expressing the hM1AA430–431 receptor were incubated with atropine (0.1 μM) 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 hM1AA430–431 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 μM) 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 hM1AA430–431 receptor after removal of atropine (0.1 μM) 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 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. After a 7-h incubation with scopolamine (0.5 μM), [3H]NMS binding to hM1AA430–431, hM1AA430–431, and hM1AA46,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. 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 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 ± S.E.M. of three experiments performed in triplicate.

![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 (○) or presence of NMS (0.2 μM) (■) or scopolamine (0.5 μM) (▲) for 7 h. Cells were washed extensively and used in intact, whole-cell [3H]NMS binding assays. Each bar represents the mean ± S.E.M. of two experiments performed in triplicate.](https://f)
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 Cells</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1862 ± 116</td>
</tr>
<tr>
<td>hM1A430–431</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>hM1AA46–47</td>
<td>263 ± 21</td>
</tr>
<tr>
<td>hM1A46,A431</td>
<td>197 ± 5</td>
</tr>
</tbody>
</table>

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).

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.

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.

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.

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 an two-way ANOVA with Bonferroni’s post hoc test.

A 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 hM1AA46–47, hM1A430–431, and hM1A46,A431 receptor mutants were only 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 (423FRDTFRPLL431) also conforms to the hydrophobic folding motif h(x)3h(x)2hh (423FRDTFRPLL431) described by Krause and co-workers (2000). To explore the role of this M₁ 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 (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 148A 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, hM1AA46–47, 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 (1300LL431) 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 (hM1AA46,A431 and hM1A47,A430) caused atropine-restoreable deficits of receptor expression on the plasma membrane comparable with those of the cis-mutations (hM1AA46–47 and hM1AA430–431). As shown in Fig. 7C (see also Fig. 3D for hM1AA6,AA31), the [3H]NMS binding values for hM1AA6,A431 and hM1A47,A430 were greatly reduced relative to wild type (7.1 and 1.2%, respectively) and comparable with those of hM1AA46–47 (2.1%) and hM1AA430–431 (8.2%). In contrast, specific binding to the double mutant hM1AA6,A431 was similar to that of the single mutant hM1AA31, 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
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 hM1A430–431 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 hM1A430–431. The pEC50 and Emax values of carbachol were both less for the hM1A430–431 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 hM1A430–431 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 hM1A430–431 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 τ that was 43% of wild type, which is similar to the estimate of the Bmax of [3H]NMS relative to wild type (63%).

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.
TABLE 3

<table>
<thead>
<tr>
<th>Receptor Construct</th>
<th>Specifically Bound [3H]NMS in Untreated Cells</th>
<th>Specifically Bound [3H]NMS in Atropine-Treated Cells</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type hM1 (4)</td>
<td>1547 ± 93</td>
<td>1807 ± 99</td>
<td>1.2 ± 0.03</td>
</tr>
<tr>
<td>hM1AA430–431 (3)</td>
<td>1261 ± 35</td>
<td>1634 ± 63**</td>
<td>1.3 ± 0.04</td>
</tr>
<tr>
<td>hM1A430 (3)</td>
<td>954 ± 21**</td>
<td>1731 ± 73**</td>
<td>1.8 ± 0.04</td>
</tr>
<tr>
<td>hM1A47 (3)</td>
<td>670 ± 47**</td>
<td>1642 ± 62**</td>
<td>2.5 ± 0.09</td>
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<tr>
<td>hM1A47,A430 (3)</td>
<td>667 ± 28**</td>
<td>1531 ± 60**</td>
<td>2.3 ± 0.08</td>
</tr>
<tr>
<td>hM1A48 (3)</td>
<td>1204 ± 28**</td>
<td>1665 ± 64**</td>
<td>1.4 ± 0.02</td>
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<tr>
<td>hM1A46–47 (3)</td>
<td>33 ± 4**</td>
<td>995 ± 91***</td>
<td>27.3 ± 0.45</td>
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<tr>
<td>hM1A423 (4)</td>
<td>808 ± 47**</td>
<td>1480 ± 111**</td>
<td>1.8 ± 0.04</td>
</tr>
<tr>
<td>hM1A47,A431 (4)</td>
<td>957 ± 121**</td>
<td>1259 ± 128**</td>
<td>1.3 ± 0.04</td>
</tr>
<tr>
<td>hM1A48,A431 (3)</td>
<td>405 ± 87**</td>
<td>854 ± 183**</td>
<td>2.1 ± 0.01</td>
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<tr>
<td>hM1A431 (3)</td>
<td>788 ± 45**</td>
<td>1319 ± 88**</td>
<td>1.7 ± 0.03</td>
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<tr>
<td>hM1A430–431 (3)</td>
<td>1019 ± 56**</td>
<td>1433 ± 75**</td>
<td>1.4 ± 0.01</td>
</tr>
<tr>
<td>hM1A430–431 (3)</td>
<td>127 ± 14**</td>
<td>971 ± 106**</td>
<td>7.6 ± 0.33</td>
</tr>
<tr>
<td>hM1A423 (3)</td>
<td>110 ± 13**</td>
<td>1107 ± 137**</td>
<td>10.1 ± 0.19</td>
</tr>
<tr>
<td>hM1A423,A431 (3)</td>
<td>19 ± 4**</td>
<td>678 ± 139**</td>
<td>36.7 ± 1.9</td>
</tr>
<tr>
<td>hM1A430–431 (3)</td>
<td>450 ± 24**</td>
<td>1140 ± 121**</td>
<td>2.5 ± 0.17</td>
</tr>
</tbody>
</table>

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. 3 The number of experiments conducted is denoted in parentheses. 4 The number of experiments conducted is denoted in parentheses. 5 The mean ± S.E.M. of data obtained from intact, whole-cell [3H]NMS binding assays conducted on untreated CHO cells. 6 The mean ± S.E.M. of data obtained from intact, whole-cell [3H]NMS binding assays conducted on atropine-treated CHO cells. 7 The mean ± S.E.M. of data obtained from intact, whole-cell [3H]NMS binding assays conducted on atropine-treated CHO cells (0.1 μM, 18 h) transiently expressing the indicated receptor constructs. 8 The mean ± S.E.M. of data obtained from intact, whole-cell [3H]NMS binding assays conducted on intact, whole-cell [3H]NMS binding assays conducted on atropine-treated CHO cells. 9 The mean ± S.E.M. of data obtained from intact, whole-cell [3H]NMS binding assays conducted on atropine-treated CHO cells (0.1 μM, 18 h) transiently expressing the indicated receptor constructs. 10 The mean ± S.E.M. of data obtained from intact, whole-cell [3H]NMS binding assays conducted on atropine-treated CHO cells (0.1 μM, 18 h) transiently expressing the indicated receptor constructs.

TABLE 4

<table>
<thead>
<tr>
<th>Receptor Construct</th>
<th>pEC50b</th>
<th>Emaxb</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type hM1 (4)</td>
<td>5.62 ± 0.11</td>
<td>8.6 ± 0.4</td>
<td>0.96 ± 0.22</td>
</tr>
<tr>
<td>hM1A430–431</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)f</td>
<td>5.16 ± 0.10*</td>
<td>8.7 ± 0.5**</td>
<td>0.82 ± 0.18</td>
</tr>
</tbody>
</table>

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

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

The was no difference in basal [3H]inositol phosphate accumulation between CHO cells expressing wild-type hM1 and hM1A430–431 receptors (3041 ± 154 dpm and 2573 ± 91 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]inositol phosphoinositides with [3H]inositol in CHO cells expressing wild-type (49,623 ± 4061 dpm) and hM1A430–431 receptors (48,316 ± 3441 dpm). Atropine treatment (0.1 μM, 18 h) did not affect labeling (48,761 ± 3474 dpm and 48,442 ± 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 hM1A430–431 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 hM1A430–431 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 hM1A430–431. We speculate that, in the ER, the hM1A430–431 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 hM1A430–431 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 hM1AA430–431 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 hM1AA430–431. If the hM1AA430–431 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; 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 hM1AA430–431 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 hM1AA430–431 mutant behaves differently from M₁ 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 β₂-adrenoceptor structure (Cherezov et al., 2007; Rosenthal et al., 2007; Duvvury et al., 2009), and the homology model of Thienen 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. Thienen 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 hM1AA430–431 receptors (i.e., V₂ receptor, L339T/L340T). They postulated, based on the observations of Wullner 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 (Thienen 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-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 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 pre-existing pool of resuable receptor in the ER as described above.

Once the hM1AA430–431 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 hM1AA430–431 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 hM1AA430–431 mutant expressed relative to that of the wild-type receptor was approximately the same as the corresponding relative Bₘₐₓ 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 hM1AA430–431 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 hM1AA430–431 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 hM1AA430–431 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

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