Constitutive Activity and Inverse Agonism at the M₂ Muscarinic Acetylcholine Receptor

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ABSTRACT

Introduction of a single-point mutation (Asn to Tyr) at position 410 at the junction between transmembrane domain 6 and the third extracellular loop of the human M₂ muscarinic acetylcholine (mACh) receptor generated a mutant receptor (N410Y) that possesses many of the hallmark features of a constitutively active mutant receptor. These included enhanced agonist binding affinity and potency, in addition to agonist-independent accumulation of [³H]inositol phosphates in cells coexpressing the chimeric G/ᵢ₅₅ protein and the N410Y mutant M₂ mACh receptor. Constitutive activity was sensitive to inhibition by a range of muscarinic ligands, including those used clinically in the management of overactive bladder (oxybutynin, tolterodine, and darifenacin), indicating that these ligands behave as inverse agonists at the N410Y mutant M₂ mACh receptor. Long-term (24-h) treatment of Chinese hamster ovary cells expressing the N410Y mutant M₂ mACh receptor with certain mACh receptor inverse agonists (atropine, darifenacin, and pirenzepine) elicited a concentration-dependent up-regulation of cell surface receptor expression. However, not all ligands possessing negative efficacy in the [³H]inositol phosphate accumulation assays were capable of significantly up-regulating receptor expression, perhaps indicating a spectrum of negative efficacies among ligands traditionally defined as mACh receptor antagonists. Finally, structurally distinct agonists exhibited differences in their relative potencies for the activation of G/ᵢ₅₅ versus G/ᵢ₃₅, consistent with agonist-directed trafficking of signaling at the N410Y mutant, but not at the wild-type M₂ mACh receptor. This indicates that the N410Y mutation of the M₂ mACh receptor alters receptor-G-protein coupling in an agonist-dependent manner, in addition to generating a constitutively active receptor phenotype.

A crucial development in our understanding of G-protein-coupled receptor (GPCR) function has been the identification of the ability of receptors to activate their cognate G-proteins in the absence of an agonist (Costa and Herz, 1989). Thus, certain ligands (termed inverse agonists and previously characterized as competitive antagonists) can inhibit agonist-independent receptor activity (Costa and Herz, 1989). Subsequent research has identified significant agonist-independent (constitutive) activity at a wide range of muscarinic ligands, including those used clinically in the management of overactive bladder (oxybutynin, tolterodine, and darifenacin), indicating that these ligands behave as inverse agonists at the M₂ mACh receptor. Long-term (24-h) treatment of Chinese hamster ovary cells expressing the N410Y mutant M₂ mACh receptor with certain mACh receptor inverse agonists (atropine, darifenacin, and pirenzepine) elicited a concentration-dependent up-regulation of cell surface receptor expression. However, not all ligands possessing negative efficacy in the [³H]inositol phosphate accumulation assays were capable of significantly up-regulating receptor expression, perhaps indicating a spectrum of negative efficacies among ligands traditionally defined as mACh receptor antagonists. Finally, structurally distinct agonists exhibited differences in their relative potencies for the activation of G/ᵢ₅₅ versus G/ᵢ₃₅, consistent with agonist-directed trafficking of signaling at the N410Y mutant, but not at the wild-type M₂ mACh receptor. This indicates that the N410Y mutation of the M₂ mACh receptor alters receptor-G-protein coupling in an agonist-dependent manner, in addition to generating a constitutively active receptor phenotype.

In the case of the muscarinic acetylcholine (mACh) receptors, a number of studies have identified constitutively activating mutations, particularly in the predominately D/ERY motif at the intracellular interface of the third transmembrane domain (TM3) and the BBXXB motif (where B is Arg or Lys) toward the C-terminal end of the third intracellular loop, have been reported to enhance agonist-independent signaling of a wide range of GPCRs (Parnot et al., 2002).

One of the most powerful tools used by researchers in this area has been the development of GPCRs harboring specific mutations known to enhance the agonist-independent coupling of receptor and G-protein [so-called constitutively active mutant (CAM) receptors] (Seifert and Wenzel-Seifert, 2002). Mutations in a number of well conserved domains, including the D/ERY motif at the intracellular interface of the third transmembrane domain (TM3) and the BBXXB motif (where B is Arg or Lys) toward the C-terminal end of the third intracellular loop, have been reported to enhance agonist-independent signaling of a wide range of GPCRs (Parnot et al., 2002).

In the case of the muscarinic acetylcholine (mACh) receptors, a number of studies have identified constitutively activating mutations, particularly in the predominately G/ᵢ₅₅-coupled M₁, M₃, and M₄ receptor subtypes (Spalding et al., 1995, 1997; Ford et al., 2002). Spalding et al. (1995) first identified that mutation of adjacent serine (Ser465) and threonine (Thr466) residues at the junction between TM6 and the third extracellular loop results in a CAM-M₄ mACh receptor. Mutation of these two conserved residues has since been demonstrated to enhance constitutive activity of all five mACh receptor family subtypes (Ford et al., 2002). The mutant receptors displayed many of the characteristic properties of CAM-GPCRs, including enhanced agonist affinity and
potency, in addition to an elevated basal functional activity (proportional to the receptor expression level), which was sensitive to the inverse agonist atropine (Ford et al., 2002).

Huang et al. (1998) had earlier investigated both double (388Ser/389Thr to Tyr/Pro) and single (388Ser to Tyr, and 389Thr to Pro) mutations in the M2 mACh receptor subtype and found that mutation of 388Ser alone was sufficient to generate a mutant receptor displaying many of the common properties exhibited by a CAM receptor (enhanced agonist potency and binding affinity). In contrast, mutation of 389Thr seemed to influence receptor-G-protein coupling fidelity, introducing multiple apparent affinity binding states in agonist competition binding experiments (Huang et al., 1999). Moreover, Spalding et al. (1997) reported that mutation of the homologous residue (465Ser) in the M5 subtype, particularly to large (Phe or Val) or basic (Arg or Lys) residues, generated receptors with significantly enhanced constitutive activity, relative to the wild type. Taken together, these data generated receptors with significantly enhanced constitutive activity, relative to large (Phe or Val) or basic (Arg or Lys) residues, generated receptors with significantly enhanced constitutive activity, relative to large (Phe or Val) or basic (Arg or Lys) residues.

Materials and Methods

Materials. [3H]NMS, myo-[3H]inositol, and [3H]tAC were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Darifenacin and tolterodine were synthesized in the laboratories of Pfizer Central Research (Sandwich, Kent, UK). All other reagents were purchased from Sigma Chemical (Poole, Dorset, UK) or Fisher Scientific Co. (Pittsburgh, PA). Goat a-s in pCDN was a generous gift from Dr. S. Rees (GlaxoSmithKline, Uxbridge, Middlesex, UK).

Generation of N410YM2 Mutant Receptor. Mutagenesis was performed using the QuiKChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the wild-type M2 mACh receptor gene in pCDNA3 as a template. The following oligonucleotide primer and its complement were used to incorporate a single amino acid change (Asn to Tyr) at position 410 (via substitution of T for A, shown in bold): 5'-GCC CCA TAC AAT GTG ATC TTC TCT TAC ACC TTT GTG GCA CCT-3'. The underlined nucleotide represents a silent mutation leading to the incorporation of an additional restriction site for the enzyme AvaII, allowing for initial confirmation of the successful mutagenesis.

Cell Culture and Transient Transfection of Chinese Hamster Ovary Cells. CHO-K1 cells were grown in minimum essential medium-a supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 g/ml streptomycin, and 2.5 g/ml amphotericin B. CHO-K1 cells stably expressing cloned human wild-type M2 or N410YM2 receptors generated in this project were grown in an identical medium to that used in the culture of CHO-K1 cells, supplemented with 500 g/ml G418 (Geneticin) selection reagent. Cells were maintained at 37°C in a humidified atmosphere of O2/CO2 (19:1) and were routinely split 1:5 every 3 to 4 days using trypsin-EDTA. Cells were transfected 48 h before experimentation using GeneJuice transfection reagent, and the medium was replaced approximately 6 h after transfection.

CHO Cell Membrane Preparation and Radioligand Binding. Confluent monolayers of CHO cells were briefly washed with HEPES-buffered saline (10 mM HEPES and 0.9% NaCl, pH 7.4) and cells lifted from the flask by the addition of HEPES-buffered saline-EDTA (10 mM HEPES, 0.9% NaCl, and 0.2% EDTA, pH 7.4) for approximately 15 min. A cell pellet was recovered by centrifugation at 1000 g for 5 min. The cell pellet was homogenized on ice in lysis buffer (10 mM HEPES and 10 mM EDTA, pH 7.4) using a Polytron homogenizer (2 20-s bursts at 4°C; Kinematica, Basel, Switzerland). The homogenate was then centrifuged (40,000 g, 15 min, 4°C), rehomogenized, and recentrifuged as described above in 10 mM HEPES and 0.1 mM EDTA, pH 7.4. The final membrane pellet was resuspended in the same buffer at a concentration of 2 mg of protein/ml and stored at −80°C until required.

Saturation binding was performed using a range of concentrations of [3H]NMS (0.03–60 nM; specific activity, 81 Ci/mmol) in the absence and presence of atropine (10 μM) to define nonspecific binding. Binding assays were performed in a final volume of 500 μl of 20 mM HEPES, pH 7.4, containing 25 to 100 μg of membrane protein for 60 min at 37°C. When monitoring the receptor expression levels after transient transfections, a single high concentration (3–4 nM) of [3H]NMS (performed in duplicate) was generally used to approximate the mACh receptor expression level. Competition binding experiments were performed using a single concentration of [3H]NMS (0.3–0.5 nM) in the absence and presence of a range of antagonist concentrations. Bound radioligand was separated from free by rapid vacuum filtration through GF/B filters (Whatman, Maidstone, UK) on a 24-well cell harvester (Brandel Inc., Gaithersburg, MD), and radioactivity was quantified by liquid scintillation counting (Nelson et al., 2004). Intact cell [3H]NMS binding assays were performed on cell monolayers on 24-well plates, as described previously (Nelson et al., 2004).

Long-Term Antagonist Treatment of CHO Cells. Where indicated, cells were incubated with putative inverse agonist ligands in culture medium for 24 h before assay. At this point, cells were thoroughly washed three times with 1 ml of KHB before being incubated in 1 ml of KHB at 37°C for 20 min. After this time, KHB was aspirated, and cells were washed with 1 ml of KHB before assay. Preliminary experiments determined that binding of atropine (300 nM) to the M2 receptor could be fully reversed using this washing protocol, so this treatment was applied to cells before all subsequent [3H]NMS phosphate ([3H]IP) accumulation experiments.

Cyclic AMP and [3H]inositol Phosphate Accumulation Assays. For cyclic AMP experiments, cells in 24-well multiwells were stimulated with forskolin (10 μM) for 10 min in the presence of agonist (agonist added 10 min before forskolin addition). Assays were stopped by aspiration and addition of ice-cold 0.5 M trichloroacetic acid (400 μl). Samples were neutralized as described previ-
ously (Nelson et al., 2004), and cyclic AMP was determined using the method of Brown et al. (1971). Where indicated, cell monolayers approaching confluence in 24-well multilwells were treated with pertussis toxin (PTx; 100 ng/ml) for 20 to 24 h before experimentation.

For [3H]IP$_x$ assays, cDNAs encoding N410Y mutant or wild-type M$_2$ mACh receptors and the chimeric G-protein Go$_{q/5}$ (Conklin et al., 1993) were cotransfected into CHO-K1 cells 24 h before experimentation. [3H]inositol (3 µCi/ml) was also added during this 24-h period. Putative inverse agonists were preincubated with [3H]inositol-prelabeled cell monolayers for 15 min before the addition of LiCl (10 mM) and continuation of the incubation for a further 15 min. [3H]inositol phosphate accumulation in the presence of various ligands was calculated as a percentage of that in the absence of ligand, after subtraction of the Li$^+$-independent accumulation (i.e., the [3H]inositol phosphate accumulation over the same time course but in the absence of Li$^+$).

**Immunoblot Analysis.** Cells were lysed with a Triton X-100-based buffer (20 mM HEPES, 200 mM NaCl, 10 mM EDTA, and 1% Triton X-100, pH 7.4) and added to an equal volume of 2× sample buffer (125 mM Tris/HCl, 4% SDS, 20% glycerol, 50 µM dithio- 

titol, and 0.01% bromphenol blue, pH 6.8) before boiling at 90°C for 5 min. Samples were subjected to electrophoresis on 10% SDS-polyacrylamide gel electrophoresis minigels with 5% stacking gels and run at 120 V for 90 min (running buffer: 25 mM Tris, 250 mM glycine, and 0.1% SDS, pH 8.0). Transfer to nitrocellulose was run at 120 V for 90 min (running buffer: 25 mM Tris, 250 mM glycine, and 0.1% SDS, pH 8.0). Transfer to nitrocellulose was achieved using a semidry apparatus (transfer buffer: 48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol, pH 9.3). Nitrocellulose membrane washing steps were performed using a modified high-stringency TBS-Tween buffer (20 mM Tris/HCl, 1 M NaCl, and 1% Tween 20, pH 7.5) and blocking with 20% milk in TBS-Tween buffer. Immunoblotting was performed using a rabbit polyclonal G$_q$ antibody (IQB; Mullaney et al., 1993). Immunoreactive proteins were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG second- 

ary antibody (Sigma-Aldrich, St. Louis, MO) and enhanced chemilu- 

minescence reagents.

**Data and Statistical Analysis.** Data are shown as mean ± S.E.M. for the indicated number of experiments. Saturation binding data were fitted with hyperbola (one-site binding) using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). $B_{\text{max}}$ and $K_d$ values were derived from these curves. Competition binding curves and functional concentration-response curves were fitted to the four-parameter logistic equation: $Y = bottom + (top - bottom)/(1 + 10^{(logEC_{50} - X) / n_{H}})$ using GraphPad Prism 3.0, where $n_{H}$ is the Hill coefficient. The best fit between a variable Hill coefficient and a Hill coefficient fixed to unity was determined using an $F$ test. IC$_{50}$ values, generated by these inhibition curves, were corrected to give binding constant ($K_i$) values for each test compound, using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

The statistical significance of differences between data were determined using either Student’s $t$ test, with $K_i$ values first being converted to the respective normally distributed negative logarithm (p$K_i$), or one-way analysis of variance with Dunnett’s post test for multiple comparisons.

**Results**

**Measurement of Agonist-Mediated Responses at Transiently Expressed Wild-Type and N410Y Mutant M$_2$ mACh Receptors.** Initial attempts to characterize pharmacologically the N410Y M$_2$ mACh receptor by measuring cyclic AMP accumulation in HEK293 or CHO cells transiently expressing either the wild type or mutant receptor proved unsuccessful. Although increases in cyclic AMP could be generated in response to forskolin (1, 3, or 10 µM) in either cell background, a range of concentrations of MCH (up to 1 mM) failed to elicit significant, reproducible inhibitions of the forskolin-stimulated cyclic AMP responses in cells expressing either wild-type or N410Y M$_2$ mACh receptors at levels >1 pmol/mg of protein (data not shown). These data contrast with the ability of MCh to cause >90% inhibition (IC$_{90}$, 230 nM) of forskolin-stimulated cyclic AMP accumulation in CHO cells stably expressing M$_2$ mACh receptors (Mistry et al., 2005).

The chimeric Go$_{q/5}$ protein [G$_{q/5}$ containing the C-terminal five amino acids (DCGLP) of Go$_{q/5}$; Conklin et al., 1993] has been shown previously to couple a variety of G$_{q/5}$-linked GPCRs, including the M$_2$ mACh receptor, to phospholipase C-β activation and phosphoinositide hydrolysis in recombinant cell systems (Conklin et al., 1993; Liu et al., 1995). Therefore, cotransfection of cDNAs coding for Go$_{q/5}$ and either the wild-type or N410Y M$_2$ mACh receptor into CHO-K1 cells allowed the measurement of [3H]IP$_x$ accumulation (under Li$^+$ block) as an index of M$_2$ mACh receptor activation. Figure 1A shows the concentration-response relationship for MCh in CHO cells coexpressing Go$_{q/5}$ and either the wild-type M$_2$ (2.74 ± 0.22 pmol/mg of protein) or N410Y M$_2$ (1.95 ± 0.09 pmol/mg of protein) mACh receptor. Both basal and MCh-stimulated [3H]IP$_x$ accumulations were linear over the time course of these experiments (data not shown), and no significant [3H]IP$_x$ response to MCh was observed in untransfected CHO cells (Fig. 1A). Maximal [3H]IP$_x$ responses to MCh were similar in wild-type and N410Y M$_2$ mACh receptor-expressing cells (53,015 ± 6283 versus 52730 ± 5123 dpm/mg of protein, respectively). However, MCh was significantly more potent (>10-fold) at the N410Y mutant than at the wild-type M$_2$ mACh receptor (pEC$_{50}$ values, 7.58 ± 0.09 versus 6.44 ± 0.11, respectively; $P < 0.05$). Go$_{q/5}$ protein overexpression relative to endogenous Go$_{q}$ levels (determined by Western blotting using a Go$_{q}$ antibody [IQB; Mullaney et al., 1993]) was found to be similar in CHO cells coexpressing wild-type M$_2$ mACh receptors (5.1 ± 0.2-fold) versus N410Y M$_2$ mACh receptor-expressing cells (4.9 ± 0.3-fold) ($P > 0.05$; $n = 3$).

**Constitutive Activity and Inverse Agonism at Wild- 

Type and Mutant N410Y M$_2$ mACh Receptors Coexpressed with Go$_{q/5}$.** Basal [3H]IP$_x$ accumulation in CHO cells expressing the N410Y M$_2$ receptor was approximately 2-fold higher than that for cells expressing wild-type receptor (Fig. 1B). In all experiments, the wild-type receptor was expressed at higher levels than the mutant M$_2$ receptor (2.66 ± 0.35 versus 1.80 ± 0.28 pmol/mg of protein); therefore, differences in receptor density cannot account for the enhanced agonist-independent activity observed for the N410Y M$_2$ receptor-expressing cells. In cells coexpressing either wild-type or N410Y M$_2$ mACh receptor with Go$_{q/5}$, addition of atropine 15 min before and throughout a 15-min incubation with Li$^+$ significantly reduced constitutive [3H]IP$_x$ accumulation (Fig. 1B; atropine-induced decrease in basal [3H]IP$_x$ accumulation: wild type (WT)-M$_2$, 2247 ± 548; N410Y M$_2$, 9171 ± 1128 dpm/mg of protein), whereas in untransfected CHO cells, atropine had no effect upon [3H]IP$_x$ accumulation (data not shown). In these experiments, therefore, atropine behaves as an inverse agonist, reducing constitutive mACh receptor activity. A number of other mACh receptor antagonists were assayed for inverse agonist activity. A maximal concentration of each ligand was selected (a concentration approximately 100-fold greater than the binding affinity for the ligand at the M$_2$
mACh receptor) and the effect on agonist-independent \([{}^3H]\)IP\(_x\) accumulation assessed. In CHO cells expressing the N\(^{410Y}\)M\(_2\) mACh receptor, all antagonists tested significantly reduced the basal \([{}^3H]\)IP\(_x\) accumulation (Fig. 2B), whereas all ligands tested, barring darifenacin, significantly inhibited basal M\(_2\) mACh receptor-dependent activity (Fig. 2A). The concentration dependence of the atropine-mediated reduction in basal \([{}^3H]\)IP\(_x\) accumulation was also investigated in both wild-type and N\(^{410Y}\)M\(_2\) mACh receptor-expressing cells (Fig. 2C). The mean pEC\(_{50}\) values for atropine at wild-type and mutant M\(_2\) receptors were 9.04 ± 0.17 and 8.42 ± 0.20. Therefore, atropine was approximately 4-fold more potent in reducing constitutive \([{}^3H]\)IP\(_x\) accumulation in cells expressing wild-type M\(_2\) than N\(^{410Y}\)M\(_2\) receptors (P < 0.05).

**Effect of Long-Term Inverse Agonist Treatment on the Expression of Transiently Transfected Wild-Type and N\(^{410Y}\)M\(_2\) mACh Receptors.** Initial observations indicated that long-term treatment (24 h) with inverse agonist (atropine) up-regulated receptor expression levels, particularly of the N\(^{410Y}\)M\(_2\) mACh receptor. Because this was consistent with previous reports on CAM-GPCRs (see Milligan and Bond, 1997), the effect of long-term treatment of CHO cells transiently expressing either wild-type or mutant receptor was investigated for a range of putative mACh receptor antagonists: atropine (Atr), darifenacin (Dari), oxybutynin (Oxy), tolterodine (Tolt), pirenzepine (Pirenz), methoctramine (Methoc), and 4-diphenylacetoxy-N-methylpiperidine. C. concentration-dependent inhibition of basal \([{}^3H]\)IP\(_x\) accumulation, in CHO-K1 cells transiently coexpressing Go\(_{\alpha}\) and wild-type M\(_2\) mACh receptor, by atropine. Results are expressed as means ± S.E.M., n ≥ 3. Statistically significant differences from control basal values are indicated as ‡, P < 0.05.
up-regulatory effect was determined for atropine at the N410YM2 mACh receptor, yielding an EC50 value of 19 nM (pEC50 value, 7.72 ± 0.18; Fig. 3C); unfortunately, the smaller maximal effect of atropine at the wild-type M2 mACh receptor precluded analysis of the concentration dependence of this response.

In contrast to the up-regulatory effects mediated by a subset of mACh receptor inverse agonists, in both wild-type and N410YM2 mACh receptor-expressing cells, 24-h treatment with the muscarinic agonist carbachol (100 μM) produced significant decreases (by 57 ± 5 and 62 ± 1%, respectively) in the receptor expression, relative to vehicle-treated cells (data not shown).

Stable Expression of Wild-Type and N410YM2 mACh Receptors in CHO Cells: Radioligand Binding Assays. A number of CHO cell clones stably expressing either wild-type or N410YM2 mACh receptors under G418 selection were next created. Single wild-type (CHO-m2 WT) and N410YM2 [CHO-m2 mutant (MUT)] mACh receptor-expressing clones (receptor expression levels, 627 ± 86 and 247 ± 51 fmol/mg of protein, respectively) were selected for further study. Data for these (and at least one other) wild-type and N410YM2 mACh receptor-expressing clone indicated that there was a trend toward [3H]NMS binding affinity (Kd) estimates being lower in cell membranes prepared from wild type (0.36 ± 0.02 nM) compared with N410YM2 (0.55 ± 0.11 nM) receptor-expressing cells, although this trend failed to achieve significance (n ≥ 3).

The binding affinities of a number of mACh receptor antagonists, demonstrated to act as inverse agonists at the N410YM2 receptor (see Fig. 3B), were determined in competition binding assays in membrane preparations from the wild-type and mutant receptor-expressing CHO cells. The mean binding affinity constants (pKi) and Hill slopes are shown in Table 1. Corrected pKi values were not significantly different between CHO-m2 WT- and MUT-derived membranes for atropine, tolterodine, pirenzepine, and methoctramine. However, darifenacin (6.2-fold) and, to a lesser extent, oxybutynin (2.2-fold), each exhibited significantly lower affinity for the N410YM2 mACh receptor compared with wild type (P < 0.05) (see Table 1). In membranes prepared from each cell line, competition binding curves for methoctramine were characterized by Hill slopes significantly greater than 1 (P < 0.05). However, for all other competition binding curves, Hill slopes did not differ significantly from unity.

Apparent binding affinity constant (pKi) estimates for four mACh receptor agonists were also determined in [3H]NMS competition binding assays at 4°C (see Table 2). MCh, oxotremorine-M (Oxo-M), and oxotremorine (Oxo) all bound with significantly higher affinity to CHO cells expressing the

**TABLE 1**

<table>
<thead>
<tr>
<th>CHO-m2 Wild Type</th>
<th>CHO-m2 N410Y Mutant</th>
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<tbody>
<tr>
<td><strong>CHO-m2 Wild Type</strong></td>
<td><strong>CHO-m2 N410Y Mutant</strong></td>
</tr>
<tr>
<td><strong>pKi</strong></td>
<td><strong>pKi</strong></td>
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<tr>
<td>Atropine</td>
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<td>Darifenacin</td>
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<td>Oxybutynin</td>
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<td>Tolterodine</td>
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<tr>
<td>Pirenzepine</td>
<td>6.36 (0.05)</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>8.51 (0.02)</td>
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</tbody>
</table>

* Significant differences between wild type and mutant (P < 0.05; Student’s t test).
** Significant difference to unity (P < 0.05; F test).
TABLE 2

Intact cell apparent binding affinity constant (pKᵢ) values and inhibition of (−PTx) and enhancement of (+PTx) forskolin-stimulated cyclic AMP accumulation in CHO cells stably expressing either wild-type or N410Y mutant M₂ mACh receptors

Data are expressed as mean (S.E.M.) values from n = 3 experiments.

<table>
<thead>
<tr>
<th>pKᵢ</th>
<th>pEC₅₀(Gi/o)</th>
<th>Eₘ₅₀(Gs)</th>
<th>pEC₅₀(Gi/o)</th>
<th>Eₘ₅₀(Gs)</th>
<th>EC₅₀(Gi/o)/EC₅₀(Gi/o)</th>
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<tr>
<td>CHO-m2 wild-type</td>
<td></td>
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<tr>
<td>MCh</td>
<td>4.97 (0.04)</td>
<td>6.66 (0.05)</td>
<td>100</td>
<td>5.01 (0.12)*</td>
<td>100</td>
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<tr>
<td>Oxo-M</td>
<td>5.58 (0.02)</td>
<td>7.37 (0.05)</td>
<td>101.7 (0.7)</td>
<td>5.70 (0.08)*</td>
<td>98.4 (4.0)</td>
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<tr>
<td>Oxo</td>
<td>6.02 (0.03)</td>
<td>7.07 (0.08)</td>
<td>99.1 (1.8)</td>
<td>5.51 (0.12)*</td>
<td>36.5 (10.1)**</td>
</tr>
<tr>
<td>Pilo</td>
<td>3.77 (0.04)</td>
<td>4.48 (0.06)</td>
<td>68.3 (5.4)**</td>
<td>N.D.</td>
<td>5.2 (1.0)**</td>
</tr>
</tbody>
</table>

CHO-m2 N410Y mutant

| MCh | 5.92 (0.07)*** | 7.63 (0.10)*** | 100 | 6.06 (0.09)*,** | 100 | 37.2 |
| Oxo-M | 6.20 (0.06)*** | 7.79 (0.10)*** | 104.6 (2.5) | 6.53 (0.04)** | 104.0 (3.8) | 18.2 |
| Oxo | 6.17 (0.03)*** | 7.76 (0.06)*** | 103.5 (0.1) | 5.82 (0.09)* | 66.5 (5.9)*** | 87.1 |
| Pilo | 3.70 (0.05) | 5.24 (0.10)*** | 83.1 (4.8)** | N.D. | 33.0 (6.0)**,** | N.D. |

N.D., not determined.
* Significant differences between pEC₅₀(Gi/o) and pEC₅₀(Gi/o) values (P < 0.05; Student’s t test).
** Eₘ₅₀ values are significantly less than 100% (P < 0.05; one-way ANOVA, Dunnett’s post-test).
*** Significant differences between wild type and mutant (P < 0.05; Student’s t test).

N₄¹⁰¹⁷M₂ mACh receptor compared with wild-type receptor, whereas pilocarpine (Pilo) displayed similar affinities in each cell line (Table 2). In all cases, Hill slopes derived from agonist competition binding curves did not differ significantly from unity (data not shown).

Functional Characterization of Wild-Type and N₄¹⁰¹⁷M₂ mACh Receptor Populations Stably Expressed in CHO Cells: Cyclic AMP Accumulation. Basal cyclic AMP accumulation was similar in CHO-m2 WT and MUT (1.4 ± 0.2 versus 1.3 ± 0.2 pmol/mg of protein, respectively; n = 6) cell lines. Cyclic AMP accumulation in response to forskolin (10 µM) was significantly lower in MUT than in WT CHO-m2 (220 ± 16 versus 855 ± 126 pmol/mg of protein, respectively; P < 0.05; n = 5). However, pretreatment with, or simultaneous addition of, a range of putative mACh receptor inverse agonists failed to enhance significantly cyclic AMP accumulation in response to either 3 or 10 µM forskolin in either CHO cell line (data not shown).

The stable CHO cell lines provide a model system in which to compare agonist pharmacology of the wild-type and N₄¹⁰¹⁷M₂ mACh receptors. The abilities of a range of mACh receptor agonists to inhibit forskolin-stimulated cyclic AMP accumulation through the Gₛ-coupled M₂ mACh receptors were assessed. In addition, after PTx pretreatment, mACh receptor agonists concentration-dependently increased forskolin-stimulated cyclic AMP accumulation via a Gₛ-dependent mechanism (Michal et al., 2001; Mistry et al., 2005). Therefore, it is possible to investigate whether differences exist in the ability of agonists to cause wild-type or N₄¹⁰¹⁷M₂ mACh receptors to couple via either Gₛ or Gᵦᵦ proteins (Fig. 4). Mean maximal inhibitory [Eₘ₅₀(Gi/o)] and stimulatory [(Eₘ₅₀(Gs))] responses (expressed as a percentage of the response to the reference agonist MCh), as well as potencies for both inhibitory [pEC₅₀(Gi/o)] and stimulatory [pEC₅₀(Gs)] responses for each agonist, are summarized in Table 2.

In the absence of PTx pretreatment, all agonists tested were more potent in inhibiting the forskolin-stimulated response in MCh compared with WT CHO-m2 cells; however, the potency difference varied considerably from >9-fold for MCh to <3-fold for Oxo-M (Table 2). MCh, Oxo-M, and Oxo caused maximal inhibitory responses in both cell lines, whereas Pilo was a full agonist in MUT compared with WT CHO-m2 cells. Comparison of inhibitory potencies on the cyclic AMP response with apparent binding affinities for the agonists [expressed as EC₅₀(Gi/o)/Ki ratios] revealed similar values for MCh (49.0 and 51.3 in WT and MUT CHO-m2 cells). For Oxo and Pilo, EC₅₀(Gi/o)/Ki values were greater in CHO-m2 MUT (38.9 and 34.7) compared with CHO-m2 WT (11.2 and 5.1). In contrast, the EC₅₀(Gi/o)/Ki ratio for Oxo-M was greater in WT than MUT CHO-m2 cells (61.7 versus 38.9).

Forskolin-stimulated cyclic AMP accumulation was significantly attenuated in both WT and MUT (129 ± 16 versus 56 ± 8 pmol/mg of protein; n = 5–8) CHO-m2 cell lines after PTx pretreatment (P < 0.05). In PTx-treated cells, EC₅₀ values for enhancements of the forskolin-stimulated response by agonists were much closer to apparent affinity (Ki) estimates. MCh (11.2-fold) and Oxo-M (6.8-fold) (but not Oxo, 2.0-fold) were significantly more potent with respect to EC₅₀(Gi/o) values in MUT versus WT CHO-m2 cells, and both were full agonists in these cell lines with respect to this response (Table 2). In contrast, Oxo and Pilo behaved as partial agonists causing maximal responses that were significantly lower than those to MCh in both cell lines (Fig. 4, C and D). Comparison of stimulatory potencies on the cyclic AMP response with apparent binding affinities for the agonists [expressed as EC₅₀(Ga)/Ki ratios] revealed values greater than unity for MCh (1.32 and 2.14 in WT and MUT CHO-m2 cells) and Oxo-M (1.10 and 1.38 in WT and MUT CHO-m2 cells), whereas those for Oxo were less than unity (0.31 and 0.45 in WT and MUT CHO-m2 cells) and could not be determined for Pilo.

Table 2 also summarizes the EC₅₀(Ga)/EC₅₀(Gi/o) ratios for MCh, Oxo-M, and Oxo, highlighting the generally lower potency observed for the stimulatory responses. In WT CHO-m2 cells, EC₅₀(Ga)/EC₅₀(Gi/o) ratios were comparable for the three agonists. In contrast, Oxo displayed a substantially larger difference in potency between stimulatory and inhibitory responses in MUT compared with WT CHO-m2 cells, whereas Oxo-M exhibited a smaller EC₅₀(Ga)/EC₅₀(Gi/o) ratio in MUT compared with WT CHO-m2 cells (Table 2).

Effect of PTx Pretreatment on Expression Levels of Wild-Type M₂ and N₄¹⁰¹⁷M₂ mACh Receptors in CHO Cells. In light of the observed differences in potency of, and maximal response to, mACh receptor agonists between cells in the absence and presence of PTx pretreatment, the binding affinities of MCh and Oxo in CHO-m2 MUT cells pretreated with PTx were also determined (data not shown). Saturation
radioligand binding analyses were also performed in both WT and MUT CHO-m2 cells pretreated with PTx. PTx pre-treatment had no effect on either [3H]NMS binding affinity (see Table 3) or \( pK_i \) estimates for MCh or Oxo in CHO-m2 MUT cells (data not shown). However, it is clear from the representative saturation binding curves shown in Fig. 5A (CHO-m2 WT) and Fig. 5B (CHO-m2 MUT) that PTx pre-treatment significantly reduced the observed \( B_{\text{max}} \) in CHO-m2 MUT cells but had no effect upon the maximal binding in CHO-m2 WT cells. \( B_{\text{max}} \) estimates are summarized in Fig. 5C and Table 3.

Cell surface receptor expression in wild-type and N410YM2 mACh receptor-expressing CHO cells was also measured in cells incubated in the absence and presence of atropine (300 nM), with and without concurrent PTx treatment (see Fig. 5C; Table 3). Atropine per se did not significantly alter N410YM2 mACh receptor expression levels in CHO cells, whereas PTx alone significantly reduced (by 33%) the cell surface expression of the mutant receptor (\( P < 0.05 \)). Inclusion of atropine (300 nM) during the PTx treatment failed to attenuate the PTx-mediated reduction in the N410YM2 mACh receptor expression level. No significant effect on wild-type receptor expression in CHO-m2 cells was observed for any treatment. In contrast to findings for transiently expressed wild-type and mutant M2 mACh receptors (see above), stably expressed N410YM2 mACh receptors exhibited a small but significantly greater affinity for [3H]NMS compared with the wild-type M2 receptor (Table 3).

**Discussion**

Some of the earliest examples of agonist-independent GPCR activity and its pharmacological reversal by receptor antagonists (subsequently reclassified as inverse agonists) were reported for the M2 mACh receptor. Thus, the ability of atropine, and a subset of other mACh receptor antagonists, to suppress constitutive activity has been reported in membrane (Hilf and Jakobs, 1992) and intact (Jakubik et al., 1995) cell preparations endogenously or recombinantly expressing M2 mACh receptors. Any constitutive activity exhibited by the wild-type receptor can often be enhanced by mutagenesis of key domains within the GPCR (Parnot et al., 2002; Seifert and Wenzel-Seifert, 2002). For the M1-M5 mACh receptor family, a single or double mutation at the TM6-e3 junction has been shown to promote constitutive activity (Spalding et al., 1995; 1997; Huang et al., 1999; Ford

![Fig. 4](image)

**Fig. 4.** Concentration-response curves for agonist-mediated inhibition or stimulation of forskolin-stimulated cyclic AMP accumulation in CHO-m2 WT (closed symbols) and CHO-m2 MUT (open symbols) cells after pre-treatment without (inhibition) or with (stimulation) PTx (100 ng/ml) for 20 to 24 h. A, MCh; B, Oxo-M; C, Oxo; D, Pilo. Results are expressed as percent maximal inhibitions or stimulations of cyclic AMP accumulation relative to the maximal responses to the reference agonist MCh. Data are expressed as means ± S.E.M., \( n \geq 3 \).

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>( K_d ) (nM)</th>
<th>( B_{\text{max}} ) (fmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atropine</td>
<td>+Atropine</td>
</tr>
<tr>
<td>CHO-m2 WT</td>
<td>0.22 (0.02)</td>
<td>1235 (62) ( () 1197 (28)</td>
</tr>
<tr>
<td>CHO-m2 WT + PTx</td>
<td>0.21 (0.02)</td>
<td>1359 (70) ( () 1283 (41)</td>
</tr>
<tr>
<td>CHO-m2 MUT</td>
<td>0.16 (0.01)*</td>
<td>637 (53) ( * ) 725 (90)</td>
</tr>
<tr>
<td>CHO-m2 MUT + PTx</td>
<td>0.16 (0.01)</td>
<td>427 (47)** ( ** ) 476 (95)</td>
</tr>
</tbody>
</table>

* Significant differences between wild type and mutant (\( P < 0.05 \); Student’s \( t \) test).
** Significant difference between untreated and PTx-pretreated cells (\( P < 0.05 \); Student’s \( t \) test).
minus atropine conditions are indicated as /H11569/H9251 receptors with the chimeric G-protein G
S.E.M., nation with PTx (100 ng/ml) for 24 h. Results are expressed as means
WT and MUT CHO-m2 cells with atropine (300 nM) alone or in combi-
more experiments. C, effect on mACh receptor expression of incubating
performed in duplicate and curves shown are representative of three or
[H]NMS saturation binding analysis in intact cells. Data points were
this system, both the N410Y mutant and wild-type M2 mACh
accumulate to be used as a readout of receptor activity. In
et al., 2002). Here, wild-type and N410Y mutant M2 mACh
receptors have been compared with respect to agonist and
antagonist (inverse agonist) actions using both transient and
stable receptor expression in CHO cells.

Cotransfection of wild-type or N410Y mutant M2 mACh
receptors with the chimeric G-protein Goq11 allowed [H]IPx
accumulation to be used as a readout of receptor activity. In
this system, both the N410Y mutant and wild-type M2 mACh
receptors exhibited atropine-inhibited constitutive activity,
with the N410YM2 receptors exhibiting a 4-fold higher level of
atropine-sensitive constitutive activity. The mutant receptor
also displayed an enhanced agonist potency (>-10-fold) relative
to wild-type receptor, consistent with previous reports on
the CAM N410Y,T411PM2 mACh receptor (Ford et al., 2002).
Although G-protein overexpression has been shown to in-
crease agonist-independent signaling for Gq/11-coupled
mACH receptors (Burstein et al., 1997), Goq11 was expressed
at similar levels in wild-type and N410Y mutant receptor-
expressing cells in our experiments. Therefore, G-protein
overexpression per se cannot account for the differences be-
tween wild-type and mutant responses seen.

The constitutive receptor activity present in the recombi-
ant M2 mACh receptor/Goq11 coexpression system allowed us
to identify inverse agonism and to investigate its short-
and long-term consequences. Of the antagonists studied, we
were particularly interested in comparing tolterodine, oxy-
butynin, and darifenacin, which are used in the management
of overactive bladder (Moreland et al., 2004; Nelson et al.,
2004). Recent estimates suggest that as many as 85% of
GPCR antagonists actually exhibit negative efficacy when
tested in constitutively active systems (Kenakin, 2004), and
the data presented here are consistent with this notion, be-
cause all seven of the mACh receptor antagonists assayed
possessed properties consistent with inverse agonism.

Although the maximal inhibition of constitutive [H]IPx
accumulation did not generally differ between wild-type and
mutant receptor-expressing cells, atropine did display a sub-
stantially higher potency at the wild-type (pEC50 9.04) com-
pared with the N410Y mutant (pEC50 8.42) M2 mACh recep-
tor. According to the extended ternary complex model,
inverse agonists may exert their effects via a selectively
higher affinity for the inactive (Ri) than for the active (Ra)
receptor species and/or by reducing the affinity of the ligand-
bound receptor for its cognate G-protein (Samama et al.,
1993; Strange, 2002). Thus, any perturbation of the system in
favor of Ra and/or RaG will result in a reduction in the
apparent binding affinity of an inverse agonist for the recep-
tor population (Costa and Herz, 1989; Samama et al.,
1993; Huang et al., 1998; Wade et al., 2001).

Given the significant level of inverse agonism observed in
the [H]IPx assays, it is surprising that only darifenacin and
oxybutynin displayed significantly lower affinities at the
CAM receptor, whereas all other inverse agonists exhibited
equivalent affinity for wild-type and CAM receptors. Previ-
uous studies have also failed to observe differences in the
binding affinity of inverse agonists between wild-type and
CAM receptors (Kjelsberg et al., 1992; Ren et al., 1993;
Ford et al., 2002). Theoretical analysis predicts that even when
50% of the total receptor population is present in the active
state, a shift of only 2-fold might be observed in the binding
affinity of an inverse agonist at a CAM receptor (Wade et al.,
2001; Strange, 2002); therefore, it is possible that the differ-
ences in the magnitude of constitutive activity between wild-
type and CAM receptors are insufficient for differences in
antagonist affinities to be detected in radioligand binding
assays. However, it remains unclear why darifenacin (and to
a lesser extent oxybutynin) exhibits such a reduced affinity
for the CAM receptor (6-fold).

The observation that long-term (24-h) treatment of
N410YM2 mACh receptor-expressing CHO-cells with certain
inverse agonists caused a significant, concentration-depen-
dent up-regulation of cell surface receptor number is consis-
tent with numerous previous reports for CAM GPCRs (see
Milligan and Bond, 1997). However, not all of the ligands
that behaved as inverse agonists in the [H]IPx assays (with

![Fig. 5. Effect of PTx pretreatment (100 ng/ml; 20–24 h) on mACh recep-
tor expression levels in WT (A) and MUT (B) CHO-m2 cells, assessed by
[H]NMS saturation binding analysis in intact cells. Data points were
performed in duplicate and curves shown are representative of three or
more experiments. C, effect on mACh receptor expression of incubating
WT and MUT CHO-m2 cells with atropine (300 nM) alone or in combi-
nation with PTx (100 ng/ml) for 24 h. Results are expressed as means ±
S.E.M., n = 3. Statistically significant differences between plus and
minus atropine conditions are indicated as *, P < 0.05.](image-url)
comparable negative efficacies) were capable of similarly facilitating an up-regulation of M2 mACH receptor expression. If receptor up-regulation is related to the negative efficacy of the ligand, these data would suggest that the ligands investigated may possess a range of different negative efficacies toward this signaling pathway. As an alternative, properties distinct from negative efficacy might also contribute to the regulation of receptor expression levels. The ability of the ligand to cross the plasma membrane and stabilize newly synthesized receptors at the endoplasmic reticulum, facilitating their maturation and endoplasmic reticulum export, might be important, as has been suggested for CAM μ-opioid receptors (Li et al., 2001). As an alternative, the enrichment of a conformational state(s) that is uncoupled from G-proteins might not necessarily stabilize the receptor at the cell surface, particularly if internalization and G-protein activation are mediated by distinct receptor conformations, as suggested by the ability of antagonists or inverse agonists to elicit internalization (Barker et al., 1994; Roettger et al., 1997).

A further intriguing difference between wild-type and N410Y mutant M2 mACH receptors emerged from studies initiated to assess Gi/o protein involvement in trafficking. PTX pretreatment of cells had no effect on the cell surface expression level of the wild-type receptor but caused a highly significant decrease in N410YM2 mACH receptor expression. A possible cause of this down-regulation of the CAM receptor could be through destabilization caused by a decreased availability of G\textsubscript{i/o} proteins. However, the failure of an inverse agonist to reverse this effect suggests that the down-regulation may not be related to the constitutive activity of the N410YM2 mACH receptor. There is some evidence that G\textsubscript{i/o} proteins play a role in the endocytosis/intracellular trafficking of proteins (Lang et al., 1995; Valentí et al., 1998), but there is little precedent for an effect of PTX treatment upon the expression of wild-type or CAM GPCRs. Indeed, Roseberry et al. (2001) reported that pretreatment of HEK293 cells stably expressing M2 mACH receptors with PTX caused a modest increase in receptor expression.

Previous studies of mACh receptor mutants (e.g., Huang et al., 1999) have found that mutations within the TM6-e3 region can have substantial effects on agonist-mediated responses. Here, the creation of wild-type or N410Y mutant M2 mACH receptor-expressing CHO cell lines has allowed agonist pharmacology also to be assessed. Consistent with the behavior of a CAM receptor, the partial agonists Pilo and Oxo displayed enhanced maximal responses at the N410YM2 mACH receptor, whereas all four agonists tested exhibited greater potencies for inhibition of forskolin-stimulated cyclic AMP accumulation at the CAM receptor. However, when the signaling of the M2 receptors through Ga\textsubscript{q} (after PTX-mediated inactivation of G\textsubscript{i/o} proteins) was investigated, only the full agonists MCh and Oxo-M displayed a greater potency at the CAM receptor.

In addition, there was some evidence for agonist-directed trafficking of signaling (ADTS) (Kenakin, 1995) at the N410Y mutant M2 receptor, where Oxo was relatively weak at activating Ga\textsubscript{q} compared with wild-type M2 receptor. In contrast, Oxo-M displayed an unexpectedly high potency for the activation of Ga\textsubscript{q} through the CAM M2 receptor, as illustrated by its relatively small EC\textsubscript{50}(Ga\textsubscript{q})/EC\textsubscript{50}(Gi/o) ratio at the mutant receptor. The EC\textsubscript{50}(Ga\textsubscript{q})/EC\textsubscript{50}(Gi/o) ratio has been used previously to investigate ADTS at a variety of other GPCRs (e.g., Berg et al., 1998), and agonist-specific trafficking has also been previously demonstrated at the wild-type M2 mACH receptor (Akam et al., 2001). However, in the present study, we found no evidence of ADTS for Ga\textsubscript{q,i} versus Ga\textsubscript{q} at the wild-type receptor, suggesting that the N410Y mutation alters the agonist-dependent signaling profile of the receptor. Malmberg and Strange (2000) similarly observed ligand-dependent alterations in receptor-G-protein coupling at the 5-HT\textsubscript{1A} receptor after mutation of sites within the i3 loop. Work with the M1 mACH receptor has indicated that mutation of the conserved threonine residue at the TM6-e3 junction influences G-protein coupling, whereas enhanced potency, affinity, and efficacy of agonists arise from mutation of the adjacent serine/asparagine residues (Huang et al., 1999). Our findings suggest that for the M2 mACH receptor, the N410Y mutation alters G-protein coupling in an agonist-dependent manner, in addition to enhancing constitutive activity and agonist affinity, potency, and maximal response.

In summary, we report that the N410Y mutation, at the TM6-e3 junction, significantly enhances agonist-independent activity of the M2 mACH receptor. Evidence has also been presented that long-term treatment with a subset of ligands, including those used in the clinical management of overactive bladder, can facilitate an increase in cell surface receptor expression. It has been proposed that inverse agonist-mediated receptor up-regulation might contribute to the development of tolerance upon long-term treatment (Smit et al., 1996). The potential for up-regulation of mACH receptors to occur in vivo, after long-term treatment with inverse agonists, therefore requires consideration in the clinical use of these ligands.

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References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.


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Huang XP, Williams FE, Peseckis SM, and Messer WS (1999) Differential modulation of agonist potency and receptor coupling by mutations of Ser388Tyr and Thr389Pro at the junction of transmembrane domain VI and the third extracellular loop of human M1 muscarinic acetylcholine receptors. Mol Pharmacol 56:775–783.


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