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Constitutive Activity and Inverse Agonism at the M_2 Muscarinic Acetylcholine Receptor

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ABBREVIATIONS: CAM, constitutively active mutant; CHO, Chinese hamster ovary; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine; KHB, Krebs-Henseleit buffer; mACh, muscarinic acetylcholine; MCh, methacholine; [^{3}H]-NMS, [*N-methyl*-^{3}H]-scopolamine methyl chloride; Oxo, oxotremorine; Oxo-M, oxotremorine-M; Pilo, pilocarpine; TM, transmembrane domain; TCA, trichloroacetic acid.

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ABSTRACT

Introduction of a single point mutation (Asn to Tyr) at position 410 at the junction between TM6 and the third extracellular loop of the human M_2 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 [^{3}H]-inositol phosphates in cells co-expressing the chimeric $G\alpha_{qi5}$ protein and the N410Y mutant M_2 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 M_2 mACh receptor. Chronic (24 h) treatment of CHO cells expressing the N410Y mutant M_2 mACh receptor with certain mACh receptor inverse agonists (atropine, darifenacin, pirenzepine) elicited a concentration-dependent up-regulation of cell-surface receptor expression. However, not all ligands possessing negative efficacy in the [^{3}H]-inositol phosphate accumulation assays were capable of significantly up-regulating receptor expression, perhaps indicating a spectrum of negative efficacies among ligands classically defined as mACh receptor antagonists. Finally, structurally distinct agonists exhibited differences in their relative potencies for the activation of $G\alpha_{i/o}$ versus $G\alpha_s$, consistent with agonist-directed trafficking of signaling at the N410Y mutant, but not at the wild-type M_2 mACh receptor. This indicates that the N410Y mutation of the M_2 mACh receptor alters receptor-G-protein coupling in an agonist-dependent manner, in addition to generating a constitutively active receptor phenotype.

Introduction

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 variety of both endogenously- and recombinantly-expressed GPCRs (for review, see Seifert and Wenzel-Seifert, 2002).

One of the most powerful tools utilized 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) towards 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 predominantly $G\alpha_{q/11}$-coupled M_1, M_3 and M_5 receptor subtypes (Spalding et al., 1995, 1997; Ford et al., 2002). Spalding and colleagues (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-M_5 mACh receptor. Mutation of these two conserved residues has since been demonstrated to enhance constitutive activity of all 5 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 and colleagues had earlier investigated both double (^{388}Ser/^{389}Thr to Tyr/Pro) and single (^{388}Ser to Tyr, and ^{389}Thr to Pro) mutations in the M_1 mACh receptor subtype and found that mutation of ^{388}Ser 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) (Huang et al., 1998). In contrast, mutation of ^{389}Thr appeared 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 (^{465}Ser) in the M_5 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 suggest that the conserved serine residue at the boundary between TM6 and the third extracellular loop is implicated in constraining the M_1 and M_5 mACh receptors in the inactive state.

The primary aim of the present study was to generate a CAM-M_2 mACh receptor by the targeted mutation of the conserved asparagine residue at position 410 of the human M_2 receptor (homologous to ^{388}Ser in M_1 and ^{465}Ser in M_5 mACh receptors) to tyrosine (to generate the N410Y mutant). To date there have been surprisingly few reports of CAM-M_2 mACh receptors. Liu et al. (1996) reported that insertion of 1-4 alanine residues into TM6, three residues *C*-terminal to the BBXXB motif of the M_2 mACh receptor, significantly enhanced the constitutive inhibition of adenylate cyclase activity. More recently, Ford et al. (2002) demonstrated that the double mutant (^{410}Asn/^{411}Thr to Tyr/Pro) CAM-M_2 receptor displayed approx. 4-5 fold higher affinity for agonist and 62% constitutive activity relative to wild-type M_2 mACh receptors expressed in a COS-7 cell background. However, Ford et al. (2002) observed no additional agonist-mediated functional response above the level of constitutive activity at the CAM-M_2 receptor and, as in the earlier study by Liu et al. (1996), further characterization of the mutant receptor beyond the establishment of constitutive activity has not been reported. Therefore, the present study aims to provide a more thorough analysis of the $^{N410Y}M_2$ mACh receptor mutant and, in particular, to define the inverse agonist properties of a number of clinically relevant ligands previously classified as mACh receptor antagonists.

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Materials and Methods

Materials. [^{3}H]-NMS, [^{3}H]-*myo*-inositol and [^{3}H]-cyclic AMP were obtained from Amersham Pharmacia (Little Chalfont, Buckinghamshire, UK). Darifenacin and tolterodine were synthesized in the laboratories of Pfizer Global Research and Development (Sandwich, UK). All other reagents were purchased from Sigma-Aldrich (Poole, Dorset, U.K.) or Fisher Scientific (Loughborough, UK). $G\alpha_{qi5}$ in pCDN was a generous gift from Dr S. Rees (GlaxoSmithKline, Stevenage, UK).

Generation of $^{N410Y}M_2$ mutant receptor. Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the wild-type M_2 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 GTC ATG GT**C** CTC ATT **T**AC ACC TTT TGT GCA CCT-3'. The underlined nucleotide represents a silent mutation leading to the incorporation of an additional restriction site for the enzyme *Ava II*, allowing for initial confirmation of the successful mutagenesis.

Cell culture and transient transfection of CHO cells. Chinese hamster ovary (CHO-K1) cells were grown in minimum essential medium-α (MEM-α) supplemented with 10% fetal calf serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} amphotericin B. CHO-K1 cells stably expressing cloned human wild-type M_2 or $^{N410Y}M_2$ receptors generated in this project were grown in an identical medium to that used in the culture of CHO-K1 cells, supplemented with 500 μg ml^{-1} Geneticin (G418) selection reagent. Cells were maintained at 37^{o}C in a humidified atmosphere of O_2/CO_2 (19:1) and were routinely split 1:5 every 3-4 days, using trypsin-EDTA. Cells were transfected 48 h prior to experimentation using GeneJuice^{TM} transfection reagent and the medium was replaced approx. 6 h post-transfection.

CHO cell membrane preparation and radioligand binding. Confluent monolayers of CHO cells were briefly washed with HEPES-buffered saline (HBS; 10 mM HEPES, 0.9% NaCl, pH 7.4) and cells lifted from the flask by the addition of HBS-EDTA (10 mM HEPES, 0.9% NaCl, 0.2% EDTA, pH 7.4) for approx. 15 min. A cell pellet was recovered by centrifugation at 1,000 x*g* for 5 min. The cell pellet was homogenized on ice in lysis buffer (10 mM HEPES, 10 mM EDTA, pH 7.4) using a Polytron (2 x 20 s bursts at 4^{o}C). The homogenate was then centrifuged (40,000 x*g*, 15 min, 4^{o}C), re-homogenized and re-centrifuged as described above in 10 mM HEPES, 0.1 mM EDTA, pH 7.4. The

final membrane pellet was re-suspended in the same buffer at a concentration of 2 mG-protein ml^{-1} and stored at -80^{o}C until required.

Saturation binding was performed using a range of concentrations of [^{3}H]-NMS (0.03–6.0 nM; specific activity 81 Ci $mmol^{-1}$) in the absence and presence of atropine (10 μM) to define non-specific binding. Binding assays were performed in a final volume of 500 μl 20 mM HEPES, pH 7.4 containing 25-100 μg membrane protein for 60 min at 37^{o}C. When monitoring the receptor expression levels following transient transfections, a single high concentration (3-4 nM) of [^{3}H]-NMS (performed in duplicate) was generally used to approximate the mACh receptor expression level. Competition binding experiments were performed using a single concentration of [^{3}H]-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 Whatman GF/B filters on a 24 well Brandel cell harvester and radioactivity quantified by liquid scintillation counting (Nelson et al., 2004). Intact cell [^{3}H]-NMS binding assays were performed on cell monolayers on 24-well plates, as previously described (Nelson et al., 2004).

Chronic antagonist treatment of CHO cells. Where indicated, cells were incubated with putative inverse agonist ligands in culture medium for 24 h prior to assaying. At this point cells were thoroughly washed 3 x 1 ml with KHB before being incubated in 1 ml KHB at 37^{o}C for 20 min. After this time, KHB was aspirated and cells were washed with 1 ml KHB before assaying. Preliminary experiments determined that binding of atropine (300 nM) to the M_2 receptor could be fully reversed using this washing protocol, so this treatment was applied to cells prior to all subsequent [^{3}H]-IP_x accumulation experiments.

Cyclic AMP and [^{3}H]-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 prior to forskolin addition). Assays were stopped by aspiration and addition of ice-cold 0.5 M TCA (400 μl). Samples were neutralized as described previously (Nelson et al., 2004) and cyclic AMP determined using the method of Brown et al. (1971). Where indicated cell monolayers approaching confluence in 24 well multiwells were treated with pertussis toxin (PTx; 100 ng ml^{-1}) for 20-24 h prior to experimentation.

For [^{3}H]-inositol phosphate ([^{3}H]-IP_x) assays, cDNAs encoding N410Y-mutant or wild-type M_2 mACh receptors and the chimeric G-protein $G\alpha_{qi5}$ (Conklin et al., 1993) were co-transfected into CHO-K1 cells 24 h prior to experimentation. [^{3}H]-inositol (3 μCi ml^{-1}) was also added during this 24 h period.

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Putative inverse agonists were pre-incubated with [^{3}H]-inositol pre-labeled cell monolayers for 15 min prior to the addition of LiCl (10 mM) and continuation of the incubation for a further 15 min. [^{3}H]-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 [^{3}H]-inositol phosphate accumulation over the same time-course, but in the absence of Li^+).

Immunoblot analysis. Cells were lysed with a Triton-X100-based buffer (20 mM HEPES, 200 mM NaCl, 10 mM EDTA, 1% Triton-X100, pH 7.4) and added to an equal volume of 2X sample buffer (125 mM Tris/HCl, 4% SDS, 20% glycerol, 50 μM dithiothreitol, 0.01% bromophenyl blue, pH 6.8) before boiling at 90^{o}C for 5 min. Samples were subjected to electrophoresis on 10% SDS-PAGE minigels with 5% stacking gels and run at 120 V for 90 min (running buffer: 25 mM Tris, 250 mM glycine, 0.1 % SDS, pH 8.0). Transfer to nitrocellulose was achieved using a semi-dry apparatus (transfer buffer: 48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol, pH 8.3). Nitrocellulose membrane washing steps were performed using a modified high stringency TBS-Tween buffer (20 mM Tris/HCl, 1 M NaCl, 1% Tween 20, pH 7.5) and blocking with 20% milk in TBS-Tween buffer. Immunoblotting was performed using a rabbit polyclonal $G\alpha_q$ antiserum ('IQB') (1:1000) raised against amino acids 119-134 of $G\alpha_q$ (Mullaney et al., 1993). Immunoreactive proteins were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich) and enhanced chemiluminescence (ECL+) reagents.

Data and statistical analysis. Data are shown as mean ± standard error of mean (s.e.m.) for the indicated number of experiments. Saturation binding data were fitted with hyperbolae (one-site binding) using GraphPad Prism 3.0 (GraphPad software, San Diego, USA). B_{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":

$$Y = Bottom + (Top - Bottom) / [1 + 10^{(\log EC50 - X).\ Hill\ coefficient}]$$

using GraphPad Prism 3.0. 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 was determined using either Student's *t*-test, with K_i values first being converted to the respective normally distributed negative logarithm (pK_i), or one-way analysis of variance (ANOVA) with Dunnett's post test for multiple comparisons.

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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^{-1} protein (data not shown). These data contrast with the ability of MCh to cause >90% inhibition (IC_{50}, 230 nM) of forskolin-stimulated cyclic AMP accumulation in CHO cells stably expressing M_2 mACh receptors (Mistry et al., 2005).

The chimeric $G\alpha_{qi5}$ protein ($G_q\alpha$ containing the *C*-terminal 5 amino acids (DCGLF) of $G_{i1\text{-}3}\alpha$; Conklin et al., 1993) has previously been shown to couple a variety of $G_{i/o}$-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). Co-transfection of cDNAs coding for $G\alpha_{qi5}$ and either the wild-type or $^{N410Y}M_2$ mACh receptor into CHO-K1 cells therefore allowed the measurement of [^{3}H]-inositol phosphate ([^{3}H]-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 co-expressing $G\alpha_{qi5}$ and either the wild-type M_2 (2.74 ± 0.22 pmol mg^{-1} protein) or $^{N410Y}M_2$ (1.95 ± 0.09 pmol mg^{-1} protein) mACh receptor. Both basal and MCh-stimulated [^{3}H]-IP_x accumulations were linear over the time-course of these experiments (data not shown), and no significant [^{3}H]-IP_x response to MCh was observed in untransfected CHO cells (Figure 1A). Maximal [^{3}H]-IP_x responses to MCh were similar in wild-type and $^{N410Y}M_2$ mACh receptor-expressing cells (53015 ± 6283 versus 52730 ± 5123 d.p.m. mg^{-1} 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; $p<0.05$). $G\alpha_{qi5}$ protein over-expression relative to endogenous $G\alpha_q$ levels (determined by western blotting using a $G\alpha_q$ antibody (IQB; Mullaney et al., 1993)) was found to be similar in CHO cells co-expressing wild-type M_2

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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 co-expressed with $G\alpha_{qi5}$. Basal [^{3}H]-IP_x accumulation in CHO cells expressing the $^{N410Y}M_2$ receptor was approx. two-fold higher than that for cells expressing wild-type receptor (Figure 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^{-1} protein) and therefore differences in receptor density cannot account for the enhanced agonist-independent activity observed for $^{N410Y}M_2$ receptor-expressing cells.

In cells co-expressing either wild-type or $^{N410Y}M_2$ mACh receptor with $G\alpha_{qi5}$, addition of atropine 15 min prior to, and throughout a 15 min incubation with Li^+ significantly reduced constitutive [^{3}H]-IP_x accumulation (Figure 1B; atropine-induced decrease in basal [^{3}H]-IP_x accumulation: WT-M_2, 2247 ± 548; $^{N410Y}M_2$, 9171 ± 1128 d.p.m. mg^{-1} protein), while in un-transfected CHO cells, atropine had no effect upon [^{3}H]-IP_x accumulation (data not shown). In these experiments, atropine therefore 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 approx. 100 fold greater than the binding affinity for the ligand at the M_2 mACh receptor) and the effect on agonist-independent [^{3}H]-IP_x accumulation assessed. In CHO cells expressing the $^{N410Y}M_2$ mACh receptor, all 'antagonists' tested significantly reduced the basal [^{3}H]-IP_x accumulation (Figure 2B), whereas all ligands tested, barring darifenacin, significantly inhibited basal M_2 mACh receptor-dependent activity (Figure 2A). The concentration-dependency of the atropine-mediated reduction in basal [^{3}H]-IP_x accumulation was also investigated in both wild-type and $^{N410Y}M_2$ mACh receptor-expressing cells (Figure 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. Atropine therefore was approx. 4-fold more potent in reducing constitutive [^{3}H]-IP_x accumulation in cells expressing wild-type M_2 than $^{N410Y}M_2$ receptors (p<0.05).

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Effect of chronic inverse agonist treatment on the expression of transiently transfected wild-type and $^{N410Y}M_2$ mACh receptors. Initial observations indicated that chronic treatment (24 h) with inverse agonist (atropine) up-regulated receptor expression levels, particularly of the $^{N410Y}M_2$ mACh receptor. As this was consistent with previous reports on CAM-GPCRs (see Milligan and Bond, 1997), the effect of chronic treatment of CHO cells transiently expressing either wild-type or mutant receptor was investigated for a range of putative mACh receptor inverse agonists (Figure 3). In CHO cells expressing the wild-type M_2 mACh receptor atropine, darifenacin, pirenzepine and methoctramine significantly enhanced receptor expression levels ($p<0.05$), whereas in cells expressing the $^{N410Y}M_2$ mACh receptor only atropine, darifenacin and pirenzepine exerted this effect ($p<0.05$) (Figure 3A, B). It is notable that the maximal effects of atropine and darifenacin were more pronounced in cells expressing $^{N410Y}M_2$ compared to wild-type receptor (approx 60% versus <40%, respectively). The concentration-dependency of the up-regulatory effect was determined for atropine at the $^{N410Y}M_2$ mACh receptor, yielding an EC_{50} value of 19 nM (pEC_{50} value, 7.72 ± 0.18; Figure 3C); unfortunately, the smaller maximal effect of atropine at the wild-type M_2 mACh receptor precluded analysis of the concentration-dependency of this response.

In contrast to the up-regulatory effects mediated by a subset of mACh receptor inverse agonists, in both wild-type and $^{N410Y}M_2$ 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 $^{N410Y}M_2$ mACh receptors in CHO cells: radioligand binding assays. A number of CHO cell clones stably expressing either wild-type or $^{N410Y}M_2$ mACh receptors under G418 selection were next created. Single wild-type (CHO-m2 WT) and $^{N410Y}M_2$ (CHO-m2 MUT) mACh receptor-expressing clones (receptor expression levels, 627 ± 86 and 247 ± 51 fmol mg^{-1} protein, respectively) were selected for further study. Data for these (and at least one other) wild-type and $^{N410Y}M_2$ mACh receptor-expressing clone indicated that there was a trend towards [^{3}H]-NMS binding affinity (K_D) estimates being lower in cell membranes prepared from wild-type (0.36 ± 0.02 nM) compared to $^{N410Y}M_2$

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(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 $^{N410Y}M_2$ receptor (see Figure 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 (pK_i) and Hill slopes are shown in Table 1. Corrected pK_i 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 $^{N410Y}M_2$ mACh receptor compared to 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 one ($p<0.05$). However, for all other competition binding curves Hill slopes did not differ significantly from unity.

Apparent binding affinity constant (pK_i) estimates for four mACh receptor agonists were also determined in [^{3}H]-NMS competition binding assays at 4^{o}C (see Table 2). MCh, Oxo-M and Oxo all bound with significantly higher affinity to CHO cells expressing the $^{N410Y}M_2$ mACh receptor compared to wild-type receptor, while 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 $^{N410Y}M_2$ 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^{-1} 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^{-1} protein, respectively; $p<0.05$; n=5). However, pre-treatment 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).

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The stable CHO cell-lines provide a model system in which to compare agonist pharmacology of the wild-type and $^{N410Y}M_2$ mACh receptors. The abilities of a range of mACh receptor agonists to inhibit forskolin-stimulated cyclic AMP accumulation through the $G_{i/o}$-coupled M_2 mACh receptors were assessed. In addition, following pertussis toxin (PTx) pre-treatment mACh receptor agonists concentration-dependently increase forskolin-stimulated cyclic AMP accumulation via a G_s-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 $^{N410Y}M_2$ mACh receptors to couple via either $G_{i/o}$ or G_s proteins (Figure 4). Mean maximal inhibitory ($E_{max}^{(Gi/o)}$) and stimulatory ($E_{max}^{(Gs)}$) responses (expressed as a percentage of the response to the reference agonist MCh), as well as potencies for both inhibitory ($pEC_{50}^{(Gi/o)}$) and stimulatory ($pEC_{50}^{(Gs)}$) responses for each agonist are summarized in Table 2.

In the absence of PTx pre-treatment all agonists tested were more potent in inhibiting the forskolin-stimulated response in MUT compared to 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, while Pilo was a fuller agonist in MUT compared to WT CHO-m2 cells. Comparison of inhibitory potencies on the cyclic AMP response with apparent binding affinities for the agonists (expressed as $EC_{50}^{(Gi/o)}/K_i$ ratios) revealed similar values for MCh (49.0 and 51.3 in WT and MUT CHO-m2 cells). For Oxo and Pilo $EC_{50}^{(Gi/o)}/K_i$ values were greater in CHO-m2 MUT (38.9 and 34.7) compared to CHO-m2 WT (11.2 and 5.1). In contrast, the $EC_{50}^{(Gi/o)}/K_i$ 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^{-1} protein; n=5-8) CHO-m2 cell-lines following PTx pre-treatment (p<0.05). In PTx-treated cells EC_{50} values for enhancements of the forskolin-stimulated response by agonists were much closer to apparent affinity (K_i) estimates. MCh (11.2 fold) and Oxo-M (6.8 fold) (but not Oxo (2.0 fold)) were significantly more potent with respect to $EC_{50}^{(Gs)}$ 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

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partial agonists causing maximal responses that were significantly lower than those to MCh in both cell-lines (Figures 4C and D). Comparison of stimulatory potencies on the cyclic AMP response with apparent binding affinities for the agonists (expressed as $EC_{50}^{(Gs)}/K_i$ 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 $EC_{50}^{(Gs)}/EC_{50}^{(Gi/o)}$ ratios for MCh, Oxo-M and Oxo, highlighting the generally lower potency observed for the stimulatory responses. In WT CHO-m2 cells, $EC_{50}^{(Gs)}/EC_{50}^{(Gi/o)}$ 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 to WT CHO-m2 cells, whereas, Oxo-M exhibited a smaller $EC_{50}^{(Gs)}/EC_{50}^{(Gi/o)}$ ratio in MUT compared with WT CHO-m2 cells (Table 2).

Effect of PTx pre-treatment on expression levels of wild-type M_2 and $^{N410Y}M_2$ 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 pre-treatment, the binding affinities of MCh and Oxo in CHO-m2 MUT cells pre-treated with PTx were also determined (data not shown). Saturation radioligand binding analyses were also performed in both WT and MUT CHO-m2 cells pre-treated with PTx. PTx pre-treatment had no effect on either [^{3}H]-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 Figure 5A (CHO-m2 WT) and 5B (CHO-m2 MUT), that PTx pre-treatment significantly reduced the observed B_{max} in CHO-m2 MUT cells, but had no effect upon the maximal binding in CHO-m2 WT cells. B_{max} estimates are summarized in Figure 5C and Table 3.

Cell surface receptor expression in wild-type and $^{N410Y}M_2$ 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 Figure 5C; Table 3). Atropine *per se* did not significantly alter $^{N410Y}M_2$ mACh receptor expression levels in CHO cells, while PTx alone

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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 $^{N410Y}M_2$ 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 M_2 mACh receptors (see above), stably expressed $^{N410Y}M_2$ mACh receptors exhibited a small, but significantly greater affinity for [^{3}H]-NMS compared to the wild-type M_2 receptor (Table 3).

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Discussion

Some of the earliest examples of agonist-independent GPCR activity, and its pharmacological reversal by receptor antagonists (subsequently re-classified as inverse agonists), were reported for the M_2 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 (Jakubík et al., 1995) cell preparations endogenously or recombinantly expressing M_2 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 M_1-M_5 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 et al., 2002). Here, wild-type and N410Y-mutant M_2 mACh receptors have been compared with respect to agonist and antagonist (inverse agonist) actions using both transient and stable receptor expression in CHO cells.

Co-transfection of wild-type or N410Y-mutant M_2 mACh receptors with the chimeric G-protein $G\alpha_{qi5}$ allowed [^{3}H]-IP_x accumulation to be used as a readout of receptor activity. In this system, both the N410Y-mutant and wild-type M_2 mACh receptors exhibited atropine-inhibited constitutive activity with the $^{N410Y}M_2$ 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 $^{N410Y,T411P}M_2$ mACh receptor (Ford et al., 2002). Although G-protein over-expression has been shown to increase agonist-independent signaling for $G_{q/11}$-coupled mACh receptors (Burstein et al., 1997), $G\alpha_{qi5}$ was expressed at similar levels in wild-type and N410Y mutant receptor-expressing cells in our experiments. Therefore, G-protein over-expression *per se* cannot account for the differences between wild-type and mutant responses seen.

The constitutive receptor activity present in the recombinant M_2 mACh receptor/$G\alpha_{qi5}$ co-expression system allowed us to identify inverse agonism and to investigate its acute and chronic consequences. Of the antagonists studied we were particularly interested in comparing tolterodine, oxybutynin 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, since all seven of the mACh receptor antagonists assayed possessed properties consistent with inverse agonism.

Although the maximal inhibition of constitutive [^{3}H]-IP_x accumulation did not generally differ between wild-type and mutant receptor-expressing cells, atropine did display a substantially higher potency at the wild-type (pEC_{50} 9.04), compared with the N410Y-mutant (pEC_{50} 8.42) M_2 mACh receptor. According to the extended ternary complex model, inverse agonists may exert their effects via a selectively higher affinity for the inactive (R_i) than for the active (R_a) 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 R_a and/or R_aG will result in a reduction in the apparent binding affinity of an inverse agonist for the receptor 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 [^{3}H]-IP_x assays it is surprising that only darifenacin and oxybutynin displayed significantly lower affinities at the CAM receptor, while all other inverse agonists exhibited equivalent affinity for wild-type and CAM receptors. Previous 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); it is therefore possible that the differences 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 chronic (24 h) treatment of $^{N410Y}M_2$ mACh receptor-expressing CHO-cells with certain inverse agonists caused a significant, concentration-dependent up-regulation of cell surface receptor number is consistent 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 [^{3}H]-IP_x assays (with comparable negative efficacies) were capable of similarly facilitating an up-regulation of M_2 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 towards this signaling pathway. Alternatively, 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 (ER), facilitating their maturation and ER-export, might be important, as has been suggested for CAM μ-opioid receptors (Li et al., 2001). Alternatively, 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 M_2 mACh receptors emerged from studies initiated to assess $G_{i/o}$ protein involvement in trafficking. PTx pre-treatment of cells had no effect on the cell-surface expression level of the wild-type receptor, but caused a highly significant decrease in $^{N410Y}M_2$ mACh receptor expression. A possible cause of this down-regulation of the CAM receptor could be through destabilization caused by a decreased availability of $G_{i/o}$ 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 $^{N410Y}M_2$ mACh receptor. There is some evidence that $G_{i/o}$ proteins play a role in the endocytosis/intracellular trafficking of proteins (Lang et al., 1995; Valenti 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 pre-treatment of HEK293 cells stably expressing M_2 mACh receptors with PTx caused a modest increase in receptor expression.

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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 M_2 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 $^{N410Y}M_2$ mACh receptor, while 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 M_2 receptors through $G\alpha_s$ (following PTx-mediated inactivation of $G\alpha_{i/o}$ 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 M_2 receptor, where Oxo was relatively weak at activating $G\alpha_s$ compared with wild-type M_2 receptor. In contrast, Oxo-M displayed an unexpectedly high potency for the activation of $G\alpha_s$ through the CAM M_2 receptor, as illustrated by its relatively small $EC_{50}^{(Gs)}/EC_{50}^{(Gi/o)}$ ratio at the mutant receptor. The $EC_{50}^{(Gs)}/EC_{50}^{(Gi/o)}$ ratio has previously been used 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 M_2 mACh receptor (Akam et al., 2001). However, in the present study we found no evidence of ADTS for $G\alpha_{i/o}$ versus $G\alpha_s$ 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_{1A} receptor after mutation of sites within the i3 loop. Work with the M_1 mACh receptor has indicated that mutation of the conserved threonine residue at the TM6-e3 junction influences G-protein coupling, while enhanced potency, affinity and efficacy of agonists arises from mutation of the adjacent serine/asparagine residues (Huang et al., 1999). Our findings suggest that for the M_2 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 M_2 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 chronic treatment (Smit et al., 1996). The potential for up-regulation of mACh receptors to occur *in vivo*, following chronic treatment with inverse agonists, therefore requires consideration in the clinical use of these ligands.

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Footnotes

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Figure Legends

Fig. 1. (**A**) MCh-stimulated [^{3}H]-inositol phosphate accumulation in untransfected CHO-K1 cells (dashed line) or CHO-K1 cells transiently co-expressing $G\alpha_{qi5}$ and either wild-type (closed symbols) or N410Y mutant (open symbols) M_2 receptor. Data are expressed as mean percent of [^{3}H]-inositol phosphate accumulation over basal ± s.e.m, n=3. (**B**) Basal [^{3}H]-inositol phosphate accumulation in the absence (WT-, MUT-) and presence (WT+, MUT+) of atropine (1 μM) in CHO-K1 cells transiently co-expressing $G\alpha_{qi5}$ and either wild-type (WT) or N410Y mutant (MUT) M_2 mACh receptor. Results are expressed as means ± s.e.m, n≥3. Statistically significant differences between WT and MUT are indicated as $^{\ddagger}p<0.05$, and between basal and plus-atropine values as $^{*}p<0.05$.

Fig. 2. Inhibition of basal [^{3}H]-inositol phosphate accumulation, in CHO-K1 cells transiently co-expressing $G\alpha_{qi5}$ and either wild-type (**A**) or N410Y mutant (**B**) M_2 mACh receptor, by a range of mACh receptor antagonists: atropine (Atr), darifenacin (Dari), oxybutynin (Oxy), tolterodine (Tolt), pirenzepine (Pirenz), methoctramine (Methoc) and 4-DAMP. (**C**) Concentration-dependent inhibition of basal [^{3}H]-IP_x accumulation, in CHO-K1 cells transiently co-expressing $G\alpha_{qi5}$ 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 $^{*}p<0.05$.

Fig. 3. Effect of long-term (24 h) treatment of CHO-K1 cells transiently co-expressing $G\alpha_{qi5}$ and either wild-type (**A**) or N410Y mutant (**B**) M_2 mACh receptor with a range of mACh receptor ligands on M_2 receptor expression. (**C**) Concentration-dependent up-regulation of M_2 mACh receptor expression by atropine in CHO-K1 cells transiently co-expressing $G\alpha_{qi5}$ and N410Y mutant M_2 mACh receptor. Results are expressed as means ± s.e.m, n≥3. Statistically significant changes from control receptor expression levels are indicated as $^{*}p<0.05$.

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 following pre-treatment without (inhibition) or with (stimulation) PTx (100 ng ml^{-1}) for 20-24 h. (**A**) Methacholine (MCh), (**B**) oxotremorine-M (Oxo-M), (**C**) oxotremorine (Oxo) and (**D**) pilocarpine (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≥3.

Fig. 5. Effect of PTx pre-treatment (100 ng ml^{-1}; 20-24 h) on mACh receptor expression levels in WT (**A**) and MUT (**B**) CHO-m2 cells, assessed by [^{3}H]-NMS saturation binding analysis in intact cells. Data points were performed in duplicate and curves shown are representative of 3 or more experiments. (**C**) Effect on mACh receptor expression of incubating WT and MUT CHO-m2 cells with atropine (300 nM) alone or in combination with PTx (100 ng ml^{-1}) 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 $^{*}p<0.05$.

Table 1 Competition [^{3}H]-NMS binding data for a range of mACh receptor antagonists in membrane homogenates prepared from CHO cells stably expressing either the wild-type or N410Y mutant M_2 mACh receptor. Data are expressed as mean (s.e.m.) values from n≥3 experiments. * indicates significant differences between wild-type and mutant (p<0.05; Student's t test). ‡ indicates a significant difference to unity (p<0.05; F-test).

	CHO-m2 wild-type		CHO-m2 N410Y mutant	
	pK_i	**Hill slope**	**pK_i**	**Hill slope**
atropine	8.56 (0.05)	1.07 (0.19)	8.51 (0.06)	0.99 (0.21)
darifenacin	7.57 (0.16)	0.96 (0.03)	6.78 (0.07)*	1.01 (0.10)
oxybutynin	7.66 (0.03)	1.05 (0.10)	7.32 (0.07)*	1.25 (0.26)
tolterodine	8.62 (0.05)	1.01 (0.02)	8.69 (0.06)	1.01 (0.09)
pirenzepine	6.36 (0.05)	0.97 (0.07)	6.41 (0.02)	0.91 (0.12)
methoctramine	8.31 (0.02)	1.64 (0.10)‡	8.13 (0.16)	1.40 (0.06)‡

Table 2 Intact cell apparent binding affinity constant (pK_i) 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 M_2 mACh receptors. Data are expressed as mean (s.e.m.) values from n≥3 experiments. * indicates significant differences between wild-type and mutant ($p<0.05$; Student's *t* test); ‡ indicates significant differences between $pEC_{50}^{(Gs)}$ and $pEC_{50}^{(Gi/o)}$ values ($p<0.05$; Student's *t* test); and # indicates that an E_{max} value is significantly less than 100% ($p<0.05$; one-way ANOVA, Dunnett's post-test).

	pK_i	$pEC_{50}^{(Gi/o)}$	$E_{max}^{(Gi/o)}$ (%)	$pEC_{50}^{(Gs)}$	$E_{max}^{(Gs)}$ (%)	$EC_{50}^{(Gs)}/EC_{50}^{(Gi/o)}$
	CHO-m2 wild-type					
MCh	4.97 (0.04)	6.66 (0.05)	100	5.01 (0.12)‡	100	44.7
Oxo-M	5.58 (0.02)	7.37 (0.05)	101.7 (0.7)	5.70 (0.08)‡	98.4 (4.0)	46.8
Oxo	6.02 (0.03)	7.07 (0.08)	99.1 (1.8)	5.51 (0.12)‡	36.5 (10.1)#	36.3
Pilo	3.77 (0.04)	4.48 (0.06)	68.3 (5.4)#	n.d.	5.2 (1.0)#	n.d.
	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)‡	66.8 (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.

Table 3 [^{3}H]-NMS binding affinity constant (K_D) and receptor expression (B_{max}) estimates at intact CHO cells expressing either wild-type (WT) or N410Y mutant (MUT) M_2 mACh receptors, with or without PTx (100 ng ml^{-1}; 20-24 h) and atropine (300 nM; 24 h) pre-treatment. Data are expressed as mean (s.e.m.) values from n≥3 experiments. * indicates significant differences between wild-type and mutant (p<0.05; Student's t test); ‡ indicates a significant difference between untreated and PTx pre-treated cells (p<0.05; Student's t test).

	K_D (nM)	**B_{max} (fmol mg^{-1} protein)**	
		- atropine	**+ atropine**
CHO-m2 WT	0.22 (0.02)	1235 (62)	1197 (28)
CHO-m2 WT + PTx	0.21 (0.02)	1359 (70)	1263 (41)
CHO-m2 MUT	0.16 (0.01)*	637 (53)	725 (90)
CHO-m2 MUT + PTx	0.16 (0.01)	427 (47)‡	476 (95)

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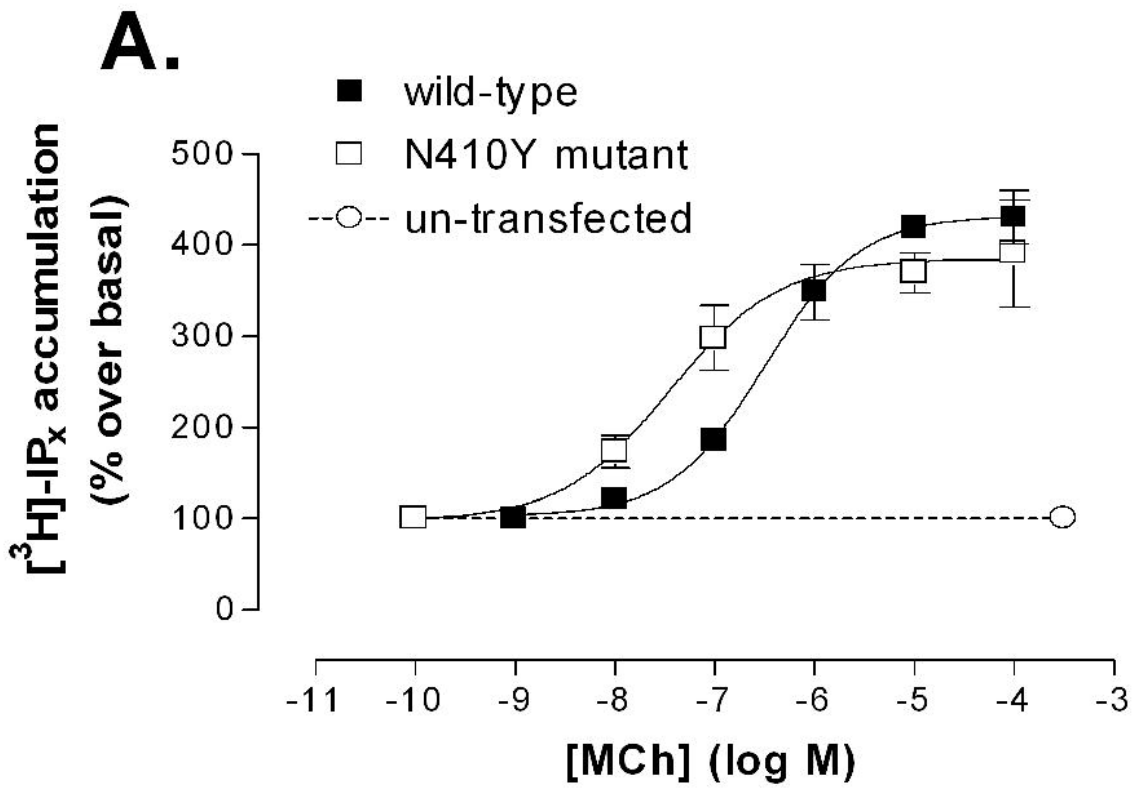
Figure 1



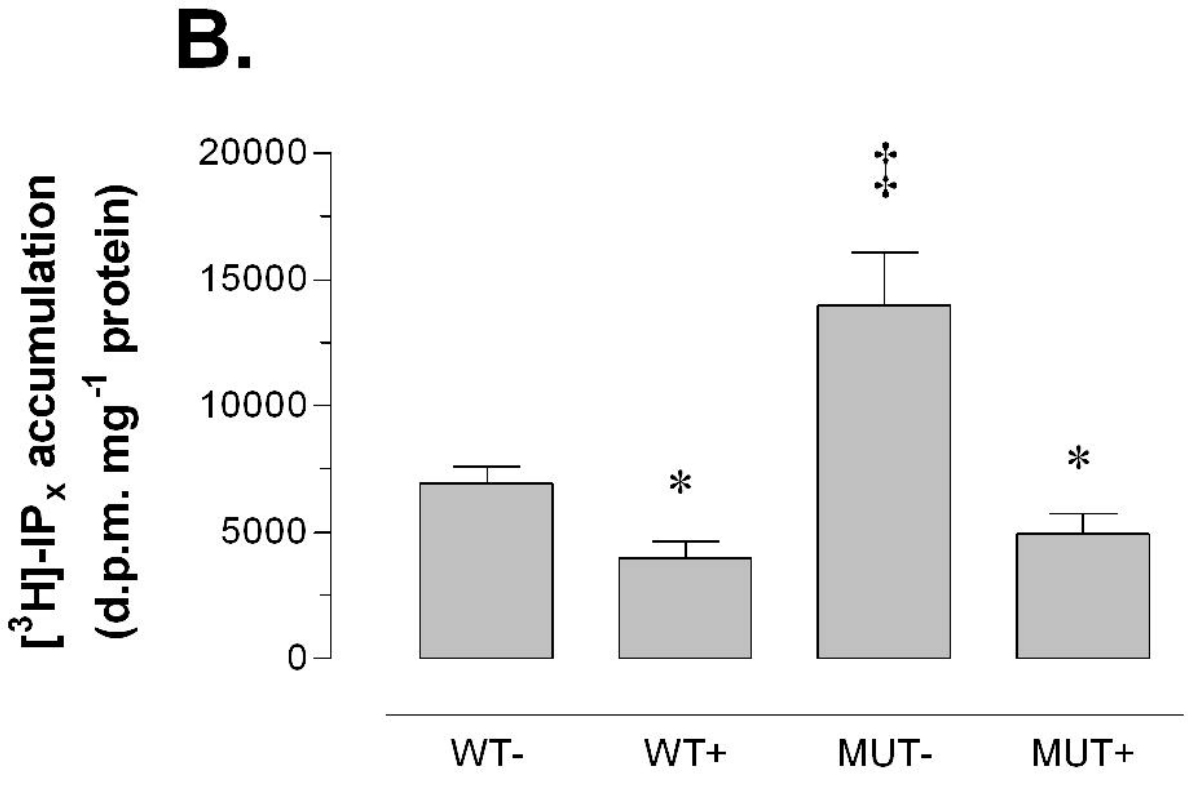


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Figure 2

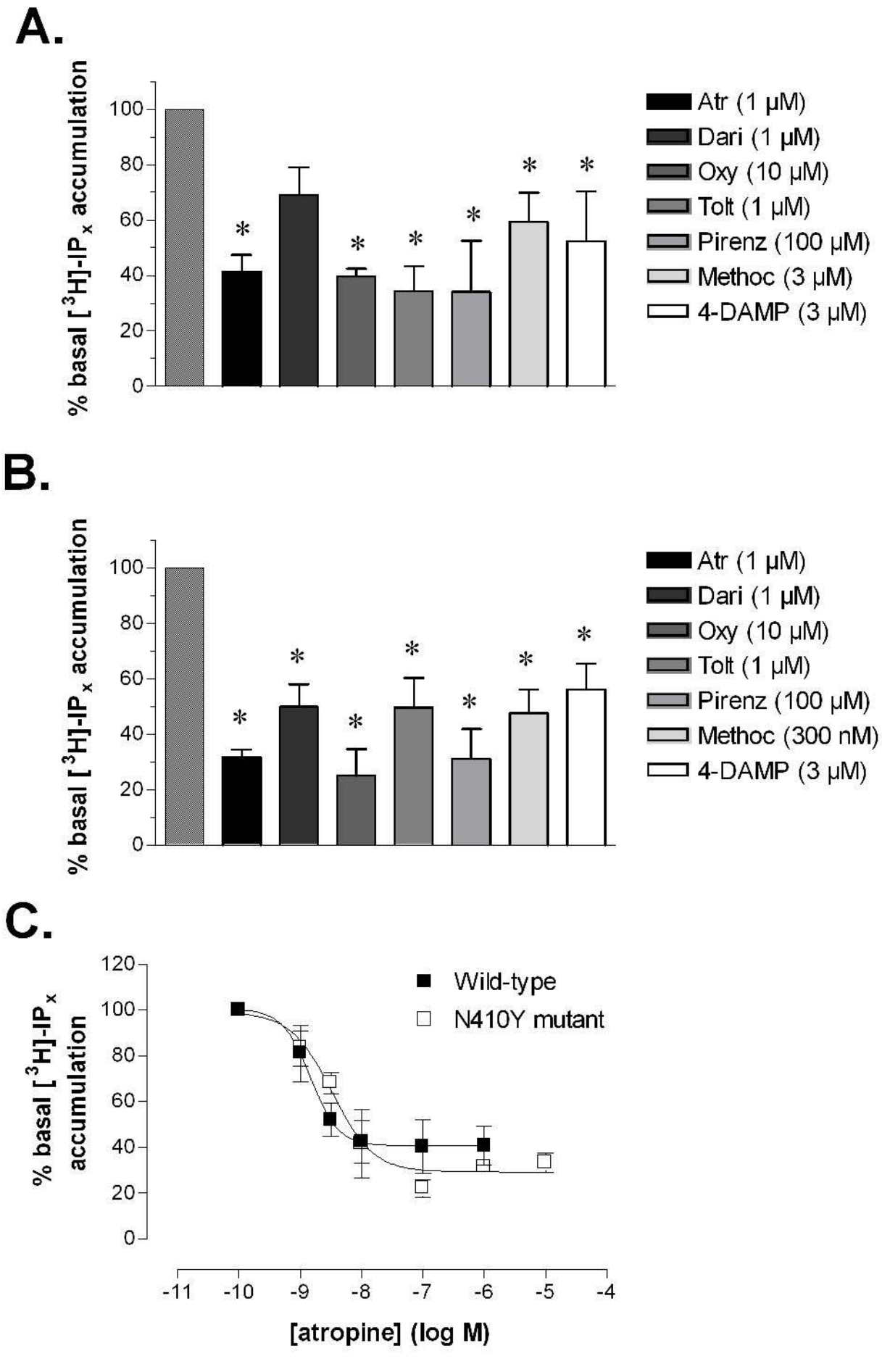

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Figure 3

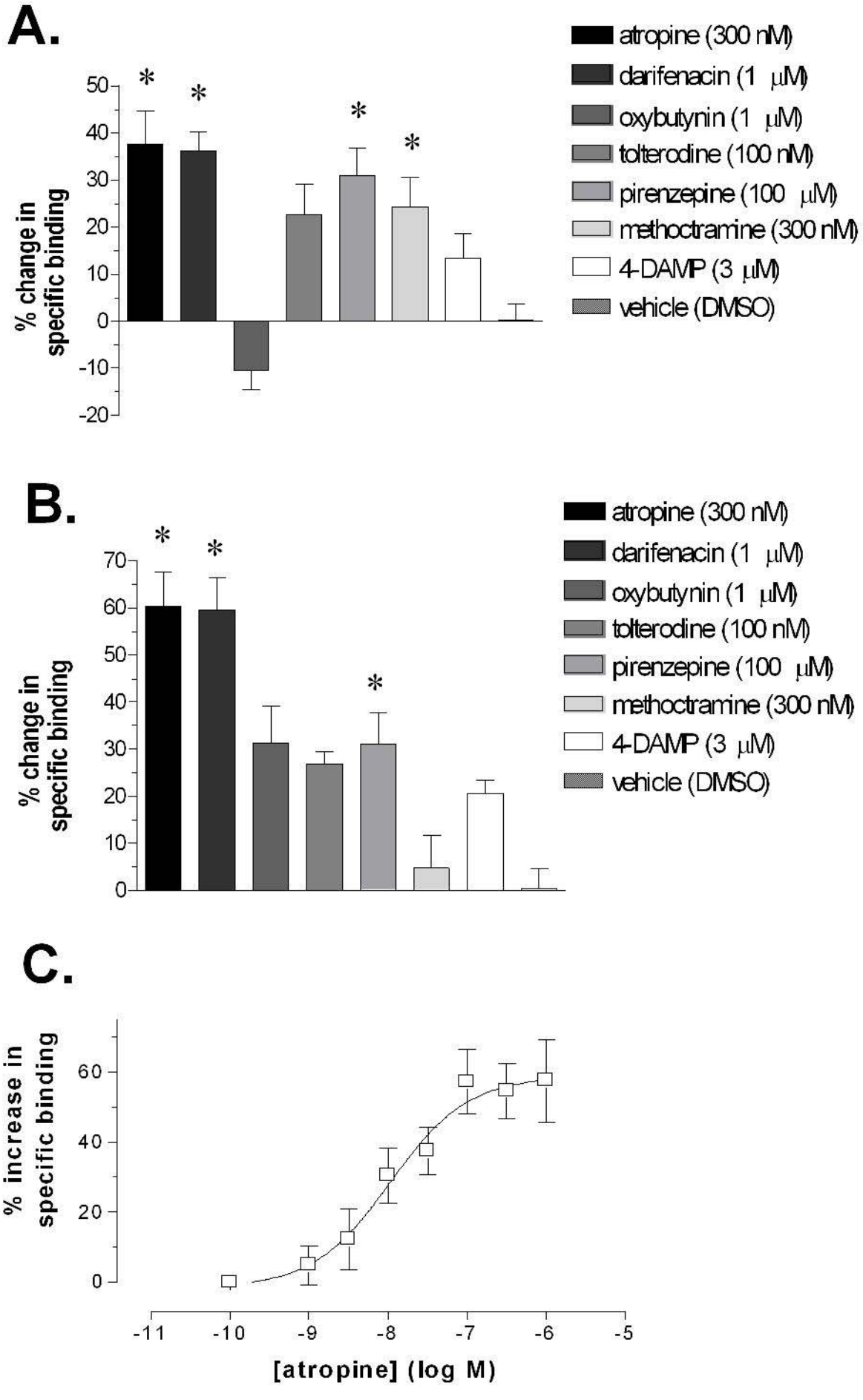

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Figure 4

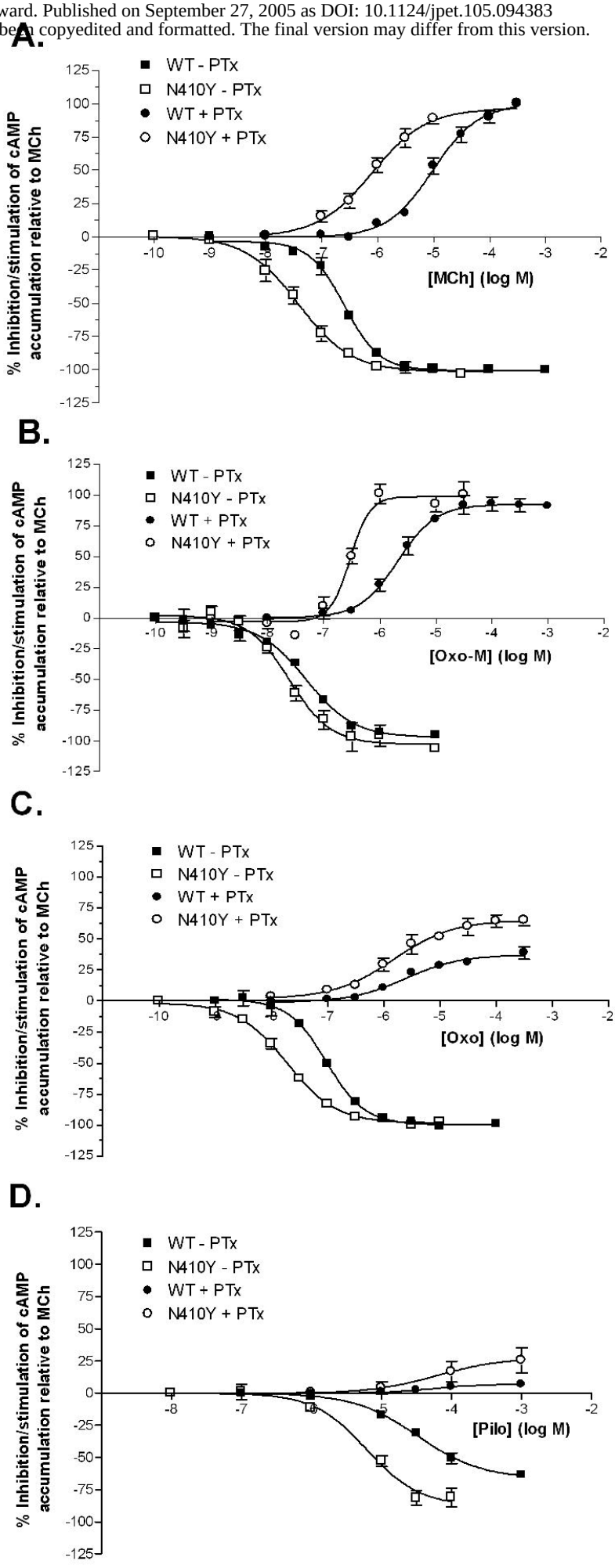

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Figure 5