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# The M1 Muscarinic Receptor Allosteric Agonists AC-42 and 1-[1′-(2-Methylbenzyl)-1,4′-bipiperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one Bind to a Unique Site Distinct from the Acetylcholine Orthosteric Site

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## ABSTRACT

Activation of M1 muscarinic receptors occurs through orthosteric and allosteric binding sites. To identify critical residues, site-directed mutagenesis and chimeric receptors were evaluated in functional calcium mobilization assays to compare orthosteric agonists, acetylcholine and xanomeline, M1 allosteric agonists AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride), TBPB (1-[1′-(2-methylbenzyl)-1,4′-bipiperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one), and the clozapine metabolite *N*-desmethylclozapine. A minimal epitope has been defined for AC-42 that comprises the first 45 amino acids, the third extracellular loop, and seventh transmembrane domain (*Mol Pharmacol* **61:**1297–1302, 2002). Using chimeric M1 and M3 receptor constructs, the AC-42 minimal epitope has been extended to also include transmembrane II. Phe77 was identified as a critical residue for maintenance of AC-42 and TBPB agonist activity. In contrast, the functional activity of *N*-desmethylclozapine did not require Phe77. To further map the binding site of AC-42, TBPB, and *N*-desmethylclozapine, point mutations previously reported to affect activities of M1 orthosteric agonists and antagonists were studied. Docking into an M1 receptor homology model revealed that AC-42 and TBPB share a similar binding pocket adjacent to the orthosteric binding site at the opposite face of Trp101. In contrast, the activity of *N*-desmethylclozapine was generally unaffected by the point mutations studied, and the docking indicated that *N*-desmethylclozapine bound to a site distinct from AC-42 and TBPB overlapping with the orthosteric site. These results suggest that structurally diverse allosteric agonists AC-42, TBPB, and *N*-desmethylclozapine may interact with different subsets of residues, supporting the hypothesis that M1 receptor activation can occur through at least three different binding domains.

## Introduction

Cholinergic dysfunction has been associated with the pathophysiology of Alzheimer's disease and psychiatric disorders, including schizophrenia. Evidence from preclinical and clinical studies suggests that overcoming the deficits in the M1 muscarinic acetylcholine receptor with mechanisms leading to selective receptor activation may contribute to improving neuropsychiatric symptoms in patients with Alzheimer's disease or schizophrenia (Langmead et al., 2008; Caccamo et al., 2009). Acetylcholinesterase inhibitors are a current first-line treatment for symptoms resulting from the decline in cognitive function in Alzheimer's disease. The M1/M4 agonist xanomeline has shown improvements in psychotic behaviors and cognitive abilities in patients with Alzheimer's disease (Bodick et al., 1997) and improving psychosis in patients with schizophrenia (Shekhar et al., 2008). The M1 muscarinic receptor belongs to a family of G-protein-coupled receptors composed of five subtypes designated M1 through M5. The M1, M3, and M5 subtypes couple preferentially through $G_q$ and M2 and M4 receptors couple through $G_o/G_i$. The M1 muscarinic receptor subtype is most abundantly expressed in prefrontal cortex and hippocampus, regions that are associated with involvement with cognitive functioning. Development of selective agonists of the muscarinic receptor subtypes has been challenging because of the high conservation of the orthosteric acetylcholine binding

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**ABBREVIATIONS:** TM, transmembrane; AC-42, 4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride; TBPB, 1-[1′-(2-methylbenzyl)-1,4′-bipiperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one; QNB, quinuclidinyl benzilate; NMS, *N*-methylscopolamine; CHONFAT, Chinese hamster ovary nuclear factor of activated T-cells; FLIPR, fluorescence imaging plate reader.

site among the five subtypes. Lack of subtype selectivity has prevented development of muscarinic agonists as therapeutics because activity on peripheral M2 and M3 muscarinic subtypes is associated with adverse side effects such as salivation, bradycardia, and gastrointestinal disturbances (Bymaster et al., 2003; Wess et al., 2003).

Significant advancement in the identification of selective M1 muscarinic receptor agonists has been realized by focusing on the discovery of allosteric modulators of the M1 receptor (Spalding et al., 2002; Jones et al., 2008; Ma et al., 2009; Marlo et al., 2009). Two types of allosteric modulators of M1 muscarinic receptors have been reported: allosteric agonists and allosteric modulators. The pharmacological action of these molecules differ in that allosteric agonists can produce receptor activation in the absence of the orthosteric agonist acetylcholine, whereas allosteric potentiators require the presence of the orthosteric agonist and exert no pharmacological action alone. Several models of the orthosteric binding site have been proposed on the basis of mutagenesis and molecular docking studies with the endogenous agonist acetylcholine and the antagonist *N*-methylscopolamine (Hulme at al., 2003; Peng et al., 2006). These studies have revealed that the binding site for acetylcholine is located within the transmembrane domains with contact residues contributed by TM3, TM4, TM6, and TM7. In contrast, both allosteric agonists and positive modulators are postulated to bind to sites distinct from the orthosteric site. The number of unique sites and molecular determinants of allosteric binding sites on M1 receptors and the proximity of allosteric sites to the orthosteric agonist site are unknown, and it is unclear whether there may be overlap among these sites for binding these ligands.

4-*n*-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride (AC-42) was the first allosteric agonist described for the M1 muscarinic receptor (Spalding et al., 2002; Langmead et al., 2006). Mutagenesis studies employing chimeric receptors and point mutations in the orthosteric acetylcholine binding site defined a minimum epitope composed of the first 45 amino acids, the third extracellular loop, and the seventh transmembrane domain. More recently, a novel structure, 1-[1′-(2-methylbenzyl)-1,4′-bipiperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one (TBPB), has also been described as an M1-selective allosteric agonist (Jones et al., 2008). We have shown previously that *N*-desmethylclozapine, a major metabolite of the atypical antipsychotic, clozapine displayed partial agonist activity on M1 receptors (Sur et al., 2003). Furthermore, *N*-desmethylclozapine was postulated to be an allosteric agonist of the M1 receptor on the basis of observations of functional activity maintenance on M1 receptors with a Y381A mutation in the orthosteric agonist site, which significantly diminished the receptor's potency for acetylcholine. However, kinetic dissociation studies demonstrated that *N*-desmethylclozapine failed to modulate the binding of orthosteric ligands and therefore did not meet the criteria for an allosteric modulator (Spalding et al., 2006). The specific binding site for *N*-desmethylclozapine was not defined in these studies.

In this study, we used mutagenesis and chimeric receptor approaches to define amino acid residues required for the binding of the agonists acetylcholine and xanomeline and the allosteric agonists AC-42, TBPB, and *N*-desmethylclozapine. This study further extends the model proposed for AC-42 binding (Spalding et al., 2002, 2006; Lebon et al., 2009); in addition, it proposes a model for the newly described M1 allosteric agonist TBPB. As a result, we define the structural relationship between orthosteric and allosteric binding sites and provide further support for M1 receptor activation occurring through at least three different binding domains.

## Materials and Methods

**Ligands.** Acetylcholine and atropine were from Sigma-Aldrich (St. Louis, MO), *N*-desmethylclozapine, clozapine and gallamine were purchased from Tocris (Ellisville, MO). AC-42, TBPB, and xanomeline were synthesized at Merck Research Labs, Medicinal Chemistry. [$^3$H]Quinuclidinyl benzilate (QNB; 30–60 Ci/mmol) and [$^3$H]*N*-methylscopolamine (NMS) (81 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA).

**DNA Constructs and Expression.** Chimeric receptor cDNAs were constructed by overlapping PCR from human M1 and M3 muscarnic cDNA templates. NC3 M1M3 corresponds to M1 amino acids 1 to 104; M3, 148 to 513; and M1, 388 to 460. NC4M1M3 corresponds to M1 amino acids 1 to 45; M3, 89 to 513; M1, 388 to 419; and M3, 546 to 590. Single point mutations of M1 and NC4M1M3 were introduced with the Stratagene Quik-Change Kit (La Jolla, CA) and confirmed by sequencing. M1, M1Y381A, NC3M1M3, and NC4M1M3 were subcloned in pcDNA3.1 and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) into CHONFATbla cells. Stable clones were selected in 1 mg/ml G418, and cells were cultured in growth media (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 25 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 units/ml penicillin/streptomycin, 2 mM glutamine, and 250 $\mu$g/ml Zeocin) and grown in 5% $CO_2$, 37°C. Point mutations of NC4M1M3 or M1 wild type were transiently transfected with Lipofectamine 2000 and analyzed in parallel with M1 wild-type and nonmutated NC4M1M3 receptors, 48 h after transfection.

**Membrane Preparations and Binding Studies.** Membranes from stably or transiently transfected CHONFAT cell lines were prepared by homogenization in 20 mM HEPES and 1 mM EDTA, pH 7.4. Membranes were resuspended in 20 mM HEPES and 5 mM $MgCl_2$, pH 7.4. The expression level of wild-type, chimeric, and mutated muscarinic receptors was determined by [$^3$H]QNB binding performed according to Ward et al. (1999) in 20 mM HEPES, 100 mM NaCl, and 1 mM $MgCl_2$, pH 7.4 at 30°C, with 20 $\mu$g of protein per tube in a final volume of 500 $\mu$l for 3 h. Nonspecific binding was determined with the use of 10 $\mu$M atropine. The assay was terminated by rapid filtration onto GFC filter plates presoaked in 0.3% polyethylenimine, followed by four washes with ice-cold assay buffer using a Brandel (Montreal, QC, Canada) 96 Harvester. Dried plates were counted in a Topcount microplate scintillation and luminescence counter with Microscint-20 (PerkinElmer Life and Analytical Sciences). Binding data represent the mean of two determinations and were analyzed by nonlinear regression analysis with the use of Prism software (GraphPad Software, Inc., San Diego, CA). Receptor expression levels for stable M1 wild-type and Y381A point mutant cell lines were 475 ± 25 and 1050 ± 50 fmol/mg, respectively. Receptor expression for stably expressed chimeric M1 M3 constructs NC3M1M3 and NC4M1M3 was 3.1 ± 0.5 and 3.3 ± 0.2 pmol/mg, respectively. Receptor expression for transiently expressed M1 wild-type and M1 point mutants are reported in Table 3.

Kinetic off-rate experiments were performed with 200 pM [$^3$H]NMS and 10 $\mu$g/tube hM1 membranes in buffer containing 20 mM HEPES, 100 mM NaCl, and 5 mM $MgCl_2$, pH 7.4. Membranes plus [$^3$H]NMS in the presence or absence of 1 $\mu$M atropine were incubated at room temperature for 1 h to achieve equilibrium. At the end of the incubation, 1 $\mu$M atropine in the absence or presence of 100 $\mu$M TBPB, 10 $\mu$M clozapine, or 1 mM gallamine was added, and the bound [$^3$H]NMS was captured at various time points on GF/C Filtermats (Skatron; Molecular Devices, Sunnyvale, CA) presoaked in 0.3% polyethylenimine by filtration on a Combi Cell Harvester

(Molecular Devices) and washing with ice-cold assay buffer. The amount of specific [$^3$H]NMS bound was calculated and analyzed by Prism using the one-phase exponential decay equation.

**Fluorescence Imaging Plate Reader Measurements.** Stable or transiently transfected CHONFAT cells were plated in clear-bottomed, poly-D-lysine–coated 384-well plates (BD Biosciences, Franklin lakes, NJ) in growth medium using a Multidrop (Thermo Fisher Scientific, Waltham, MA). The plated cells were grown overnight at 37°C in the presence of 5% $CO_2$. The following day, the cells were washed three times with 100 $\mu$l of assay buffer (Hanks' balanced salt solution containing 20 mM HEPES, 2.5 mM probenecid, and 0.1% bovine serum albumin) using a Skatron Embla cell washer (Molecular Devices). The cells were incubated with 1 $\mu$M Fluo-4AM (Invitrogen, Carlsbad, CA) for 1 h at 37°C and 6% $CO_2$. The extracellular dye was removed by washing as described above. Calcium flux was measured using Molecular Devices $FLIPR_{384}$ fluorometric imaging plate reader. For dose response curves, cells were incubated for 4 min in assay buffer before addition of various concentrations of agonists, and calcium flux was measured for 4 min after agonist addition. Functional data represent the means of at least three determinations performed in triplicate and were analyzed using PRISM software (GraphPad Software, Inc.).

**M1 Receptor Homology Model Building.** The homology model of M1 was built using the rhodopsin X-ray crystal structure (Palczewski et al., 2000) as a template. Alignment of the seven transmembrane helices was accomplished using established conserved residues, which are hallmarks of class A G-protein-coupled receptors (Fredriksson et al., 2003). Residues Leu225-Lys353, comprising intracellular loop 3 of M1, were deleted to facilitate model building. One thousand initial models were built using MOE 2005.06 (Chemical Computing Group, Montreal, QC, Canada) and the 10 lowest energy models were taken into the Prime module of Maestro (Schrodinger, LLC, Portland, OR) for loop refinement (Jacobson et al., 2004), while maintaining the disulfide bond between Cys98 (3.25) and the second extracellular loop Cys178. Amino acids in the model are referred to according to their M1 sequence position and supplemented by the nomenclature of Ballesteros and Weinstein (1995). Rotamer sampling using the software vendor's rotamer libraries was allowed during all phases of refinement.

**Small Molecule Docking Protocol.** Initial ligand docking was accomplished using the induced fit protocol as implemented in the Maestro interface (Sherman et al., 2006) using GlideXP with enlarged inner and outer boxes and a distance-dependent dielectric constant ($\varepsilon$ = 4). All ligand poses within 25 kcal/mol of the global minima (irrespective of score) were kept and further flexibly refined using MMFF94s (Halgren, 1999) as implemented in our in-house MIX (modeling in UNIX) system. During this stage of docking, all protein side chains within an 8-Å radius and the backbone of the extracellular loops were allowed mobility while maintaining the disulfide bond between Cys98 (3.25) and the second extracellular loop Cys178. After this round of refinement, ligand poses within the lowest 10 kcal/mol of their global in vacuo minima were inspected visually, and the partner protein conformer from each pair was inspected for side-chain/backbone geometries that fell within the allowed Ramachandran space. Those with many Ramachandran violations were rejected. Individual side-chain rotamers were sampled using the rotamer library in PyMol (http://www.pymol.org/) and reminimized using the above protocol.

## Results

The M1 muscarinic receptor agonists profiled in these studies included the orthosteric agonist acetylcholine, the muscarinic M1/M4 agonist xanomeline, the clozapine metabolite *N*-desmethylclozapine, the M1 allosteric agonist AC-42, and a newly described M1 allosteric agonist, TBPB (Fig. 1). The functional activities of these ligands were first evaluated on stably expressed wild-type M1 and Y381A receptors in calcium mobilization assays measured by FLIPR. Mutation of the highly conserved Tyr381(6.51) in transmembrane VI (TMVI) has been previously shown to drastically reduce the ability of orthosteric ligands to bind and activate the M1 receptor (Ward et al., 1999). The Y381A point mutation was also used in the first in vitro characterization of AC-42 by Spalding et al., (2002), where in contrast to acetylcholine, functional agonist activity was observed to be maintained, suggesting that the binding site for AC-42 on M1 receptors may be different from the orthosteric agonist site. We have also previously shown that *N*-desmethylclozapine, a major metabolite of the atypical antipsychotic clozapine, retained functional activity on the Y381A mutant and exhibited increased potency and efficacy (Sur et al., 2003). This is in contrast to a marked decreased activity observed with acetylcholine on the Y381A mutant. TBPB, an M1 allosteric agonist (Jones et al., 2008) was also found to exhibit an increased potency of ~10-fold and increased efficacy on the Y381A mutant compared with wild-type M1 receptors (Table 1). Compared with these observations with TBPB, AC-42, and *N*-desmethylclozapine, the functional potency and maximal efficacy of xanomeline was found to be slightly reduced on the Y381A mutant.

To further investigate the allosteric interactions of TBPB at M1 receptors, kinetic experiments of [$^3$H]NMS dissociation were carried out on CHO-hM1 cell membranes. As reported in Fig. 2, the orthosteric ligand atropine (1 $\mu$M) alone displaced bound [$^3$H]NMS with a $k_{off}$ of 0.060 ± 0.004/min (mean ± S.E.M.) equivalent to a $t_{1/2}$ of 12 min. In contrast, the classic muscarinic allosteric compound gallamine drastically slowed the atropine-induced dissociation of [$^3$H]NMS as evidence by a $t_{1/2}$ of 97 min and $k_{off}$ of 0.0071 ± 0.0016/min. This result was expected and consistent with what has been previously reported for gallamine (Langmead et al., 2006; Spalding et al., 2006). In comparison, TBPB (100 $\mu$M) showed a significant retardation of [$^3$H]NMS dissociation, $t_{1/2}$ of 20 min, and $k_{off}$ of 0.034 ± 0.002/min. This effect of TBPB was similar to that reported for AC-42, where a significant retardation of the [$^3$H]NMS dissociation rate was observed (Langmead et al., 2006; Spalding et al., 2006). In contrast to AC-42, Spalding et al.,

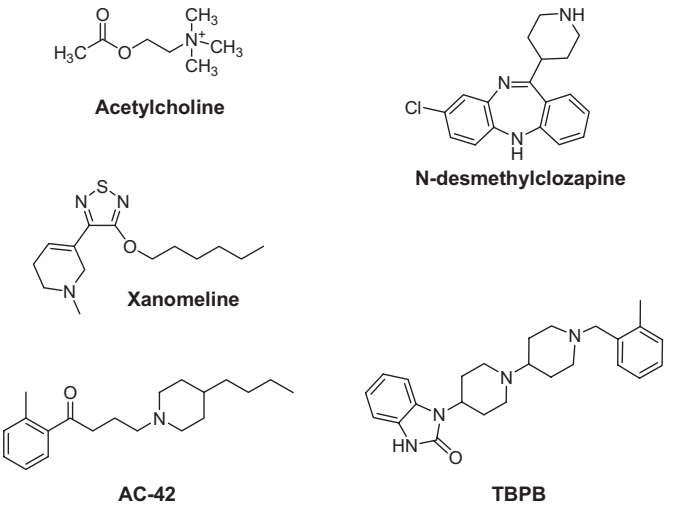


**Fig. 1.** Structures of M1 muscarinic receptor orthosteric and allosteric agonists.

(2006) demonstrated that neither *N*-desmethylclozapine nor clozapine produced retardation of the [$^3$H]NMS dissociation rate. Similarly to previous published reports, clozapine was found to lack a significant effect ($t_{1/2}$ = 12 min.; $k_{off}$ = 0.057 ± 0.004/min (Fig. 2).

The functional activity of the M1 muscarinic agonists was further profiled on chimeric receptors constructed between M1 and M3 muscarinic receptor subtypes in the attempt to determine binding epitopes for the various ligands. In a series of elegant experiments, Spalding et al. (2002) employed chimeric receptors composed of M1 and M5 muscarinic receptor sequences and defined a minimal binding epitope for AC-42 composed of the first 45 amino acid residues of M1 corresponding to the amino terminus and TMI, the third extracellular loop and TMVII domains. In the present study, the M3 muscarinic receptor subtype was chosen to generate chimeric receptors with M1, because the M3 receptor exhibits less homology with M1 sequences compared with the M5 receptor subtype. Evaluation of M1–M3 chimeras could potentially identify additional critical residues for binding of AC-42 and other M1 agonists that may have been obscured by the high degree of homology between M1 and M5 receptor amino acid sequences. The chimeric constructs were designated NC3M1M3 and NC4M1M3. NC3M1M3 consisted of the M3 receptor with substitution of the first 45 amino acids of the M1 sequence (amino terminus and TMI), the third extracellular loop and TMVII of M1. NC3M1M3 contained additional M1 sequence compared with NC4M1M3 and included the first intracellular loop and TMII of M1 and the carboxyl terminal tail of M1. Stable clones of NC3M1M3 and NC4M1M3 were established, and the functional activities of the M1 agonists were measured on the chimeric receptors in calcium mobilization assays measured by FLIPR and compared with stably expressed wild-type M1 receptors (Table 2). Cell lines were selected for evaluation and comparison of functional activities on the basis of having comparable expression levels. The functional potency and maximum response of acetylcholine, xanomeline and *N*-desmethylclozapine were found to be similar between wild-type M1, NC3M1M3, and NC4M1M3 chimeric receptors (Table 2). In contrast to the profiles observed with acetylcholine, xanomeline, and *N*-desmethylclozapine, a dramatic reduction in activity was determined for both AC-42 and TBPB ($EC_{50}$ >100 μM) on the NC4M1M3 chimeric receptor (Table 2). In comparison, a partial restoration of activity was observed for AC-42 and TBPB on the NC3M1M3 chimera. These results indicated a role for amino acid residues within TMII of the M1 receptors for maintaining functional potency and efficacy of AC-42 and TBPB and suggested that the binding sites for the allosteric agonists were differentiated from acetylcholine, xanomeline, and *N*-desmethylclozapine.

To further elucidate the amino acid residues in TMII involved in binding AC-42 and TBPB, single point mutations were introduced into the NC4M1M3 construct in the attempt to generate a "gain of function" mutant and identify amino acid residues that may contribute restoration of activity with AC-42 and TBPB on the chimeric receptor. To guide selection of amino acids for mutagenesis, the sequences comprising TMII, intracellular loop 1 and extracellular loop 1 were aligned between M1, M3, and M5 receptor subtypes to locate differences between the sequences, specifically, those amino acids in M3 that differed from M1 and M5 (Fig. 3). A comparison with M5 sequences was included because Spalding et al. (2002) showed that AC-42 activity was retained in a corresponding chimera, NC4M1M5, composed of M1 and M5 sequences. Therefore, amino acids for mutagenesis were selected on the basis of being conserved in both M1 and M5 sequences in the designated region of the NC4 chimera yet were different in the M1 and M3 subtypes. Using this distinction, three new chimeric constructs were generated to individually replace amino acids in NC4M1M3 originating from M3 sequence with corresponding residues derived from the M1 TMII sequence. Specifically, amino acids corresponding to M3 TM sequences Ile120 (2.56), Asn132, and Asn138 in the extracellular loops were identified for mutagenesis, substituted with the analogous M1 amino acid sequence Phe77 (2.56), Gly89, and Thr95, respectively, in the NC4M1M3 chimeric construct, and evaluated for the potential role in restoring activity of AC-42 and TBPB. The constructs were evaluated on transiently expressed receptors with the functional calcium mobilization assay in FLIPR (Fig. 4) Resultant expression levels were comparable for the three transiently expressed constructs (5.8 ± 0.9, 9.1 ± 1.6, and 7.3 ± 0.2 pmol/mg for Phe, Gly, and Thr substitutions, respectively). The functional potency and maximum response of acetylcholine was similar for the mutated NC4M1M3 constructs; acetylcholine $EC_{50}$ = 2.1 ± 0.4, 3.0 ± 0.9, and 5.2 ± 1.5 nM for Phe, Gly, and Thr substitutions, respectively. Substitution of the extracellular loop residues, Asn89 or Asn95 with corresponding M1 residues glycine or threonine, respectively, had no effect on restoring potency or efficacy of AC-42 or TBPB on the NC4M1M3 chimera. In contrast, substitution of Phe77 in NC4M1M3 for isoleucine, resulted in an increase in both AC-42 and TBPB potencies and maximal response; however, this substitution did not completely restore the agonist potencies to that observed on M1 wild-type receptors. $EC_{50}$ values were determined to be $EC_{50}$ = 3602 ± 331 nM, max percentage = 75 ± 4% for AC-42 and $EC_{50}$ = 361 ± 80 nM, max percentage = 55 ± 6% for TBPB and reflected 4- and 16-fold lower potency for AC-42 and TBPB, respectively, com-

TABLE 1

Functional activity of muscarinic agonists at wild-type and Y381A mutated M1 receptors

$EC_{50}$ values and percentage of maximum acetylcholine response are presented as mean ± S.E.M. of three to six determinations. Shift is $EC_{50}$ value on Y381A mutant/$EC_{50}$ value on wild-type M1.

| | hM1-WT | | Y381A | | Shift |
|---|---|---|---|---|---|
| | $EC_{50}$ | Max | $EC_{50}$ | Max | |
| | *nM* | % | *nM* | % | |
| Acetylcholine | 21 ± 6 | 100 | 3837 ± 677 | 100 | 183 |
| Xanomeline | 19 ± 7 | 81 ± 3 | 42 ± 7 | 69 ± 3 | 2.2 |
| AC-42 | 805 ± 251 | 71 ± 2 | 220 ± 16 | 83 ± 3 | 0.27 |
| TBPB | 22 ± 2 | 77 ± 2 | 2 ± 1 | 90 ± 1 | 0.09 |
| *N*-Desmethylclozapine | 115 ± 28 | 50 ± 5 | 14 ± 2 | 96 ± 2 | 0.12 |

pared with values measured on wild-type M1 receptors (Table 1). In addition, maximal activity of AC-42 and TBPB was found to be improved with the introduction of the phenylalanine residue into the NC4M1M3 chimeric construct. Nonetheless, these results implicate the contribution of Phe77 in TMII of the M1 receptor in supporting the functional activity of AC-42 and TBPB.

To further understand the molecular components of the binding pocket for M1 agonists and the relationship between orthosteric and allosteric agonist binding sites, a homology model of the M1 receptor was generated using the bovine rhodopsin crystal structure as a template. In this model, the side chain of Phe77 (2.56) identified to be a critical residue for maintenance of AC-42 and TBPB functional potency was determined to be positioned facing inward into a pocket composed of hydrophobic residues contributed from TMIII, TMVI, and TMVII (Fig. 5). The orientation of Phe77 is similar to that observed in the analogous residue (Phe83, 2.56) of the squid rhodopsin X-ray crystal structure (Murakami et al., 2008). In contrast, Gly89 and Thr95, residues that were found to have no effect on restoring AC-42 and TBPB activity in the NC4M1M3 chimera, were located in the first extracellular loop spatially distinct from the orthosteric site. The Phe77 residue in M1 was found to reside at the base of this hydrophobic channel of amino acid residues leading up to the Asp105 (3.32), previously identified by mutagenesis to be a critical residue for binding acetylcholine and postulated to participate in a salt bridge with the choline head group of the orthosteric agonist (Page et al., 1995; Hulme et al., 2003). Furthermore, the orthosteric binding pocket has been proposed to be composed of a hydrophobic network of aromatic residues contributed by the exofacial domains of TMIV, TMVII, TMIII, and TMVI. Site-directed mutagenesis of the M1 muscarinic receptor was previously employed to evaluate the role of specific amino acids contributed by TMIII and TMVI in binding acetylcholine (Matsui et al., 1995; Lu and Hulme, 1999; Lu et al., 2001). From these studies, Trp101 (3.28), Tyr404 (7.39) and Tyr408 (7.43) have been reported to affect orthosteric agonist binding, because mutagenesis of these residues resulted in decreases for acetylcholine binding affinity. We further explored the consequence of mutating these specific amino acids on the functional activity of AC-42, TBPB, xanomeline, and *N*-desmethylclozapine and evaluated the role of hydrophobic side chains by substitution of either phenylalanine or alanine for the wild-type aromatic residue to determine the functionality of the hydroxyl group separately from the phenyl ring. Using the M1 homology model, Tyr179 and Phe374 (6.44) were also identified as residues that could participate in the allosteric binding pocket and were included in the mutagenesis studies. It is noteworthy that a corresponding tyrosine epitope was identified on the M2 receptor, Tyr177 to be important for subtype selectivity of alkane-bisammonium-type and caracurine V-type allosteric modulators (Voigtländer et al., 2003).

The functional activities of the muscarinic agonists acetylcholine, xanomeline, AC-42, TBPB, and *N*-desmethylclozapine on transiently expressed receptors containing the selected point mutations are summarized in Table 3. Because differences in receptor expression could affect interpretation of the functional activity results measured for the point mutations, and mutagenesis of specific amino acids in M1 has been previously reported to decrease M1 receptor expression (Hulme et al., 2003), the level of expression was measured for all the mutant receptors by [$^3$H]QNB binding, and values are included in Table 3. The majority of the point mutants gen-

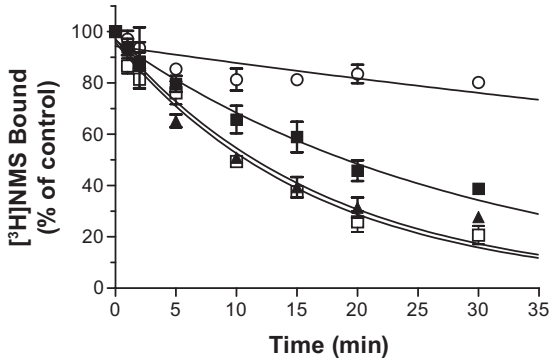


**Fig. 2.** Effect of TBPB (100 $\mu$M; ■), gallamine (1 mM; ○), and clozapine (10 $\mu$M; △) on the atropine-induced dissociation of [$^3$H]NMS binding to human M1 receptor expressed on CHO-K1 membranes. Atropine alone (1 $\mu$M; □) was measured as a control. The $k_{off}$ values measured in the presence of TBPB ($k_{off}$ = 0.034 ± 0.002 min$^{-1}$), gallamine ($k_{off}$ = 0.0071 ± 0.0016 min$^{-1}$), and clozapine ($k_{off}$ = 0.057 ± 0.004 min$^{-1}$) compared with atropine alone ($k_{off}$ = 0.060 ± 0.004 min$^{-1}$).

TABLE 2

Functional activity of muscarinic agonists at wild-type M1, NC4M1M3, and NC3M1M3 chimeric receptors measured in calcium mobilization assays by FLIPR

$EC_{50}$ values and percentage of maximum acetylcholine activity are presented as mean ± S.E.M. of three individual experiments performed in triplicate.

| | hM1-WT | | NC4M1M3 | | NC3M1M3 | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ | Max | $EC_{50}$ | Max | $EC_{50}$ | Max |
| | *nM* | % | *nM* | % | *nM* | % |
| Acetylcholine | 5 ± 0.2 | 100 | 3.4 ± 1.5 | 100 | 40 ± 14 | 100 |
| Xanomeline | 3.2 ± 0.2 | 81 ± 3 | 18 ± 4 | 82 ± 2 | 44 ± 9 | 69 ± 5 |
| AC-42 | 128 ± 7 | 74 ± 1 | >100,000 | | 922 ± 11 | 48 ± 3 |
| TBPB | 5.3 ± 0.2 | 70 ± 0.4 | >100,000 | | 117 ± 42 | 36 ± 4 |
| N-desmethylclozapine | 20 ± 1 | 65 ± 0.4 | 59 ± 18 | 43 ± 2 | 83 ± 5 | 47 ± 3 |

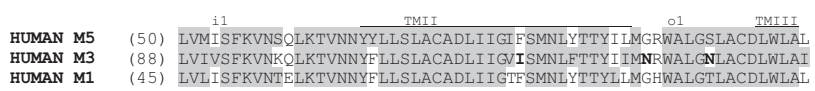


**Fig. 3.** Sequence alignment of the first intracellular loop (i1), transmembrane II (TMII), extracellular loop 1 (o1), and transmembrane III (TMIII) of human M1, M3, and M5 muscarinic receptors. Gray shading indicates 100% identity. Bolded residues indicate residues modified in NC4M1M3 chimeric receptor by site-directed mutagenesis.

erated were found to express at levels (2–3 pmol/mg) comparable with levels measured for wild-type M1 receptors. In contrast, the mutants F374A and Y408A were determined to have decreased levels of expression of less than 4 to 5% of wild-type receptors. No expression of the D105A (3.32) mutant could be measured by [$^3$H]QNB binding (data not shown); therefore, no functional response with any of the muscarinic receptor ligands could be detected. The compromised activity measured for acetylcholine measured on mutants Y404F, Y408F, and W101F and lack of activity with the D105A mutant was anticipated on the basis of previous studies where these mutations were shown to exhibit decreases in acetylcholine binding affinities (Lu et al., 2001). Compared with the acetylcholine, the functional potencies of AC-42, TBPB, and *N*-desmethylclozapine were found to be less severely affected by the mutations studied; in addition, the potencies of AC-42, TBPB, and *N*-desmethylclozapine were found to be increased on a number of mutants compared with that measured on wild-type receptors.

Substitution of Trp101 (3.28) in TMIII with an alanine residue resulted in a dramatic decrease in acetylcholine functional potency. Replacement of Trp101 with phenylalanine was better tolerated with only a 2.6-fold reduction in potency measured compared with wild-type M1 receptors. The potency for xanomeline was also observed to be decreased on the W101A mutant (26-fold), whereas the potency was found to be only slightly decreased on W101F mutant. In contrast to the decreased activity measured for acetylcholine and xanomeline on substitutions at Trp101, the affinities of both AC-42 and TBPB were found to be increased. The potencies of AC-42 and TBPB were increased by 77- and 5-fold, respectively, on W101A. Substitution of phenylalanine for Trp101 resulted in less marked changes with only a 1.7- and 2.5-fold increase in potency measured for AC-42 and TBPB, respectively. In comparison, the affinity of *N*-desmethylclozapine was only slightly changed on W101A and W101F, where a 2-fold decrease and 1.25-fold increase in potency were measured, respectively.

Substitution of Tyr179 with either alanine or phenylalanine resulted in only minor changes in functional potencies

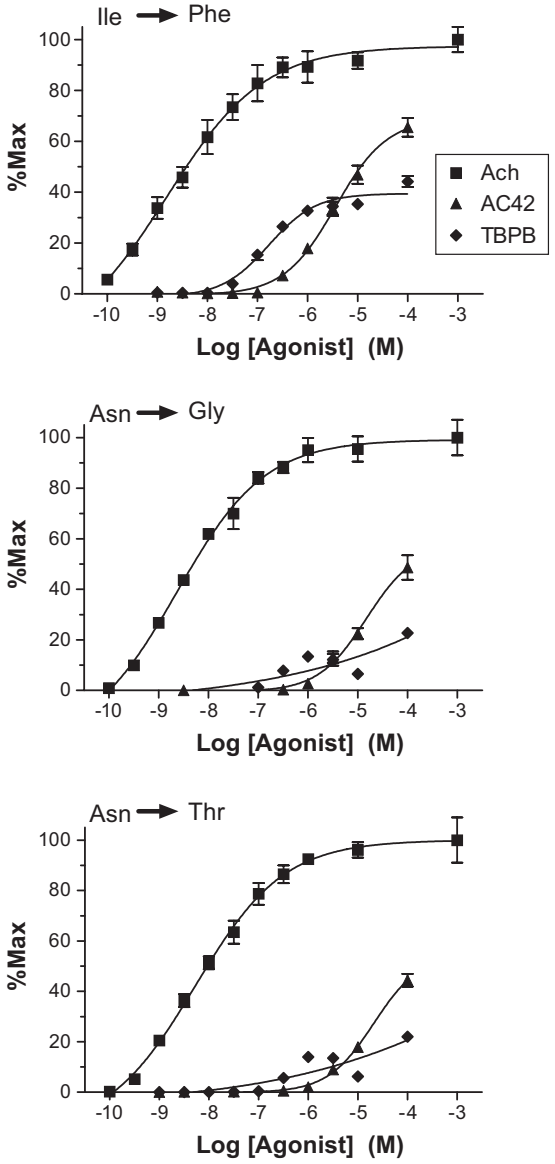


**Fig. 4.** Functional activity of acetylcholine (■), AC-42 (▲), and TBPB (♦) on point mutations selectively introduced into chimeric receptor NC4M1M3 measured by calcium mobilization assay in FLIPR. Results are expressed as percentage of maximum acetylcholine activity and are the means ± S.E.M. from three individual experiments performed in triplicate.

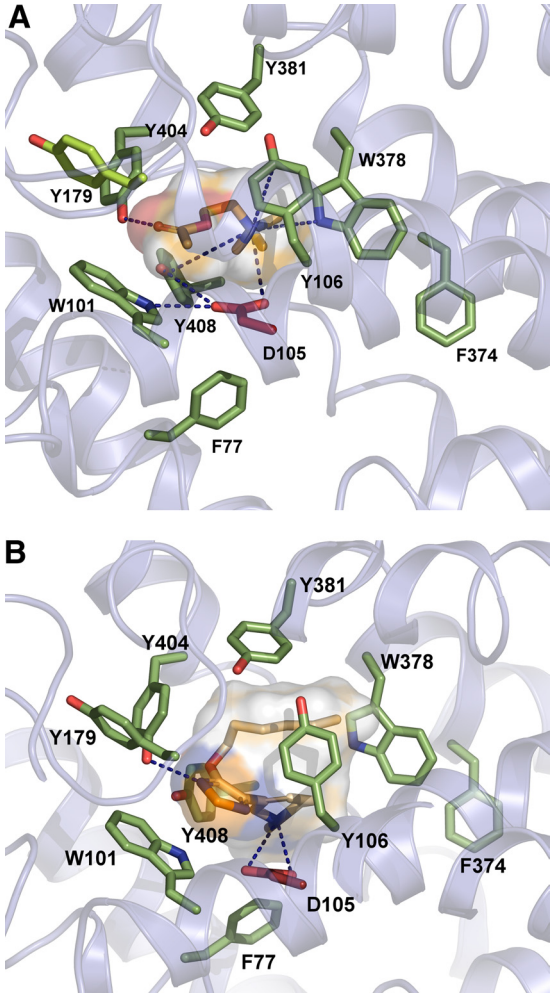


**Fig. 5.** Models (gold sticks) of acetylcholine (A) and xanomeline (B) bound to M1 receptor. Residues labeled have favorable interactions with either the acetylcholine or xanomeline.

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TABLE 3
Functional activity of muscarinic agonists on M1 point mutations measured in calcium mobilization assays with FLIPR
Values for $EC_{50}$ and percentage of maximum acetylcholine response represent the mean of five to seven determinations ± S.E.M. measured on transiently expressed receptors. The shift represents the $EC_{50}$ measured for the mutated receptor divided by the $EC_{50}$ measured for the wild-type (WT) receptor. If the response was too low to quantify, $EC_{50}$ and percentage of maximum acetylcholine is designated N.D. (not determined). Receptor expression levels for wild-type and point mutant receptors were measured with [$^3$H]QNB binding on membranes prepared from transfected CHONFAT cells.

| Receptor | Acetylcholine | | | Xanomeline | | | AC-42 | | | TBPB | | | NDMC | | | $B_{max}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $EC_{50}$ | Max | Shift | $EC_{50}$ | Max | Shift | $EC_{50}$ | Max | Shift | $EC_{50}$ | Max | Shift | $EC_{50}$ | Max | Shift | |
| | *nM* | *%* | | *nM* | *%* | | *nM* | *%* | | *nM* | *%* | | *nM* | *%* | | *pmol/mg protein* |
| M1 WT | 5.0 ± 0.2 | 100 | | 3.2 ± 0.2 | 81 ± 3 | | 128 ± 7 | 74 ± 1 | | 5.3 ± 0.2 | 70 ± 0.4 | | 20 ± 1 | 65 ± 0.4 | | 2.7 ± 1 |
| W101A | 15175 ± 823 | 100 | 3035 | 82 ± 26 | 77 ± 2 | 26 | 1.7 ± 0.2 | 73 ± 2 | 0.013 | 1.1 ± 0.1 | 57.5 ± 4 | 0.2 | 43 ± 6 | 68 ± 3 | 2 | 1.9 ± 0.1 |
| W101F | 12.8 ± 0.9 | 100 | 2.6 | 4.0 ± 0.5 | 89 ± 2 | 1.2 | 73 ± 7 | 72 ± 1.5 | 0.6 | 2.1 ± 0.3 | 77 ± 1.5 | 0.4 | 16 ± 2 | 78 ± 3 | 0.8 | 3.8 ± 0.4 |
| Y179A | 12.4 ± 0.9 | 100 | 2.5 | 3.6 ± 0.3 | 83 ± 1 | 1.1 | 101 ± 14 | 72 ± 2 | 0.8 | 1.8 ± 0.2 | 75 ± 2 | 0.34 | 12 ± 1 | 72 ± 2 | 0.6 | 2.9 ± 0.2 |
| Y179F | 10.5 ± 0.6 | 100 | 2 | 4.5 ± 0.5 | 84 ± 0.3 | 1.4 | 359 ± 67 | 74 ± 3 | 2.8 | 5.4 ± 0.1 | 65 ± 1 | 1 | 37 ± 6 | 66 ± 1 | 1.8 | 3.0 ± 0.0 |
| F374A | 90 ± 4 | 100 | 18 | 19.4 ± 1.5 | 79 ± 2 | 6 | 470 ± 37 | 69 ± 1.6 | 3.7 | 0.4 ± 0.0 | 77 ± 2 | 0.08 | 106 ± 14 | 70 ± 3 | 5.2 | 0.12 ± 0.01 |
| Y404A | 18660 ± 498 | 100 | 3732 | 85 ± 13 | 80 ± 1 | 26 | 2.2 ± 0.1 | 76 ± 0.7 | 0.017 | 1.7 ± 0.1 | 59 ± 1 | 0.2 | 63 ± 6.1 | 71 ± 3 | 3.1 | 0.93 ± 0.06 |
| Y404F | 809 ± 12 | 100 | 162 | 20 ± 3 | 55 ± 1.0 | 6 | 75 ± 8 | 62 ± 0.5 | 0.6 | 2.1 ± 0.3 | 63 ± 0.5 | 0.4 | 25.4 ± 2.7 | 49 ± 1 | 1.25 | 1.8 ± 0.8 |
| Y408A | 8500 ± 358 | 100 | 1700 | 252 ± 34 | 74 ± 3 | 79 | N.D. | N.D. | | 1304 ± 52 | 34 ± 1 | 246 | 63 ± 8.5 | 34 ± 1 | 3.1 | 0.13 |
| Y408F | 75 ± 4 | 100 | 35 | 1.8 ± 0.4 | 84 ± 3 | 0.6 | 1339 ± 16 | 55 ± 5 | 10.5 | 47 ± 2 | 59 ± 4 | 9 | 9.7 ± 0.7 | 67 ± 4 | 0.48 | 1.2 ± 0.4 |

for all the M1 agonists studied. The affinity of acetylcholine was decreased by only 2.5- and 2-fold on Y179A and Y179F, respectively. The affinity for xanomeline was essentially unchanged on either mutant. The affinity for AC-42 and TBPB were increased on Y179A by 1.25- and 2.9-fold, respectively. In comparison, AC-42 exhibited a 2.8-fold decrease in affinity on Y179F, whereas the affinity for TBPB was unchanged compared with wild-type M1 receptors.

The functional potencies for acetylcholine, xanomeline, AC-42, and *N*-desmethylclozapine were all reduced on F374A compared with wild-type receptors. This mutant was found to be expressed at low levels (4% of wild-type M1) and may account for the decreased potency determined in the functional assay. In comparison, TBPB exhibited a 12.5-fold increase in affinity on F374A, but this result may be confounded by the low mutant protein expression levels.

The reduction of acetylcholine binding affinity on Y404A and Y408A has been reported by Lu et al., (2001). The activity of the M1 agonists were evaluated in functional assays on these mutations; in addition, substitutions with phenylalanine (Y404F and Y408F) were generated to evaluate the contribution of the hydroxyl group separately from the phenyl ring in binding AC-42, TBPB, *N*-desmethylclozapine, and xanomeline. For acetylcholine, large reductions in potency were measured on both Y404A and Y408A consistent with previous published reports. Substitution of the phenylalanine ring was better tolerated; however, reductions of 162- and 35-fold were observed with acetylcholine on Y404F and Y408F, respectively. The potency of xanomeline was also reduced on Y404A and Y408A, 26- and 79-fold, respectively. Similar to acetylcholine, a smaller decrease in xanomeline potency was observed on Y404F (6-fold) compared with wild-type receptors. Compared with acetylcholine, the activity of xanomeline was found to be slightly increased on Y408F. In contrast to the reductions observed for acetylcholine and xanomeline on the Y404A and Y404F mutants, the potencies of both AC-42 and TBPB were observed to be increased. AC-42 exhibited a 59-fold increase on Y404A and a smaller 1.7-fold increase on Y404F. TBPB affinity was also found to be increased on both Y404A (5-fold) and Y404F (2.5-fold); in comparison, however, the magnitude was not observed to be as large as that observed with AC-42. In contrast, only small decreases in potency were observed with *N*-desmethylclozapine on either Y404A or Y404F mutants.

In contrast to the observed increase in AC-42 and TBPB potencies resulting from modifications of Tyr404, substitution of either alanine or phenylalanine at Tyr408 resulted in a reduction in AC-42 and TBPB activities. The Y408A mutant was found to be expressed at only 5% of the wild-type receptor levels, and a 1700-fold reduction in acetylcholine affinity was determined. The reduced receptor expression is consistent with what has been reported previously (Hulme et al., 2003). On this mutant, AC-42 activity could not be detected; in addition, the affinity of TBPB was reduced by 246-fold. Affinities for xanomeline and *N*-desmethylclozapine were less severely affected, yet showed reductions of 79- and 3-fold, respectively. It is likely that the low level of receptor expression may have contributed to the reduction of agonist activity measured on this mutant. In contrast, the Y408F mutant was expressed at receptor levels similar to the wild-type M1 receptor. The functional potencies of AC-42 and TBPB on Y408F were found to be decreased by 10.5- and 9-fold, respec-

tively. In comparison, a small increase was observed with xanomeline (1.7-fold) and *N*-desmethylclozapine (2-fold).

To define the binding sites for the M1 agonists evaluated in this study, the ligands were docked into the M1 receptor homology model (Fig. 5), using the mutagenesis results to support the orientation of the ligands. Multiple orientations of each ligand were docked into a variety of sites using flexible docking methods until a set of internally self-consistent sites was identified. Additional hand and computational refinement was then carried out for the final models presented in this work. The model supported the docking of acetylcholine consistent with previously published reports (Hulme et al., 2003; Peng et al., 2006). In comparison, xanomeline was also found to occupy a site similar to that occupied by acetylcholine. The orthosteric agonists acetylcholine and xanomeline interact with Asp105 to stabilize the anion (Fig. 5). Both ligands were found to make specific interactions with the hydroxyl group of Tyr404 (7.39). Cation-$\pi$ interactions between acetylcholine and the aromatic residues Trp101 (3.28), Tyr106 (3.33), Trp378 (6.48), Tyr404, and Tyr408 (7.43) stabilize the ligand (Fig. 5A). Xanomeline was found to make favorable hydrophobic contacts between its lipophilic tail and Tyr106, Trp378 (6.48), and Tyr381 (6.51) (Fig. 5B). In contrast to the orthosteric ligands, the allosteric agonists AC-42 and TBPB were determined to occupy a binding site adjacent to the orthosteric site on the opposite face of Trp101 (Fig. 6). The binding pocket is analogous to the hydrophobic channel between TMs I, III, and VII, with specific polar interactions contributed by Thr83 (2.62) and van der Waals interactions contributed by the residues lining the channel. Phe77 (2.56) lies at the base of the allosteric agonist binding site and acts as a lid at the base of the channel.

The docking of *N*-desmethylclozapine was challenging as the functional activity was largely unaffected by the mutants explored in this study. The only truly discernible effects were observed for mutations to Tyr404. After repeated rounds of docking to plausible sites, it is hypothesized that *N*-desmethylclozapine is expected to bind proximal to the orthosteric site (Fig. 7). The highly hydrophobic nature of this ligand drives the chloro group toward the hydrophobic pocket formed by Ile180, Thr189 (4.39), and Trp400 (6.50). The phenyl group para to the chlorine is stabilized by an interaction with Cys 407 (7.42), a face contact with Tyr381, and an edge-to-face interaction with Trp378. The polar piperazine makes contacts with Asp105, Tyr404 and Tyr408. As discussed above, this is the most speculative of the modeled small molecules because of the lack of mutagenesis data indicating a clear impact on binding and functional activity.

## Discussion

Selective targeting of M1 muscarinic receptors for the development of novel therapeutics has proven challenging because of the high degree of homology of the orthosteric agonist binding site between the five muscarinic receptor subtypes. Allosteric modulation of G protein-coupled receptors represents an attractive alternative for identifying subtype selective ligands because it is expected that allosteric binding sites, unlike the site for orthosteric agonists, are less conserved or not conserved among subtypes. An increasing number of examples have been reported for G protein-coupled receptors, including M1 muscarinic receptors subtypes

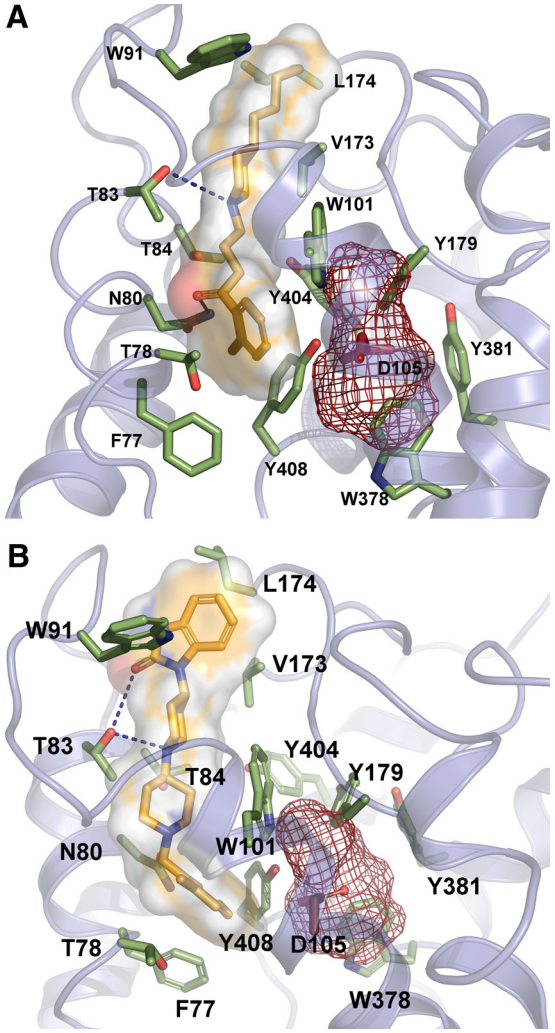


**Fig. 6.** Models (gold sticks) of AC-42 (A) and TBPB (B) bound to M1 receptor. Residues labeled have favorable interactions with either the ligand or acetylcholine. Mesh representation of acetylcholine binding is shown for reference.

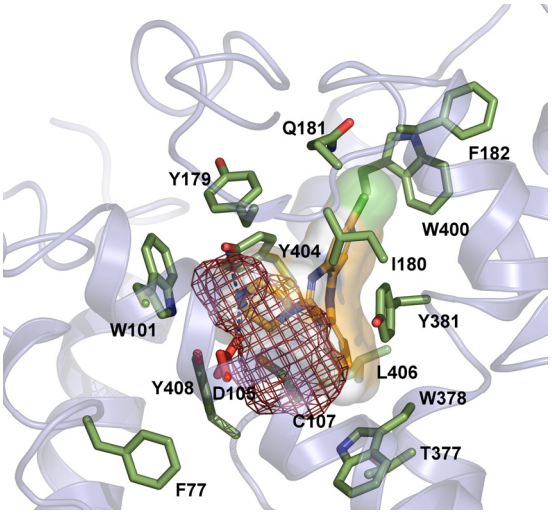


**Fig. 7.** Model of *N*-desmethylclozapine (gold sticks) bound to M1 receptor. Residues labeled have favorable interactions with either the ligand or acetylcholine. Mesh representation of acetylcholine binding is shown for reference.

and have recently been reviewed (Langmead and Christopoulos, 2006; May et al., 2007; De Amici et al., 2010).

Activation of M1 muscarinic receptors has been shown to occur with orthosteric agonists and allosteric potentiators requiring the presence of orthosteric agonists to produce activation (Matsui et al., 1995; Lazareno et al., 1998; Birdsall and Lazareno, 2005; Ma et al., 2009; Marlo et al., 2009). Furthermore, selective M1 receptor allosteric agonists, exemplified by AC-42 (Spalding et al., 2002; Langmead et al., 2006) and TBPB (Jones et al., 2008), modulate receptor activation without requirement for orthosteric agonists. Mutagenesis studies have focused on the orthosteric binding site for acetylcholine and the antagonist *N*-methylscopolamine to define residues comprising the binding sites. Several orthosteric binding site models have been proposed on the basis of mutagenesis and molecular docking studies using homology models constructed from the crystallography structure of bovine rhodopsin (Hulme et al., 2003; Peng et al., 2006). These studies revealed that the acetylcholine binding site was located within the transmembrane domains with residues contributed by TM3, TM4, TM6, and TM7. With the discovery that orthosteric agonist activation of the M1 muscarinic receptor could be modulated by allosteric potentiators such as brucine and allosteric inhibitors, such as gallamine, mutagenesis studies focused on residues within the extracellular loops, because it was hypothesized that allosteric sites resided outside of the transmembrane domains (Matsui et al., 1995; Birdsall et al., 2001). 1-(4-Methoxybenzyl)-4-oxo-1,4-dihydro-3-quinoline carboxylic acid, a highly selective M1 allosteric potentiator, has been described previously (Ma et al., 2009) and is proposed, on the basis of site-directed mutagenesis and molecular modeling, to bind to an extracellular site between transmembranes IV and V. With the recent discovery of M1 allosteric agonists AC-42 and its analogs, mutagenesis studies have begun to explore residues for their role in binding and define the proximity of allosteric sites to orthosteric sites on the receptor (Spalding et al., 2006; Lebon et al., 2009). In this report, we have provided further evidence supporting a model in which AC-42 binds at an allosteric site distinct from the orthosteric site and demonstrated that the structural diverse allosteric agonist TBPB binds to a similar site on the M1 receptor.

In this study, we compared the activity of the M1 orthosteric agonists acetylcholine and xanomeline, the allosteric agonist AC-42 and TBPB, and an active metabolite of clozapine, *N*-desmethylclozapine, on chimeric M1M3 receptors and point mutants of M1 receptors to elucidate their binding sites. An M1 receptor homology model was constructed on the basis of the rhodopsin crystallography structure and the orientation of the M1 ligands were supported by the changes in functional potency observed on the mutant constructs. As a result, we have determined that AC-42 and TBPB share a similar binding pocket adjacent to the orthosteric binding site. In contrast, results supported *N*-desmethylclozapine binding to a site distinct from both AC-42 and TBPB that overlaps with the orthosteric site.

In the present study, the allosteric mode of action of TBPB was evaluated in dissociation kinetic binding assays. Similar to AC-42 (Langmead et al., 2006; Spalding et al., 2006), TBPB was determined to reduce the [$^3$H]NMS dissociation rate from the M1 receptor. In contrast, Spalding et al. (2006) also showed that *N*-desmethylclozapine did not exhibit allosteric properties on M1 in dissociation binding studies. These findings further established the functional similarity of AC-42 and TBPB and difference with *N*-desmethylclozapine.

Both AC-42 and TBPB have been shown to retain functional activity on a point mutant, Y381A in TMVI of the M1 receptor, which in contrast results in diminishing the receptor's potency for the orthosteric agonist acetylcholine. It was postulated, therefore, that AC-42 and TBPB might bind to a different site than acetylcholine. In comparison, we reported previously that *N*-desmethylclozapine retained functional activity on Y381A mutant, suggesting that it also acted at a different site from acetylcholine; however, it was unclear without further investigation whether *N*-desmethylclozapine bound to a site on the M1 receptor similar to or different from that to which AC-42 and TBPB bind. In the homology model, Tyr381 contributes to the stabilization of the hydrophobic cage proposed by Hulme et al., (2003), surrounding the critical Asp105 reside in the orthosteric acetylcholine binding site. Our model also predicts that the allosteric binding pocket for AC-42 and TBPB resides at the opposite interface of Trp101 and Tyr408; therefore, Tyr381, being far removed from this site, would not be expected to participate in the binding of AC-42 and TBPB. In contrast to AC-42 and TBPB, the binding site for *N*-desmethylclozapine is proposed to overlap with the orthosteric agonist binding site; in part, hydrophobic contacts are contributed by Tyr381. The substitution of alanine for phenylalanine at position 381 would be expected to allow *N*-desmethylclozapine to more effectively occupy the hydrophobic cage. The homology model supports the functional activity data measured with AC-42, TBPB, and *N*-desmethylclozapine on the Y381A mutant whereby, in contrast to orthosteric agonists, an increase in potency and efficacy was observed with all three ligands.

The minimal epitope for AC-42 binding to the M1 receptor was first described by Spalding et al. (2002) to be composed of the first 45 amino acids of M1 corresponding to the amino terminus and TMI, the third extracellular loop and TMVII. Our data using M1M3 chimeric receptors now extends this binding domain to include residues within TMII, specifically Phe77 for both AC-42 and TBPB. Phe77 was not previously implicated in the first report describing AC-42 because M1-M5 chimeric receptors were used in these studies. The phenylalanine residue at this position is conserved between M1 and M5 muscarinic receptor subtypes and was obscured in the initial AC-42 mapping using the M1M5 chimeras. In the present study, the importance of the phenylalanine residue was realized because the corresponding position contributed by the M3 sequence was an isoleucine substitution. It is noteworthy that the Phe77 residue resides at the base of a hydrophobic network of amino acids contributed among TMs II, VI, and VII that constitutes the binding site for AC-42 and TBPB and may function by providing stabilization to the receptor-ligand complex.

In this report, we evaluated the impact of substitution of Trp101 or Tyr404 residues with phenylalanine or alanine to examine the contribution of the hydroxyl group compared with the aromatic side chain on the ability of the allosteric agonists, TBPB and AC-42 to produce receptor activation. The greater effect observed on functional potency when Trp101 and Tyr404 were mutated to alanine compared with that seen with phenylalanine substitution suggests that there is an enhancement in accessing the allosteric agonist

binding pocket for TBPB and AC-42 when the steric constraint of the aromatic ring is removed. Hulme et al., (2003) have suggested that a hydrophobic cage exists around Asp105, a critical residue required to form a salt bridge with the choline head group of acetylcholine composed of residues contributed by TM3 (Trp101), TM6 (Tyr381), and TM7 (Tyr404, Tyr408). It is postulated that an activated state of the receptor is favored by rearrangement of the hydrophobic cage, allowing for AC-42 or TBPB to participate in the hydrophobic cage to favor an activated conformation of the receptor.

Lebon et al. (2009) have proposed a similar location for AC-42 binding to the M1 receptor. In comparison with our study, a homology model based on the $\beta$2-adrenergic receptor was employed to interpret the mutagenesis studies and the mapping of TBPB was not evaluated. Lebon et al. (2009) also found a similar large increase in the functional potency for AC-42 when Trp101 was mutated to alanine. In contrast, on the Y404A mutant, we observed significant enhancement of both AC-42 and TBPB, whereas Lebon et al. (2009) reported only a slight enhancement of activity.

In summary, the results of this study further defined the binding site for the allosteric agonists AC-42 and TBPB to be at a similar site distinct from the orthosteric agonist site. Comparing the functional activity of AC-42, TBPB, and *N*-desmethylclozapine and the orthosteric agonists acetylcholine and xanomeline on mutant constructs, the ligands were found to generally cluster into three groups, supporting the hypothesis that the M1 receptor activation can occur though at least three different binding domains (Spalding et al., 2006). Additional evidence is now provided from the homology model that illustrates the location of the three binding domains: the orthosteric agonist site for acetylcholine and xanomeline stabilized by charge interactions, a defined allosteric site proximal to the orthosteric site for AC-42 and TBPB stabilized by hydrophobic interactions, and a third site positioned above the orthosteric binding site for binding *N*-desmethylclozapine. Modeling allosteric binding sites on M1 muscarinic receptors could facilitate the optimization and development of novel treatments for psychiatric disorders and cognition impairment in Alzheimer's disease.

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