Differential Modulation of Agonist Potency and Receptor Coupling by Mutations of Ser388Tyr and Thr389Pro at the Junction of Transmembrane Domain VI and the Third Extracellular Loop of Human M₁ Muscarinic Acetylcholine Receptors

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ABSTRACT

Transmembrane domain VI of muscarinic acetylcholine receptors plays an important role in ligand binding and receptor function. A human M₁ (HM₁) mutant receptor, HM₁(S388Y, T389P), displayed significantly enhanced agonist potency, binding affinity, and G protein coupling. The mutations are located at the top of transmembrane domain VI and about two helical turns above Tyr381 and Asn382, which are important for ligand binding and receptor function. To determine the functional role of individual mutations of Ser388Tyr and Thr389Pro, we created stable A9 L cell lines expressing HM₁(S388Y) or HM₁(T389P) receptors. In phosphatidylinositol hydrolysis assays, muscarinic agonists showed greater potency at the HM₁(S388Y) and HM₁(S388Y, T389P) mutants compared with the wild-type and HM₁(T389P) receptors. Acetylcholine demonstrated 105-fold higher potency at HM₁(S388Y) receptors than at HM₁(T389P) receptors. Choline (30 μM, the concentration found in Dulbecco’s modified Eagle’s medium) exhibited 90% stimulation at HM₁(S388Y) receptors but was inactive at HM₁(T389P) receptors. In ligand binding experiments, mutation of Ser388Tyr resulted in significantly increased agonist binding affinity. In contrast, mutation of Thr389Pro did not change agonist binding affinity but rendered multiple agonist binding sites, and the high-affinity binding was sensitive to GTP analogs. These results demonstrate that the Ser388Tyr mutation is responsible for enhanced agonist potency and binding affinity, whereas the Thr389Pro mutation alters G protein interactions. The data suggest that Ser388 and Thr389 are potential targets for modulation of agonist binding and G protein coupling.

Muscarinic acetylcholine (ACh) receptors (mAChRs; M₁–M₅; Caulfield and Birdsall, 1998) are members of the G protein-coupled receptor family and represent important targets for drug design and development. Functionally, M₁, M₃, and M₅ subtypes couple to the activation of phospholipase Cβ (PLCβ) through the pertussis toxin-insensitive G₃/₁₁ family of G proteins; and M₂ and M₄ subtypes couple to the inhibition of adenylyl cyclase through the pertussis toxin-sensitive Gαo family of G proteins (Hulme et al., 1990; Caulfield, 1993). Previous molecular modeling studies (Ward et al., 1992; Nordvall and Hacksell, 1993) and site-directed mutagenesis and pharmacological studies (Fraser et al., 1989; Wess et al., 1991, 1992; Blüm et al., 1994; Huang et al., 1999) have identified several highly conserved residues critical for agonist binding and receptor activation; however, the molecular mechanisms by which receptors are activated on acetylcholine (ACh) binding are still not clear.

A mutant human M [HM₅(S465Y, T466P)] receptor showed significant constitutive activity, increased agonist potency, and binding affinity (Spalding et al., 1995). The HM₅ Ser465 is conserved in M₁ receptors and is an Asn residue in M₂, M₃, and M₄ receptor subtypes; whereas the HM₅ Thr466 is conserved in all five subtypes of mAChRs (Ser388 and Thr389 in

ABBREVIATIONS: ACh, acetylcholine; mAChR, muscarinic acetylcholine receptor; CCh, carbachol; Gpp(NH)p, guanosine-5’- (β,γ-imido)triphosphate; PI, phosphatidylinositol; HM₁, human muscarinic acetylcholine receptor subtype 1; NMS, N-methylscopolamine; Oxo, oxotremorine; Oxo-M, oxotremorine-M; PLC, phospholipase C; (R)-QNB, (R)-3-quinoxalinyl benzoate; Ne3, N-terminal region of the third extracellular loop; TM, transmembrane domain; R-SAT, receptor selection and amplification technology; WT, wild-type.
HM₁ receptors; see Fig. 1). In previous studies (Huang et al., 1998), we demonstrated that an equivalent mutant HM₁ receptor [HM₁(S388Y, T389P), a mutant HM₁ receptor with Ser388 replaced by Tyr and Thr389 replaced by Pro], stably expressed in A9 L cells, showed significantly enhanced agonist potency, binding affinity, and G protein coupling. The enhancement is neither expression level nor cell line dependent but rather is an intrinsic property of the mutant receptor (X.-P. H., F. E. W., S. M. P. and W. S. M. O., submitted for publication). In contrast to the high level of constitutive activity observed for HM₅(S465Y, T466P) receptors, HM₁(S388Y, T389P) receptors showed limited constitutive activity (~20%) at high expression levels, but not at low expression levels (X.-P. H., F. E. W., S. M. P. and W. S. M. O., submitted for publication). However, HM₁(S388Y, T389P) receptors can be activated by choline, which is found in Dulbecco’s modified Eagle’s medium, and unknown agonists in FBS (Huang et al., 1998). The differences observed between mutant HM₁ and HM₅ receptors might be due to several factors, such as subtype (HM₁ versus HM₅ receptors), cell line (A9 L versus NIH 3T3 cells), receptor functional assay (phosphatidylinositol (PI) hydrolysis assay versus receptor selection and amplification technology (R-SAT)), expression mode (stable versus transient expression), or expression level. However, the common features, enhanced agonist potency and binding affinity, observed at HM₅(S465Y, T466P) and HM₁(S388Y, T389P) receptors suggest that the junction of transmembrane domain (TM) VI and the N-terminal of the third extracellular loop (Ne3) has a conserved functional role in mAChRs. This is supported by preliminary results indicating that equivalent mutations (AsnThr-to-TyrPro mutations) in M₂ and M₃ receptors also result in increased agonist binding affinity (Ellis et al., 1998).

It is widely accepted that ACh binds to a binding cavity, about two helical turns below the membrane surface, formed within the TM domains (Hulme et al., 1990; Wess 1993, 1996). Ser388 and Thr389 are located at the junction of TM VI and Ne3 and about two helical turns above the Tyr381 and Asn382 residues in TM VI (see Fig. 1), which are critical residues involved in ACh binding and/or receptor activation (Ward and Hulme, 1997; Huang et al., 1999). Therefore, Ser388 and Thr389 probably are not the primary ligand binding sites. In fact, several single mutations at Ser465 of HM₅ receptors cause varying degrees of constitutive activity (measured by R-SAT) in which basic and bulky substitutions are more effective than acidic and small substitutions (Spalding et al., 1997). The mutations at Ser465 or Ser465 and Thr466 appear to cause the formation of active receptor states (R*; Spalding et al., 1995, 1997) in accordance with the allosteric ternary complex model for G protein-coupled receptors (Samama et al., 1993). Mutations of Ser388 and Thr389 to Tyr and Pro, respectively, in HM₁ receptors may induce the mutant receptor to form structurally and conformationally flexible states that are favorable for agonist binding and activation (Huang et al., 1998), as suggested recently in mutant β₂-adrenergic receptors (Kobilka et al., 1998).

Fig. 1. A model of the HM₁ receptor amino acid sequence depicting seven TMs. Residues involved in agonist binding and receptor activation are highlighted in gray, including Asp105 in TM III, Thr192 in TM V, and Tyr381 and Asn382 in TM VI. The Ser388 and Thr389 residues are circled. The equivalent residues in other subtypes of mAChRs are listed for comparison.
To determine the functional role of individual substitutions in HM1(S388Y, T389P) receptors, we created A9 L cell lines stably expressing HM1(S388Y) or HM1(T389P) receptors and characterized the mutant receptors. Here we present results indicating that the Ser388Tyr mutation is responsible for enhanced agonist potency and agonist binding affinity, whereas the Thr389Pro mutation is responsible for altering G protein interactions.

### Materials and Methods

Materials and methods used in this study were as reported previously (Huang et al., 1998, 1999) or described below.

Muscarinic Agonists, Chemicals, and Other Materials. Muscarinic agonists bethanecol and methacholine were purchased from Research Biochemical Inc. (Natick, MA). GTP sodium salt was ordered from Sigma Chemical Co. (St. Louis, MO). Filtermate 196, UniFilter GF/B, MicroScint 20, TopSeal A, and Backing Tape were purchased from Packard Instrument Company (Meriden, CT). Deep-well microtiter plates (96-well and 1.2 ml/well) were obtained from Marshall Biomedical Products, Inc. (Rochester, NY).

**Mutation Strategy.** Mutations of Ser388 to Tyr or Thr389 to Pro were carried out using the QuickChange kit (Stratagene, La Jolla, CA) using HM1pcD1 provided by Dr. Tom I. Bonner (Bonner et al., 1998). All primers used in mutations were synthesized by Life Technologies (Grand Island, NY). The sense primer for the Ser388Tyr mutation was 5′-GCTTCTGGTGTACCTCTGCAAGG-3′ (with the changed base in bold), and the antisense primer was 5′-CCTTGCAGAAGGACCCCAACGACC-3′. The sense primer for the Thr389Pro mutation was 5′-GTTGCTGTGGTCCCCCTGGTCAAGG-3′ (with the mutated base in bold), and the antisense primer was 5′-CCTTGCAAGGAGGGGACACCAACGACC3′. The mutations were confirmed by dyeode nucleotide sequencing using the T7 Sequenase sequencing kit from Amersham Life Science (Arlington Heights, IL).

**Creation of Stable A9 L Cell Lines.** A9 L cells (American Type Culture Collection, Rockville, MD) were cotransfected with HM1(S388Y)pcD1 or HM1(T389P)pcD1 and pNEOgal (Stratagene) according to the calcium phosphate method (Chen and Okayama, 1987). Transfected A9 L cells were selected in the presence of 800 μg/ml G418 (Fisher, Pittsburgh, PA) and screened as described previously (Huang et al., 1998). A9 L cells stably expressing HM1(S388Y) or HM1(T389P) receptors were subcultured for functional and binding studies.

**Radioligand Binding Assays—TopCount NXT System.** [3H](R)-3-Quinuclidinyl benzilate [3H](R)-QNB saturation binding assays and ligand inhibition binding assays were performed as described previously (Huang et al., 1998), except that deep-well microtiter plates (96-well) were used instead of glass tubes. Reactions were initiated by the addition of membrane proteins to mixtures of reagents. The plates were sealed with Parafilm and incubated at room temperature for 2 h. The incubation was terminated by transfer to a 96-well UniFilter (GF/B) using Filtermate 196. The UniFilter was washed 4 times with 1 ml of cold binding buffer (25 mM sodium phosphate, pH 7.4, containing 5 mM magnesium chloride). The UniFilter then was dried in a fume hood for at least 1 h, and its back was sealed with Backing Tape. To each well was added 50 μl of MicroScint 20, and the top of the plate was sealed with TopSeal A. The filter was soaked overnight, and the radioactivity was counted in the TopCount NXT system (Packard) running Microsoft Windows NT 4.0. There were no differences between results obtained from the TopCount NXT system and a traditional liquid scintillation count system (data not shown).

### Results

**Receptor Expression and Antagonist Binding Properties.** HM1(S388Y) and HM1(T389P) receptors were stably expressed in A9 L cells at levels of 2600 ± 160 (mean ± S.E.M.) and 1200 ± 210 fmol/mg, respectively. They both showed a small (1.8- to 3.8-fold) but significant (P < 0.05) reduction in binding affinity for [3H](R)-QNB compared with HM1[wild-type (WT)] and/or HM1(S388Y, T389P) receptors. HM1(T389P) receptors also showed a 1.7-fold lower (P < 0.01) binding affinity for [3H](R)-QNB than HM1(S388Y) receptors (Table 1). When other classic muscarinic antagonists were examined, HM1(S388Y) and HM1(T389P) receptors showed the same antagonist binding profiles as HM1(WT) and/or HM1(S388Y, T389P) receptors (P > 0.05). In contrast, HM1(T389P) receptors displayed varying degrees of reduced affinity for these antagonists (2.8- to 16-fold; P < 0.05) compared with HM1(WT) and/or HM1(S388Y, T389P) receptors.

In the previous study of HM1(S388Y, T389P) receptors, we found that a freezing/thawing treatment, but not incubation at 37°C, significantly reduced [3H](R)-QNB binding activity in membrane homogenates (Huang et al., 1998). Because HM1(S388Y) receptors stably expressed in A9 L cells showed similar functional properties as HM1(S388Y, T389P) receptors (see below), we examined the effects of a freezing/thawing treatment (−70°C overnight) on total [3H](R)-QNB binding activity. In a similar fashion to that observed on HM1(S388Y, T389P) receptors, total specific binding activity of frozen membranes of HM1(S388Y) receptors was decreased by 26 ± 9.3% (n = 4) compared with nonfrozen membrane homogenates. The frozen membranes showed a binding affinity for [3H](R)-QNB of 60 ± 18 pM (n = 4) versus 67 ± 16 pM.
Pharmacology of HM$_1$(S388Y) and HM$_1$(T389P) Receptors. 
Muscarinic agonists used to characterize HM$_1$(S388Y, T389P) receptors displayed similar dose-response profiles (maximal responses and/or potencies) at HM$_1$(T389P) receptors (Fig. 2, A–E) as at WT receptors but a full agonist at HM$_1$(S388Y, T389P) receptors (Huang et al., 1998) and exhibited weak agonist activity at HM$_1$(S388Y) receptors than at HM$_1$(T389P) or WT receptors. These data indicated that the mutation of Ser388Tyr resulted in enhanced agonist potency, an intrinsic property observed at HM$_1$(S388Y, T389P) receptors (Huang et al., 1998). We also measured the activity of four other muscarinic agonists (bethanechol, methacholine, methlycarbachol, and pilocarpine) at a concentration of 10 μM for stimulation of PI hydrolysis at HM$_1$(S388Y) receptors. Consistent with the observation of enhanced agonist activity at HM$_1$(S388Y) receptors, these four agonists stimulated PI hydrolysis by 340 ± 53, 340 ± 17, 390 ± 7.2, and 330 ± 12% (n = 3), respectively; responses comparable or close to the maximal responses by ACh and CCh.

HM$_1$(S388Y, T389P) receptors exhibited limited constitutive activity and the constitutive activity (~20%) was inhibited by muscarinic antagonists (Huang et al., 1998). To determine whether HM$_1$(S388Y) or HM$_1$(T389P) receptors also showed constitutive activity, inhibition of basal PI hydrolysis was measured by i-hyoscyamine (the active enantiomer of atropine). The basal PI hydrolysis was reduced at HM$_1$(S388Y) receptors (~14 ± 10%, n = 5) but not HM$_1$(T389P) receptors (4.7 ± 4.8%, n = 3). When two other muscarinic antagonists, pirenzepine and N-methylscopolamine (NMS), were examined on HM$_1$(S388Y) receptors, similar maximal inhibition of basal PI hydrolysis was observed for pirenzepine (~22 ± 9.3%, n = 4) and NMS (~20 ± 5.6%, n = 3). These results indicate that HM$_1$(S388Y) receptors were constitutively activated to a limited degree, similar to that observed for HM$_1$(S388Y, T389P) receptors (Huang et al., 1998).

To determine whether endogenous G protein and/or PLC activities were changed in transfected A9 L cells and whether such changes could account for the enhanced agonist potency for the mutant receptors compared with WT receptors, we measured PI hydrolysis mediated by 20 mM NaF. The PI hydrolysis in A9 L cells expressing HM$_1$(S388Y) receptors (160 ± 33%, n = 6) was comparable with untransfected A9 L cells or transfected A9 L cells expressing HM$_1$(WT) receptors or HM$_1$(N382A) mutant receptors (Huang et al., submitted for publication). Therefore, the enhanced agonist potency observed at HM$_1$(S388Y) receptors does not appear to be due to changes in endogenous G protein and/or PLC activities. In contrast, the response elicited by 20 mM NaF in A9 L cells expressing HM$_1$(T389P) receptors was 75 ± 13% (n = 5). The reduced PI response in A9 L cells expressing HM$_1$(T389P) receptors may account for lower maximal responses of agonists at HM$_1$(T389P) receptors (Table 2) than at HM$_1$(WT) receptors expressed at lower levels (Huang et al., 1998).

**Agonist Binding Properties at HM$_1$(S388Y) and HM$_1$(T389P) Receptors.** Consistent with enhanced agonist activity at HM$_1$(S388Y) receptors, muscarinic agonists also displayed significantly increased binding affinities at HM$_1$(S388Y) receptors compared with HM$_1$(WT) receptors (Huang et al., 1998, 1999) or HM$_1$(T389P) receptors (Table 3 and Fig. 3). The smallest changes were observed for choline.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>HM$_1$(S388Y)</th>
<th>HM$_1$(S388Y, T389P)$^a$</th>
<th>HM$_1$(T389P)</th>
<th>HM$_1$(WT)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,600 ± 160 fmol/mg</td>
<td>2,600 ± 690 fmol/mg</td>
<td>1,200 ± 210 fmol/mg</td>
<td>260 ± 29 fmol/mg</td>
</tr>
<tr>
<td>$S_{max}$</td>
<td>%</td>
<td>nM</td>
<td>%</td>
<td>nM</td>
</tr>
<tr>
<td>ACh</td>
<td>410 ± 23</td>
<td>8.3 ± 3.6</td>
<td>550 ± 95</td>
<td>4.8 ± 1.6</td>
</tr>
<tr>
<td>CCh</td>
<td>430 ± 27</td>
<td>130 ± 53</td>
<td>520 ± 77</td>
<td>59 ± 5.5</td>
</tr>
<tr>
<td>Choline</td>
<td>390 ± 31</td>
<td>160,000 ± 85,000</td>
<td>420 ± 68</td>
<td>200,000 ± 20,000</td>
</tr>
<tr>
<td>Oxo-M</td>
<td>420 ± 22</td>
<td>26 ± 9.9</td>
<td>510 ± 45</td>
<td>12 ± 5.4</td>
</tr>
<tr>
<td>Oxo</td>
<td>440 ± 46</td>
<td>13 ± 5.3</td>
<td>500 ± 100</td>
<td>18 ± 2.8</td>
</tr>
<tr>
<td>Arecoline</td>
<td>330 ± 4.8</td>
<td>120 ± 65</td>
<td>510 ± 100</td>
<td>60 ± 14</td>
</tr>
</tbody>
</table>

$^a$ Data are from Huang et al. (1998) and included for comparison.

$S_{max}$ (%) represents the maximal stimulation expressed as the percentage above basal levels. N.A., not applicable.
which showed essentially the same binding affinity for HM₁(S388Y) receptors as HM₁(WT) receptors, yet 2.7-fold higher affinity than for HM₁(T389P) receptors. Except for Oxo-M, which showed more than 15-fold higher binding affinity for HM₁(S388Y) receptors than for HM₁(S388Y, T389P) receptors (Huang et al., 1998), all other agonists (ACh, CCh, Oxo-M, arecoline, and choline) showed similar binding affinity at HM₁(S388Y) receptors and HM₁(S388Y, T389P) receptors, and the difference in binding affinity (one-site binding model) was within 3.5-fold.

In addition to the significantly increased binding affinity of agonists at HM₁(S388Y) receptors, differences were observed in the nature of agonist binding profiles between HM₁(S388Y) and HM₁(T389P) receptors (Table 3 and Fig. 3). ACh interacted with three binding sites at HM₁(S388Y, T389P) and HM₁(WT) receptors (Huang et al., 1998). High-affinity binding of ACh at HM₁(S388Y, T389P) receptors was lost at HM₁(S388Y) receptors, as indicated by a rightward shift of binding curve between 0.1 and 10 nM ACh (Fig. 3A). In addition, ACh binding at HM₁(S388Y) receptors was insensitive to GTP modulation as

![Graphs showing pharmacology of muscarinic agonists](image-url)

**Fig. 2.** Pharmacology of muscarinic agonists at HM₁(S388Y) and HM₁(T389P) receptors. Data for HM₁(WT) and/or HM₁(S388Y, T389P) receptors are from Huang et al. (1998) and are included for comparison. PI hydrolysis assays were conducted in Krebs-Henseleit buffer. Data represent the mean ± S.E. from a minimum of three assays, each performed in duplicate. A, ACh. B, CCh. C, Oxo-M. D, Oxo. E, arecoline. F, choline.
### TABLE 3
Agonist binding properties at HM1(S388Y) and HM1(T389P) receptors

Binding assays were conducted on membrane homogenates in the presence of [3H]-QNB (0.1 nM for HM1(S388Y) receptors or 0.3 nM for HM1(T389P) receptors). Data represent the mean ± S.E. from a minimum of three assays, each performed in triplicate. High (K_H) and low (K_L) binding affinities were assigned in two-site models, whereas K_M (medium binding affinity), and K_N were assigned in three-site models.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>One Site</th>
<th>Two or Three Sites</th>
<th>One Site</th>
<th>Two or Three Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without GTP</td>
<td>With Gpp(NH)p (100 μM) or GTP (400 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S388Y</td>
<td>42 ± 5.5</td>
<td>15 ± 5.2</td>
<td>7.0 ± 2.6</td>
<td>230 ± 50</td>
</tr>
<tr>
<td>STYP*</td>
<td>13 ± 2.7</td>
<td>0.09 ± 0.028</td>
<td>28 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>T389P</td>
<td>630 ± 250</td>
<td>7.4 ± 3.7</td>
<td>2,800 ± 1,200</td>
<td>680,000 ± 590,000</td>
</tr>
<tr>
<td>WT*</td>
<td>510 ± 60</td>
<td>17 ± 5.4</td>
<td>3,000 ± 300</td>
<td></td>
</tr>
<tr>
<td>CCh</td>
<td>230 ± 22</td>
<td>78 ± 31</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>S388Y</td>
<td>230 ± 20</td>
<td>32 ± 1.3</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>STYP*</td>
<td>4,300 ± 1,500</td>
<td>41 ± 32</td>
<td>18,000 ± 2,300</td>
<td>250,000 ± 48,000</td>
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<tr>
<td>T389P</td>
<td>20,000 ± 4,100</td>
<td>1,600 ± 760</td>
<td>18,000 ± 7,100</td>
<td></td>
</tr>
<tr>
<td>WT*</td>
<td>220 ± 50</td>
<td>79 ± 40</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Arecoline</td>
<td>S388Y</td>
<td>240 ± 30</td>
<td>570</td>
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<tr>
<td>STYP*</td>
<td>380 ± 140</td>
<td>320 ± 190</td>
<td>N.A.</td>
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</tr>
<tr>
<td>T389P</td>
<td>4,200 ± 410</td>
<td>320 ± 190</td>
<td>14,000 ± 2,100</td>
<td>88,000 ± 13,000</td>
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<tr>
<td>WT*</td>
<td>230,000 ± 55,000</td>
<td>320 ± 190</td>
<td>N.D.</td>
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<tr>
<td>Choline</td>
<td>210,000 ± 39,000</td>
<td>320 ± 190</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>S388Y</td>
<td>4,200 ± 410</td>
<td>320 ± 190</td>
<td>N.A.</td>
<td></td>
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<tr>
<td>STYP*</td>
<td>620,000 ± 190,000</td>
<td>320 ± 190</td>
<td>N.A.</td>
<td></td>
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<tr>
<td>T389P</td>
<td>200,000 ± 710</td>
<td>320 ± 190</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>Oxo-M</td>
<td>S388Y</td>
<td>130 ± 37</td>
<td>N.A.</td>
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<tr>
<td>STYP*</td>
<td>72 ± 21</td>
<td>13 ± 8.8</td>
<td>N.A.</td>
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<tr>
<td>T389P</td>
<td>2,200 ± 400</td>
<td>1,100 ± 300</td>
<td>1,800 ± 440</td>
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<tr>
<td>WT*</td>
<td>58 ± 15</td>
<td>13 ± 8.8</td>
<td>N.A.</td>
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<tr>
<td>Oxo</td>
<td>S388Y</td>
<td>3.8 ± 0.40</td>
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<tr>
<td>STYP*</td>
<td>490 ± 150</td>
<td>15 ± 8.4</td>
<td>N.A.</td>
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<tr>
<td>T389P</td>
<td>180 ± 20</td>
<td>15 ± 8.4</td>
<td>N.A.</td>
<td></td>
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</table>

* Most of these data are from Huang et al. (1998) and are included for comparison. H% or M% represent the percentage of the sites with K_H or K_M, respectively. The remainder is the percentage of the sites with K_L (total binding is 100%). The K_H and H% in a two-site model are not necessarily equivalent to the K_H and H% in a three-site model. Binding affinity is expressed in nM. N.A., not applicable; N.D., not determined. S388Y and T389P are for Ser388-to-Tyr and Thr389-to-Pro mutations. STYP is for Ser388Thr389-to-TyrPro mutations, whereas WT is for wild-type receptors.
reflected by overlapping binding curves in the absence and presence of 100 μM guanosine-5′-(β,γ-imido)triphosphate [Gpp(NH)p]. In contrast, ACh binding to HM1(T389P) receptors was shifted to the right in the presence of 100 μM Gpp(NH)p, although ACh still interacted with three binding sites (Table 3 and Fig. 3A). Similarly, CCh bound to two sites at HM1(S388Y) receptors as at HM1(WT) and HM1(S388Y, T389P) receptors, yet three sites at HM1(T389P) receptors (Table 3 and Fig. 3B). The high binding affinity of CCh to HM1(T389P) receptors was abolished by the addition of 100 μM Gpp(NH)p or 400 μM GTP, as indicated by a rightward shift, resulting in a binding curve similar to HM1(WT) receptors (Fig. 3B). In contrast to HM1(WT) receptors, multiple binding sites were observed for partial agonists such as arecoline (Fig. 3C) and Oxo (Fig. 3D) at HM1(T389P) receptors. The high-affinity binding of arecoline to HM1(T389P) receptors was sensitive to GTP modulation (Fig. 3C).

**Discussion**

Mutations of Ser388Thr389 to TyrPro produced enhancement of agonist potency, binding affinity, and G protein coupling (Huang et al., 1998); however, because both residues were replaced concurrently, studies of the double mutations could not identify the relative roles of individual residues. In this study, we characterized two mutant receptors with single substitutions: HM1(S388Y) and HM1(T389P) receptors. Mutation of either Ser388 or Thr389 did not change the overall structure of the mutant receptors, as indicated by generally similar antagonist binding profiles for HM1(S388Y) and HM1(T389P) receptors as found previously for HM1(S388Y, T389P) receptors and HM1(WT) receptors. The greatest reduction was observed for NMS, with a 16-fold lower binding affinity for HM1(T389P) than for HM1(WT) receptors, suggesting that the binding pocket for NMS may extend to the Thr389 region.

In general, HM1(S388Y) receptors functioned much like HM1(S388Y, T389P) receptors, whereas HM1(T389P) receptors more closely resembled HM1(WT) receptors in PI hydrolysis assays. Agonists were much more potent at HM1(S388Y) receptors than at HM1(T389P) receptors. Antagonists slightly inhibited basal PI hydrolysis at HM1(S388Y) receptors but not at HM1(T389P) receptors. Consistent with the functional similarity between HM1(S388Y) and HM1(S388Y, T389P) receptors or between HM1(T389P) and HM1(WT) receptors, HM1(S388Y) receptors had dramatically enhanced agonist binding affinity that resembled HM1(S388Y, T389P) receptors, whereas HM1(T389P) receptors showed similar agonist binding affinity

![Fig. 3.](https://molpharm.aspetjournals.org)
to HM₁(WT) receptors. These data indicate that greatly enhanced agonist binding affinity may be a major contributor to enhanced agonist potency in HM₁(S388Y) receptors. In addition, the potency difference varied widely for different agonists and was not restricted to agonists with permanent positive charges in the amine head group. Agonists without hydrophobic side chains (e.g., CCh and choline) displayed lower increases in potency at HM₁(S388Y) receptors compared with WT receptors.

Bulk or basic substitutions (such as Phe, Arg, or Lys) at Ser465 of HM₁ receptors favor the formation of active receptor states leading to significant increases in agonist potency (measured by R-SAT) and high levels of constitutive activity (Spalding et al., 1997). However, the single Ser465Tyr mutation in HM₁ receptors was not identified or characterized. In the present study, the Ser388Tyr mutation did not result in a high level of constitutive activity in PI hydrolysis assays but rather produced significant increases in agonist potency and binding affinity. These data indicate that HM₁(S388Y) receptors are probably not in an active conformational state (Kobilka et al., 1998).

HM₁(S388Y) receptors had enhanced agonist binding affinity similar to that of HM₁(S388Y, T389P) receptors but without a GTP-sensitive high-affinity binding site. For example, ACh interacted with three sites at HM₁(S388Y, T389P) receptors with the highest affinity binding sensitive to Gpp(NH)p, yet bound to two sites at HM₁(S388Y) receptors, with the high-affinity site insensitive to GTP. Choline and Oxo interacted with multiple sites at HM₁(S388Y, T389P) receptors but with only a single site at HM₁(S388Y) receptors. In contrast to HM₁(S388Y) receptors, HM₁(T389P) receptors had similar agonist binding affinities as HM₁(WT) receptors but gained an extra GTP-sensitive high-affinity binding site. For example, Oxo and arecoline bound to a single site at HM₁(WT) receptors, whereas two sites were observed for all tested agonists at HM₁(S388Y, T389P) receptors (Huang et al., 1998). In contrast, CCh, Oxo-M, and Oxo displayed three sites at HM₁(T389P) receptors, as did ACh at HM₁(WT) and HM₁(S388Y, T389P) receptors. The high-affinity binding sites of ACh, CCh, and arecoline were shifted or abolished by GTP or Gpp(NH)p, indicating that the extra high-affinity binding sites on HM₁(T389P) receptors were associated with G protein interactions. The underlying molecular mechanisms of the modulation of receptor-G protein coupling are not clear at this point. Because the mutation was at the extracellular face of TM VI, it is possible that the mutation might produce conformational changes at the cytoplasmic side of TM VI, which is a critical determinant for G protein coupling (Wess, 1996). This is consistent with the recent finding that the third extracellular loop of the β₂-adrenergic receptor can modulate receptor-G protein interactions (Zhao et al., 1998).

HM₁(S388Y) receptors lost a GTP-sensitive high-affinity site compared with HM₁(S388Y, T389P) receptors, but muscarinic agonists showed similar maximal responses and potencies at these mutant receptors. In contrast, HM₁(T389P) receptors gained an extra GTP-sensitive high-affinity site and were expressed at a higher level compared with HM₁(WT) receptors, yet most agonists had similar activities. In fact, arecoline and Oxo-M displayed reduced potencies by 3.6- and 4.9-fold, respectively, at HM₁(T389P) receptors compared with HM₁(WT) receptors. Therefore, the changes in G protein coupling with HM₁(S388Y) or HM₁(T389P) receptors apparently were not associated with functional changes in PI hydrolysis. These data suggest that the effects of mutations on G protein coupling and receptor activity may be independent. The detected changes in G protein coupling may not reflect association with Gs proteins but with other G proteins. Further investigations are necessary to address the molecular mechanisms underlying the changes in G protein coupling of the mutant receptors and the possibility that other G proteins are involved in coupling with the mutant receptors.

These data indicate a potential role for the junction of TM VI and Ne3 consistent with the proposed switch function of TM VI in receptor activation processes (Spalding et al., 1998). In addition, Ser388Thr389 may harbor allosteric binding sites that regulate the binding of ligands at the primary site, although HM₁(T389A) receptors did not exhibit changes in binding affinities for NMS, ACh, or an allosteric ligand, galamine (Matsui et al., 1995). The Thr389 residue is highly conserved in mACRs, and it is expected that equivalent mutations would cause similar effects in other members of the mACR family.

The importance of the region in ligand binding and receptor function is not restricted to the mACR family. A similar critical involvement of residues at equivalent positions in ligand binding and/or receptor function has been reported in many other G protein-coupled receptors (sequence alignment information is available from the database of mutants of family A G protein-coupled receptor (http://www-grad.fgmed.uio.no/GRAP/homepage.html; Kristiansen et al., 1996; Edvardsen and Kristiansen, 1997)). For example, Glu297 in κ-opioid receptors (Hjorth et al., 1995; Jones et al., 1998) and the corresponding Trp284 in δ-opioid receptors (Valiquette et al., 1996) appear equivalent to Ser388 and are critical for the selectivity of opioid ligands. Asp268 in the B₂ bradykinin receptors (Kyle et al., 1994; Nardone and Hogan, 1994; Novotny et al., 1994) and the corresponding Gly273 in NK₁ receptors (Bhogal et al., 1994; also equivalent to Ser388), and Asp263Val264 of AT₁ receptors (Hjorth et al., 1994) and the corresponding Phe268Asp267 in human Y₁ neuropeptide Y receptors (Walker et al., 1994; Sautel et al., 1995) are important for agonist binding. The naturally occurring mutation of Ala593Pro in the human luteinizing hormone receptor (Ala593 corresponding to Thr389) does not change hormone binding affinity but abolishes Gs coupling (Kremer et al., 1995). The Tyr272 in NK₁ receptors (equivalent to Thr389) is also important in the selective binding of nonpeptide antagonists (Gether et al., 1993, 1994; Huang et al., 1994), and mutations of Tyr272 did not affect substance P binding but did decrease the Hill slope (Gether et al., 1994). In addition, mutations at Pro271 (equivalent to Ser388) in NK₁ receptors increased substance P binding affinity and decreased the Hill slope (Gether et al., 1994), suggesting that mutations in this region improve G protein coupling, in a similar fashion to the Ser388Tyr mutation in HM₁ receptors.

Taken together, the data indicate that the Ser388Tyr mutation is the major source of enhanced agonist potency and binding affinity, whereas the Thr389Pro mutation more subtly modified receptor interactions with G proteins. Enhanced agonist binding affinity, but not G protein coupling, appears to be fully responsible for the increased agonist potency ob-
served at HM$_2$(S388Y) receptors. Ser388 and Thr389 are located about two helical turns above Tyr381 and Asn382, which are important for ACh binding and receptor function. This study demonstrates that Ser388 and Thr389 are potential targets for indirect modulation of agonist binding and G protein coupling, respectively. A combination of medicinal chemistry and pharmacological approaches could help identify ligands that can bind and induce receptor conformational changes to enhance endogenous ACh activity. This type of ligand might prove useful as a lead compound in the development of new treatments for neurological disorders such as Alzheimer’s disease.

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