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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 M_1 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 M_1 (HM_1) mutant receptor, HM_1(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 HM_1(S388Y) or HM_1(T389P) receptors. In phosphatidylinositol hydrolysis assays, muscarinic agonists showed greater potency at the HM_1(S388Y) and HM_1(S388Y, T389P) mutants compared with the wild-type and HM_1(T389P) receptors. Acetylcholine demonstrated 105-fold higher potency at HM_1(S388Y) receptors than at HM_1(T389P) receptors. Choline (30 μM, the concentration found in Dulbecco's modified Eagle's medium) exhibited 90% stimulation at HM_1(S388Y) receptors but was inactive at HM_1(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; M_1–M_5; Caulfield and Birdsall, 1998) are members of the G protein-coupled receptor family and represent important targets for drug design and development. Functionally, M_1, M_3, and M_5 subtypes couple to the activation of phospholipase C_β (PLC_β) through the pertussis toxin-insensitive $G_{q/11}$ family of G proteins; and M_2 and M_4 subtypes couple to the inhibition of adenylyl cyclase through the pertussis toxin-sensitive $G_{i/o}$ 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; Blüml 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 [HM_5(S465Y, T466P)] receptor showed significant constitutive activity, increased agonist potency, and binding affinity (Spalding et al., 1995). The HM_5 Ser465 is conserved in M_1 receptors and is an Asn residue in M_2, M_3, and M_4 receptor subtypes; whereas the HM_5 Thr466 is conserved in all five subtypes of mAChRs (Ser388 and Thr389 in

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ABBREVIATIONS: ACh, acetylcholine; mAChR, muscarinic acetylcholine receptor; CCh, carbachol; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; PI, phosphatidylinositol; HM_1, human muscarinic acetylcholine receptor subtype 1; NMS, *N*-methylscopolamine; Oxo, oxotremorine; Oxo-M, oxotremorine-M; PLC, phospholipase C; (*R*)-QNB, (*R*)-3-quinuclidinyl benzilate; Ne3, N-terminal region of the third extracellular loop; TM, transmembrane domain; R-SAT, receptor selection and amplification technology; WT, wild-type.

HM_1 receptors; see Fig. 1). In previous studies (Huang et al., 1998), we demonstrated that an equivalent mutant HM_1 receptor [HM_1(S388Y, T389P), a mutant HM_1 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 HM_5(S465Y, T466P) receptors, HM_1(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, HM_1(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 HM_1 and HM_5 receptors might be due to several factors, such as subtype (HM_1 versus HM_5 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 HM_5(S465Y, T466P) and HM_1(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 M_2 and M_3 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 HM_5 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 HM_1 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 β_2-adrenergic receptors (Kobilka et al., 1998).

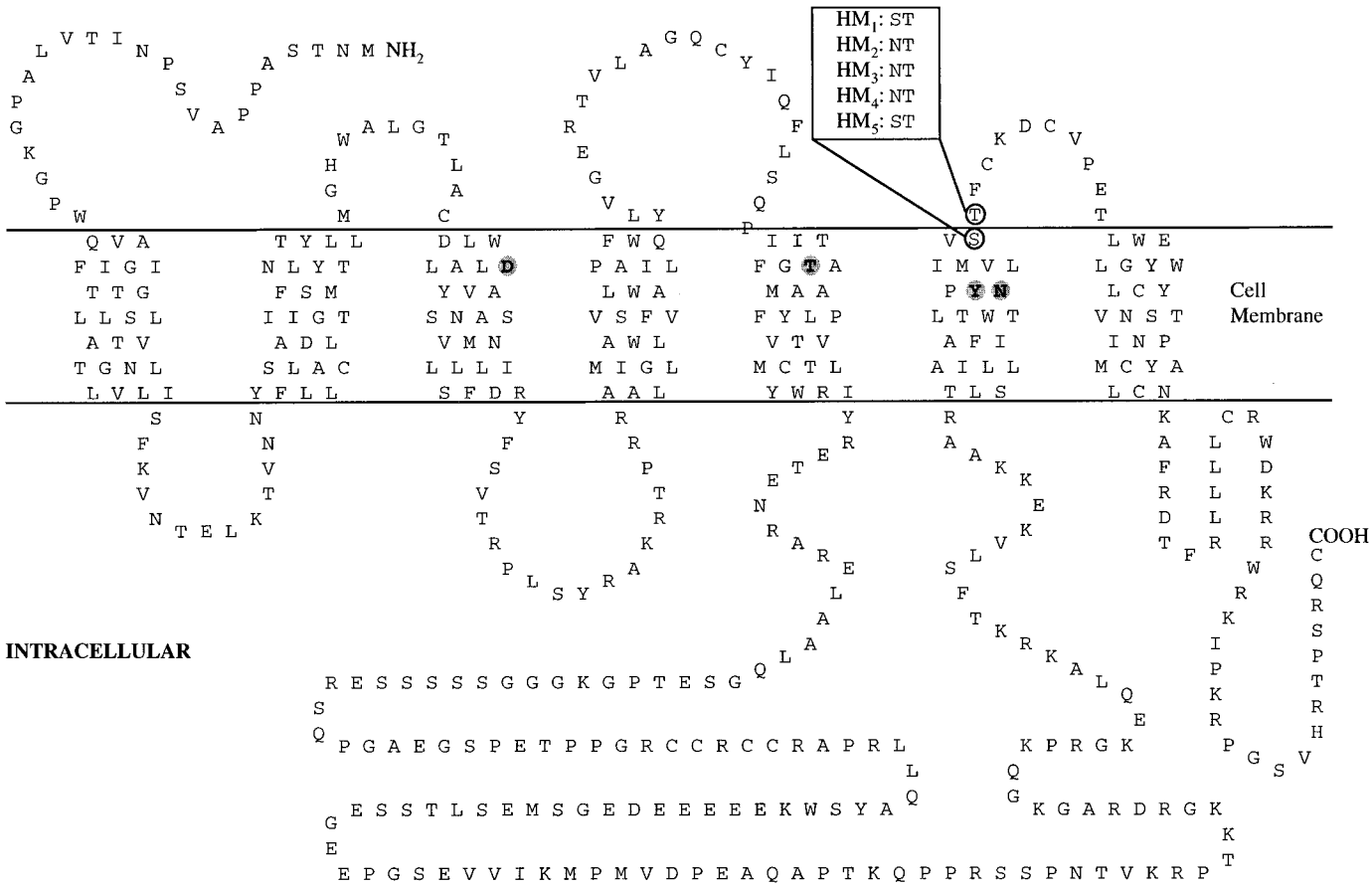

Fig. 1. A model of the HM_1 receptor amino acid sequence depicting seven TMs. Residues involved in agonist binding and receptor activation are highlighted in gray, including Asp105 in TM III, Thr192 in TM V, and Tyr381 and Asn382 in TM VI. The Ser388 and Thr389 residues are circled. The equivalent residues in other subtypes of mAChRs are listed for comparison.

To determine the functional role of individual substitutions in HM_1(S388Y, T389P) receptors, we created A9 L cell lines stably expressing HM_1(S388Y) or HM_1(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 bethanechol 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 Marsh 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 HM_1pcD1 provided by Dr. Tom I. Bonner (Bonner et al., 1988). All primers used in mutations were synthesized by Life Technologies (Grand Island, NY). The sense primer for the Ser388Tyr mutation was 5′-GGTGCTGGTGT**A**CACCTTCTGCAAGG-3′ (with the changed base in bold), and the antisense primer was 5′-CCTTGCAGAAGGTGTACACCAGCACC-3′. The sense primer for the Thr389Pro mutation was 5′-GGTGCTGGTGTCC**C**CCTTCTGCAAGG-3′ (with the mutated base in bold), and the antisense primer was 5′-CCTTGCAGAAGGGGGACACCAGCACC-3′. The mutations were confirmed by dideoxy 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 HM_1(S388Y)pcD1 or HM_1(T389P)pcD1 and pNEOβGAL (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 HM_1(S388Y) or HM_1(T389P) receptors were subcultured for functional and binding studies.

Radioligand Binding Assays–TopCount NXT System. [^{3}H](R)-3-Quinuclidinyl benzilate [(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. HM_1(S388Y) and HM_1(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 ($P < .05$) reduction in binding affinity for [^{3}H](R)-QNB compared with HM_1[wild-type (WT)] and/or HM_1(S388Y, T389P) receptors. HM_1(T389P) receptors also showed a 1.7-fold lower ($P < .01$) binding affinity for [^{3}H](R)-QNB than HM_1(S388Y) receptors (Table 1). When other classic muscarinic antagonists were examined, HM_1(S388Y) receptors showed the same antagonist binding profiles as HM_1(WT) and/or HM_1(S388Y, T389P) receptors ($P > .05$). In contrast, HM_1(T389P) receptors displayed varying degrees of reduced affinity for these antagonists (2.8- to 16-fold; $P < .05$) compared with HM_1(WT) and/or HM_1(S388YY, T389P) receptors.

In the previous study of HM_1(S388Y, T389P) receptors, we found that a freezing/thawing treatment, but not incubation at 37°C, significantly reduced [^{3}H](R)-QNB binding activity in membrane homogenates (Huang et al., 1998). Because HM_1(S388Y) receptors stably expressed in A9 L cells showed similar functional properties as HM_1(S388Y, T389P) receptors (see below), we examined the effects of a freezing/thawing treatment (−70°C overnight) on total [^{3}H](R)-QNB binding activity. In a similar fashion to that observed on HM_1(S388Y, T389P) receptors, total specific binding activity of frozen membranes of HM_1(S388Y) receptors was decreased by 26 ± 9.3% ($n = 4$) compared with nonfrozen membrane homogenates. The frozen membranes showed a binding affinity for [^{3}H](R)-QNB of 60 ± 18 pM ($n = 4$) versus 67 ± 16

TABLE 1

Antagonist binding properties at HM_1(S388Y) and HM_1(T389P) receptors

Binding assays were conducted on membrane homogenates. Inhibition binding assays were performed in the presence of 0.1 nM [^{3}H](R)-QNB for HM_1(S388Y) receptors or 0.3 nM [^{3}H](R)-QNB for HM_1(T389P) receptors.

Antagonist	HM_1(S388Y)	HM_1(T389P)	HM_1(WT)[a]	HM_1(S388Y, T389P)[a]
[^{3}H](R)-QNB	0.063 ± 0.016*	0.11 ± 0.025**	0.029 ± 0.0067	0.060 ± 0.019
	(10.23 ± 0.11)	(9.93 ± 0.09)	(10.57 ± 0.11)	(10.28 ± 0.14)
l-Hyoscyamine	0.29 ± 0.11*	0.46 ± 0.12*	0.088 ± 0.0099	0.21 ± 0.030
	(9.59 ± 0.15)	(9.42 ± 0.18)	(10.06 ± 0.04)	(9.69 ± 0.06)
(−)-NMS	0.040 ± 0.0078	0.35 ± 0.13**	0.022 ± 0.0025	0.052 ± 0.031
	(10.42 ± 0.09)	(9.55 ± 0.23)	(10.61 ± 0.03)	(10.44 ± 0.26)
Pirenzepine	13 ± 5.9	33 ± 11**	4.4 ± 0.74	12 ± 0.2
	(7.99 ± 0.21)	(7.53 ± 0.14)	(8.37 ± 0.08)	(7.91 ± 0.01)
Trihexyphenidyl	0.27 ± 0.10	N.D.	0.10 ± 0.0027	0.32 ± 0.056
	(9.66 ± 0.22)		(10.02 ± 0.11)	(9.51 ± 0.08)

Data represent the mean (± S.E.) from two or three assays, each performed in triplicate. Concentrations are in nM. N.D., not determined. Values in parentheses are pK_d or pK_i (−log[K_d] or −log[K_i], respectively).

[a] Binding data for HM_1(WT) and HM_1(S388Y, T389P) receptors are from Huang et al. (1998) and are included for comparison.

* $P < .05$ compared with HM_1(WT) receptors.

** $P < .05$ compared with HM_1(WT) and HM_1(S388Y, T389P) receptors.

pM ($n = 5$) for fresh membranes. These results indicated that HM_1(S388Y) receptors had similar vulnerability to freezing/thawing as HM_1(S388Y, T389P) receptors. The effects of freezing/thawing treatment on HM_1(T389P) receptors were not examined.

Pharmacology of HM_1(S388Y) and HM_1(T389P) Receptors. Muscarinic agonists used to characterize HM_1(S388Y, T389P) receptors (Huang et al., 1998) were also examined at HM_1(S388Y) and HM_1(T389P) receptors to compare maximal responses and potencies in stimulating PI hydrolysis. All PI hydrolysis experiments were conducted in Krebs-Henseleit buffer instead of media to exclude the effects of choline and other potential muscarinic agonists in animal serum (Huang et al., 1998).

In general, muscarinic agonists showed much higher potencies at HM_1(S388Y) receptors than at HM_1(T389P) receptors. HM_1(S388Y) and HM_1(S388Y, T389P) receptors displayed similar pharmacological profiles, whereas HM_1(T389P) receptors functioned more like HM_1(WT) receptors in PI hydrolysis assays with classic muscarinic agonists (Table 2 and Fig. 2). Specifically, muscarinic agonists showed similar dose-response profiles (maximal responses and/or potencies) at HM_1(T389P) receptors (Fig. 2, A–E) as at WT receptors (Huang et al., 1998). Choline, a component of Dulbecco's modified Eagle's medium, was inactive 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(T389P) receptors with low potency. In contrast, all muscarinic agonists exhibited much stronger activity at HM_1(S388Y) receptors than at HM_1(T389P) or WT receptors. This was reflected by large increases in agonist potencies, such as over 100-fold increases for ACh, oxotremorine (Oxo)-M (Oxo-M), and arecoline and 28- to 70-fold increases for carbachol (CCh) and Oxo, respectively. Choline functioned as a full agonist at HM_1(S388Y) receptors with an maximal response comparable with ACh and CCh and with a potency comparable with that at HM_1(S388Y, T389P) receptors (200 ± 20 μM; Huang et al., 1998), which is about 17-fold higher than that at HM_1(T389P) receptors. These data indicate 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, methylcarbachol, 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 $330 \pm 12\%$ ($n \geq 3$), 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 *l*-hyoscyamine (the active enantiomer of atropine). The basal PI hydrolysis was reduced at HM_1(S388Y) receptors ($-14 \pm 10\%$, $n = 5$) but not HM_1(T389P) receptors ($4.7 \pm 4.8\%$, $n = 3$). 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 ($-22 \pm 9.3\%$, $n = 4$) and NMS ($-20 \pm 5.6\%$, $n = 3$). 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 ($160 \pm 33\%$, $n = 6$) 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 $75 \pm 13\%$ ($n = 5$). 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 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) eceptors 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 2

Pharmacology of HM_1(S388Y) and HM_1(T389P) receptors

Receptor expression levels were determined from [^{3}H](*R*)-QNB saturation binding assays on membrane homogenates prepared from stably transfected A9 L cells. PI hydrolysis was carried out in KH buffer to exclude potential effects of choline in media and unknown muscarinic agonists in serum. Data represent the mean ± S.E. from a minimum of three assays, each performed in duplicate.

Ligand	HM_1(S388Y) 2,600 ± 160 fmol/mg		HM_1(S388Y, T389P)[a] 2,600 ± 690 fmol/mg		HM_1(T389P) 1,200 ± 210 fmol/mg		HM_1(WT)[a] 260 ± 29 fmol/mg	
	S_{max}	EC_{50}	S_{max}	EC_{50}	S_{max}	EC_{50}	S_{max}	EC_{50}
	%	*nM*	%	*nM*	%	μM	%	μM
ACh	410 ± 23	8.3 ± 3.6	550 ± 95	4.8 ± 1.6	320 ± 77	0.87 ± 0.71	370 ± 66	1.5 ± 0.19
CCh	430 ± 27	130 ± 53	520 ± 77	59 ± 3.5	300 ± 89	3.7 ± 0.69	350 ± 55	13 ± 1.1
Choline	390 ± 31	160,000 ± 85,000	420 ± 68	200,000 ± 20,000	110 ± 5.8	2,700 ± 1,500	2.6 ± 7.7	N.A.
Oxo-M	420 ± 22	26 ± 9.9	510 ± 45	12 ± 5.4	330 ± 89	3.9 ± 2.0	310 ± 29	0.80 ± 0.15
Oxo	440 ± 46	13 ± 5.3	500 ± 100	18 ± 2.8	180 ± 46	0.91 ± 0.33	110 ± 28	0.22 ± 0.030
Arecoline	330 ± 4.8	120 ± 65	510 ± 100	60 ± 14	250 ± 46	13 ± 5.4	150 ± 26	3.6 ± 0.70

[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 HM_1(S388Y) receptors as HM_1(WT) receptors, yet 2.7-fold higher affinity than for HM_1(T389P) receptors. Except for Oxo-M, which showed more than 15-fold higher binding affinity for HM_1(S388Y) receptors than for HM_1(S388Y, T389P) receptors (Huang et al., 1998), all other agonists (ACh, CCh, Oxo-M, arecoline, and choline) showed similar binding affinity at HM_1(S388Y) receptors and HM_1(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 HM_1(S388Y) receptors, differences were observed in the nature of agonist binding profiles between HM_1(S388Y) and HM_1(T389P) receptors (Table 3 and Fig. 3). ACh interacted with three binding sites at HM_1(S388Y, T389P) and HM_1(WT) receptors (Huang et al., 1998). High-affinity binding of ACh at HM_1(S388Y, T389P) receptors was lost at HM_1(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 HM_1(S388Y) receptors was insensitive to GTP modulation as

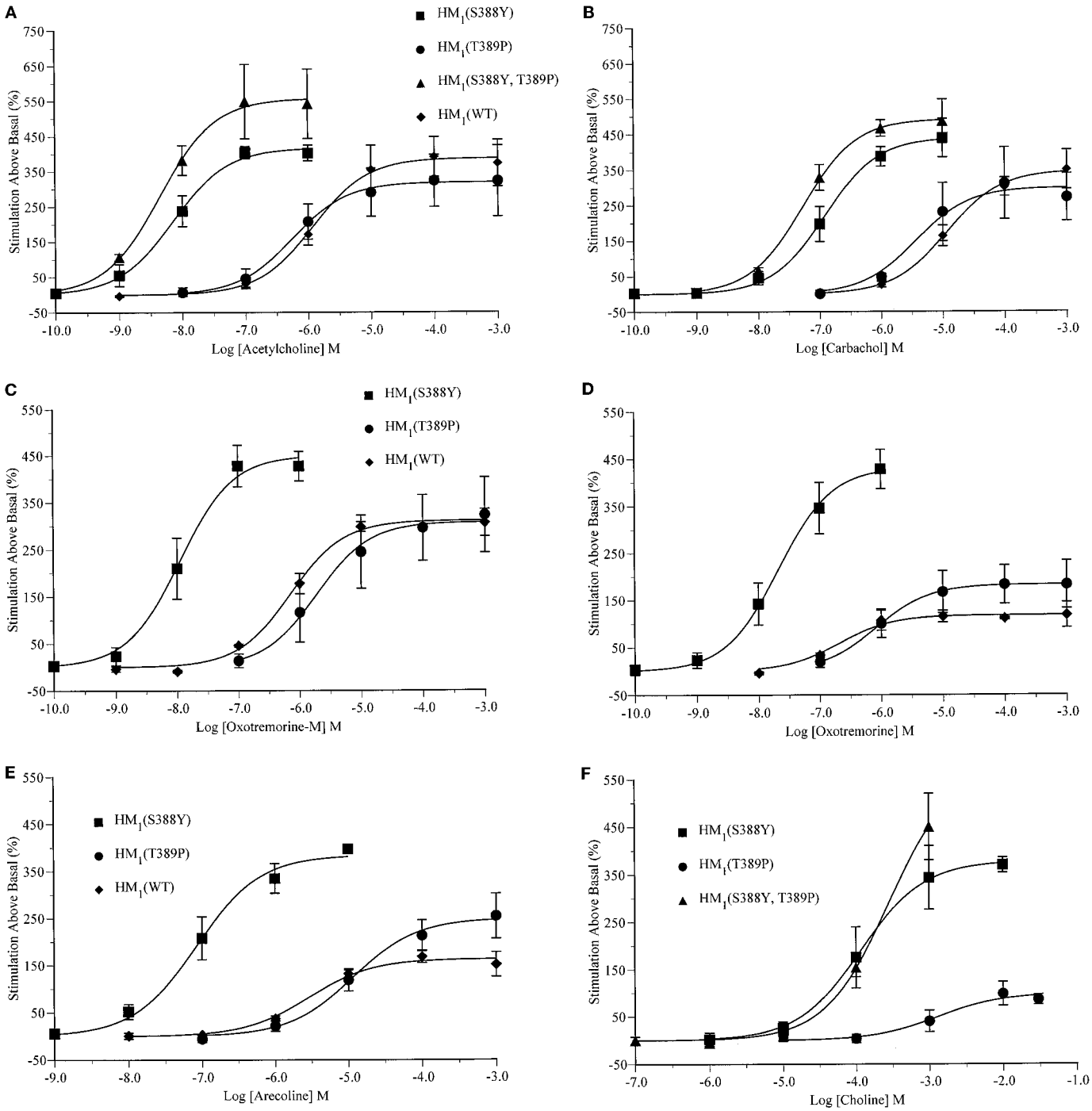

Fig. 2. Pharmacology of muscarinic agonists at HM_1(S388Y) and HM_1(T389P) receptors. Data for HM_1(WT) and/or HM_1(S388Y, T389P) receptors are from Huang et al. (1998) and are included for comparison. PI hydrolysis assays were conducted in Krebs-Henseleit buffer. Data represent the mean ± S.E. from 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 HM_1(S388Y) and HM_1(T389P) receptors

Binding assays were conducted on membrane homogenates in the presence of [^{3}H](*R*)-QNB [0.1 nM for HM_1(S388Y) receptors or 0.3 nM for HM_1(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_H, K_M (medium binding affinity), and K_L were assigned in three-site models.

	Mutation	Without GTP				With Gpp(NH)p (100 μM) or GTP (400 μM)			
		One Site	Two or Three Sites			One Site	Two or Three Sites		
		K_i	K_H (H%)	K_M (M%)	K_L	K_i	K_H (H%)	K_M (M%)	K_L
ACh	S388Y	42 ± 5.5	15 ± 5.2 (59 ± 10)	N.A.	500 ± 180	28 ± 8.1	7.0 ± 2.6 (56 ± 4.7)	N.A.	230 ± 50
	STYP[a]	13 ± 2.7	0.09 ± 0.028 (23)	16 ± 4.5 (59)	1,300 ± 820	37 ± 5.7	N.A.	N.A.	N.A.
	T389P	630 ± 250	7.4 ± 3.7 (15 ± 6.5)	480 ± 92 (50 ± 13)	72,000 ± 11,000	2,800 ± 1,200	330 ± 130 (22 ± 8.6)	20,000 ± 19,000 (52 ± 2.0)	680,000 ± 590,000
	WT[a]	510 ± 60	17 ± 5.4 (30)	1,100 ± 330 (54)	39,000 ± 14,000	3,000 ± 300	1,400 ± 570 (55)	N.A.	13,000 ± 4,400
CCh	S388Y	230 ± 22	78 ± 31 (56 ± 8.4)	N.A.	2,800 ± 1,000	N.D.	N.D.	N.D.	N.D.
	STYP[a]	230 ± 80	3.2 ± 1.3 (18)	N.A.	930 ± 260	N.D.	N.D.	N.D.	N.D.
	T389P	4,300 ± 1,500	41 ± 32 (16 ± 2.2)	5,600 ± 1,700 (51 ± 4.3)	360,000 ± 180,000	18,000 ± 2,300	6,700 ± 2,300 (52 ± 2.5)	N.A.	250,000 ± 48,000
	WT[a]	20,000 ± 4,100	1,600 ± 760 (28)	N.A.	83,000 ± 21,000	18,000 ± 7,100	N.A.	N.A.	N.A.
Arecoline	S388Y	220 ± 50	79 ± 40 (51 ± 13)	N.A.	1,500 ± 650	N.D.	N.D.	N.D.	N.D.
	STYP[a]	240 ± 30	8.6 ± 7.0 (21)	N.A.	550 ± 60	570	N.A.	N.A.	N.A.
	T389P	380 ± 140	320 ± 190 (38 ± 0.71)	N.A.	33,000 ± 8,600	14,000 ± 2,100	3,800 ± 1,200 (47 ± 4.9)	N.A.	88,000 ± 13,000
	WT[a]	4,200 ± 410	N.A.	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.
Choline	S388Y	230,000 ± 55,000	N.A.	N.A.	N.A.	N.D.	N.D.	N.D.	N.A.
	STYP[a]	210,000 ± 39,000	16,000 ± 14,000 (19)	N.A.	400,000 ± 68,000	360,000 ± 41,000	19,000 ± 750 (9.5)	N.A.	480,000 ± 51,000
	T389P	620,000 ± 190,000	N.A.	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.
	WT[a]	200,000 ± 710	N.A.	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.
Oxo-M	S388Y	130 ± 37	33 ± 13 (48 ± 15)	N.A.	1,200 ± 400	N.D.	N.D.	N.D.	N.D.
	STYP	72 ± 21	13 ± 8.8 (37 ± 8.9)	N.A.	1,200 ± 400	N.D.	N.D.	N.D.	N.D.
	T389P	760 ± 120	5.5 ± 2.0 (20 ± 5.0)	1,100 ± 300 (49 ± 0.61)	64,000 ± 9,900	N.D.	N.D.	N.D.	N.D.
	WT	2,200 ± 400	1,100 ± 300 (66 ± 4.4)	N.A.	33,000 ± 8,300	1,800 ± 440	N.A.	N.A.	N.A.
Oxo	S388Y	58 ± 15	N.A.	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.
	STYP[a]	3.8 ± 0.40	0.70 ± 0.29 (46)	N.A.	18 ± 3.4	N.D.	N.D.	N.D.	N.D.
	T389P	490 ± 150	15 ± 8.4 (21 ± 4.0)	1,200 ± 900 (40 ± 12)	43,000 ± 800	N.D.	N.D.	N.D.	N.D.
	WT[a]	180 ± 20	N.A.	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.

[a] 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 HM_1(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 HM_1(S388Y) receptors as at HM_1(WT) and HM_1(S388Y, T389P) receptors, yet three sites at HM_1(T389P) receptors (Table 3 and Fig. 3B). The high binding affinity of CCh to HM_1(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 HM_1(WT) receptors (Fig. 3B). In contrast to HM_1(WT) receptors, multiple binding sites were observed for partial agonists such as arecoline (Fig. 3C) and Oxo (Fig. 3D) at HM_1(T389P) receptors. The high-affinity binding of arecoline to HM_1(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: HM_1(S388Y) and HM_1(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 HM_1(S388Y) and HM_1(T389P) receptors as found previously for HM_1(S388Y, T389P) receptors and HM_1(WT) receptors. The greatest reduction was observed for NMS, with a 16-fold lower binding affinity for HM_1(T389P) than for HM_1(WT) receptors, suggesting that the binding pocket for NMS may extend to the Thr389 region.

In general, HM_1(S388Y) receptors functioned much like HM_1(S388Y, T389P) receptors, whereas HM_1(T389P) receptors more closely resembled HM_1(WT) receptors in PI hydrolysis assays. Agonists were much more potent at HM_1(S388Y) receptors than at HM_1(T389P) receptors. Antagonists slightly inhibited basal PI hydrolysis at HM_1(S388Y) receptors but not at HM_1(T389P) receptors. Consistent with the functional similarity between HM_1(S388Y) and HM_1(S388Y, T389P) receptors or between HM_1(T389P) and HM_1(WT) receptors, HM_1(S388Y) receptors had dramatically enhanced agonist binding affinity that resembled HM_1(S388Y, T389P) receptors, whereas HM_1(T389P) receptors showed similar agonist binding affinity

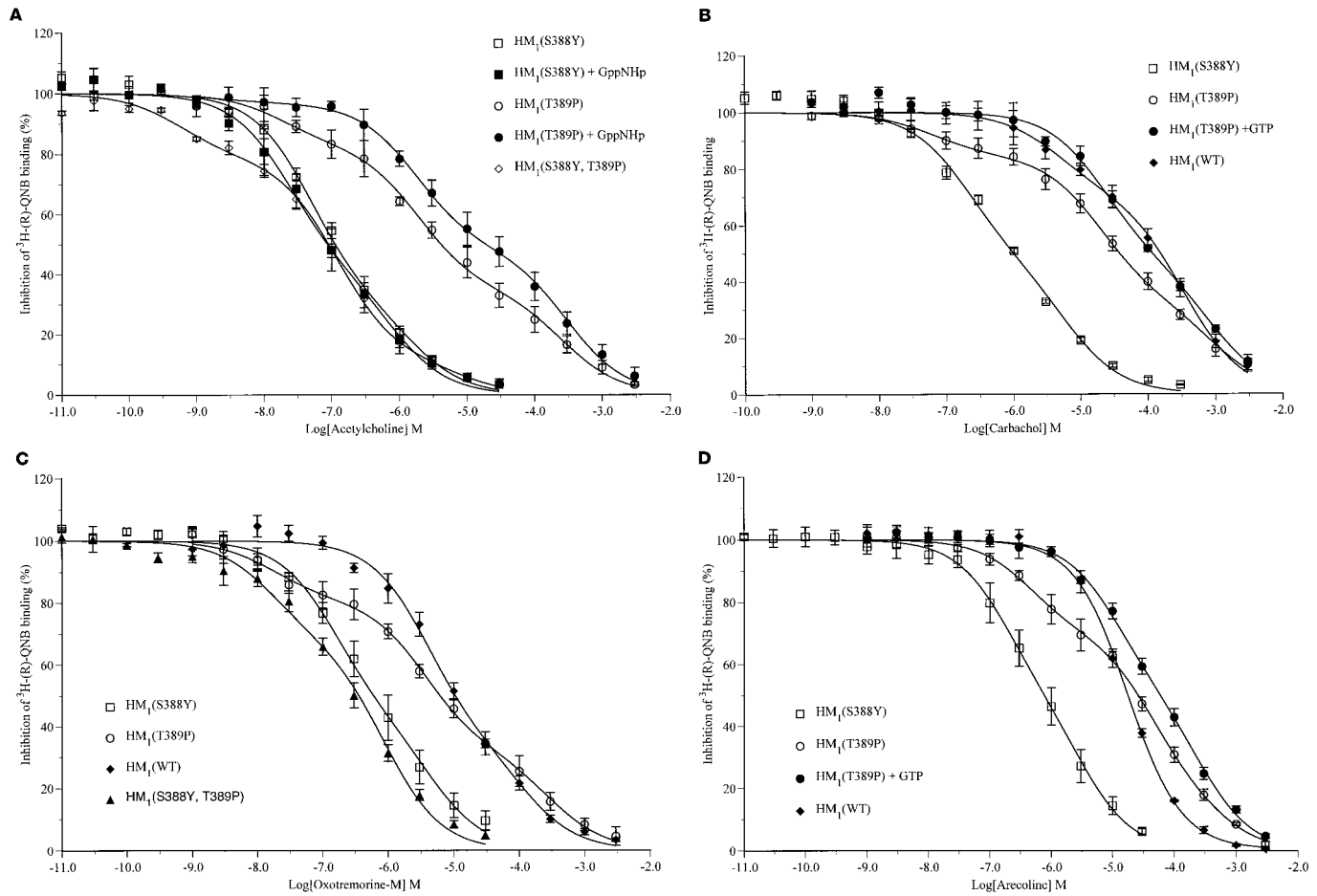

Fig. 3. Muscarinic agonist binding properties at HM_1(S388Y) and HM_1(T389P) receptors. Agonist binding data (except Oxo-M) for HM_1(WT) and HM_1(S388Y, T389P) receptors are from Huang et al. (1998) and are included for comparison. Inhibition binding assays were conducted on membrane homogenates in the presence of 0.1 nM [^{3}H](R)-QNB for HM_1(S388Y) receptors and 0.3 nM [^{3}H](R)-QNB for HM_1(T389P) receptors. Data represent the mean ± S.E.M. from a minimum of three assays, each performed in triplicate. A, ACh binding in the absence and presence of Gpp(NH)p (100 μM). B, CCh binding in the absence and presence of GTP (400 μM). C, Oxo-M in the absence of GTP analogs. D, arecoline binding in the absence and presence of GTP (400 μM).

to HM_1(WT) receptors. These data indicate that greatly enhanced agonist binding affinity may be a major contributor to enhanced agonist potency in HM_1(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 HM_1(S388Y) receptors compared with WT receptors.

Bulky or basic substitutions (such as Phe, Arg, or Lys) at Ser465 of HM_5 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 HM_5 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 HM_1(S388Y) receptors are probably not in an active conformational state but in a state favorable for agonist binding and activation as suggested recently for the mutant β_2-adrenergic receptor (Kobilka et al., 1998).

HM_1(S388Y) receptors had enhanced agonist binding affinity similar to that of HM_1(S388Y, T389P) receptors but without a GTP-sensitive high-affinity binding site. For example, ACh interacted with three sites at HM_1(S388Y, T389P) receptors with the highest affinity binding sensitive to Gpp(NH)p, yet bound to two sites at HM_1(S388Y) receptors, with the high-affinity site insensitive to GTP. Choline and Oxo interacted with multiple sites at HM_1(S388Y, T389P) receptors but with only a single site at HM_1(S388Y) receptors. In contrast to HM_1(S388Y) receptors, HM_1(T389P) receptors had similar agonist binding affinities as HM_1(WT) receptors but gained an extra GTP-sensitive high-affinity binding site. For example, Oxo and arecoline bound to a single site at HM_1(WT) receptors, whereas two sites were observed for all tested agonists at HM_1(S388Y, T389P) receptors (Huang et al., 1998). In contrast, CCh, Oxo-M, and Oxo displayed three sites at HM_1(T389P) receptors, as did ACh at HM_1(WT) and HM_1(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 HM_1(T3989P) 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 β_2-adrenergic receptor can modulate receptor-G protein interactions (Zhao et al., 1998).

HM_1(S388Y) receptors lost a GTP-sensitive high-affinity site compared with HM_1(S388Y, T389P) receptors, but muscarinic agonists showed similar maximal responses and potencies at these mutant receptors. In contrast, HM_1(T389P) receptors gained an extra GTP-sensitive high-affinity site and were expressed at a higher level compared with HM_1(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 HM_1(T389P) receptors compared with HM_1(WT) receptors. Therefore, the changes in G protein coupling with HM_1(S388Y) or HM_1(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 $G_{q/11}$ 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 HM_1(T389A) receptors did not exhibit changes in binding affinities for NMS, ACh, or an allosteric ligand, gallamine (Matsui et al., 1995). The Thr389 residue is highly conserved in mAChRs, and it is expected that equivalent mutations would cause similar effects in other members of the mAChR family.

The importance of the region in ligand binding and receptor function is not restricted to the mAChR 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-grap.fagmed.uit.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 B_2 bradykinin receptors (Kyle et al., 1994; Nardone and Hogan, 1994; Novotny et al., 1994) and the corresponding Gly273 in NK_2 receptors (Bhogal et al., 1994; also equivalent to Ser388), and Asp263Val264 of AT_1 receptors (Hjorth et al., 1994) and the corresponding Phe286Asp287 in human Y_1 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 G_s coupling (Kremer et al., 1995). The Tyr272 in NK_1 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 NK_1 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 HM_1 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_1(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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References

Bhogal N, Donnelly D and Findlay JB (1994). The ligand binding site of the neurokinin 2 receptor: Site-directed mutagenesis and identification of neurokinin A binding residues in the human neurokinin 2 receptor. *J Biol Chem* **269:**27269–27274.

Blüml K, Mutschler E and Wess J (1994) Functional role in ligand binding and receptor activation of an asparagine residue present in the sixth transmembrane domain of all muscarinic acetylcholine receptors. *J Biol Chem* **269:**18870–18876.

Bonner TI, Young AC, Brann MR and Buckley NJ (1988) Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1:**403–410.

Caulfield MP (1993) Muscarinic receptors–characterization, coupling and function. *Pharmacol Ther* **58:**319–379.

Caulfield MP and Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50:**279–290.

Chen C and Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:**2745–2752.

Edvardsen O and Kristiansen K (1997) Computerization of mutant data: The tinyGRAP mutant database. *7TM J* **6:**1–6.

Ellis J, Porter DJ and Seidenberg M (1998) Homologous mutations increase agonist affinity at m1, m3 and m5 muscarinic receptors. *Soc Neurosci* **24:**624.

Fraser CM, Wang CD, Robinson DA, Gocayne JD and Venter JC (1989) Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: Conserved aspartic acids play important roles in receptor function. *Mol Pharmacol* **36:**840–847.

Gether U, Johansen TE, Snider RM, Lowe JA, Nakanishi S and Schwartz TW (1993) Different binding epitopes on the NK1 receptor for substance P and non-peptide antagonist. *Nature (Lond)* **362:**345–348.

Gether U, Nilsson L, Lowe JA and Schwartz TW (1994) Specific residues at the top of transmembrane segment V and VI of the neurokinin-1 receptor involved in binding of the nonpeptide antagonist CP 96,345 [corrected; published erratum appears in *J Biol Chem* (1994) **269:**32708]. *J Biol Chem* **269:**23959–23964.

Hjorth SA, Schambye HT, Greenlee WJ and Schwartz TW (1994) Identification of peptide binding residues in the extracellular domains of the AT1 receptor. *J Biol Chem* **269:**30953–30959.

Hjorth SA, Thirstrup K, Grandy DK and Schwartz TW (1995) Analysis of selective binding epitopes for the κ-opioid receptor antagonist nor-binaltorphimine. *Mol Pharmacol* **47:**1089–1094.

Huang XP, Nagy PI, Williams FE, Peseckis SM and Messer WS Jr (1999) Roles of threonine 192 and asparagine 382 in agonist and antagonist interactions with m1 muscarinic acetylcholine receptors. *Br J Pharmacol* **126:**735–745.

Huang XP, Williams FE, Peseckis SM and Messer WS Jr (1998) Pharmacological characterization of human m1 muscarinic acetylcholine receptors with double mutations at the junction of TM VI and the third extracellular domain. *J Pharmacol Exp Ther* **286:**1129–1139.

Huang XY, Morielli AD and Peralta EG (1994) Molecular basis of cardiac potassium channel stimulation by protein kinase A. *Proc Natl Acad Sci USA* **91:**624–628.

Hulme EC, Birdsall NJ and Buckley NJ (1990) Muscarinic receptor subtypes. *Annu Rev Pharmacol Toxicol* **30:**633–673.

Jones RM, Hjorth SA, Schwartz TW and Portoghese PS (1998) Mutational evidence for a common kappa antagonist binding pocket in the wild-type kappa and mutant mu[K303E] opioid receptors. *J Med Chem* **41:**4911–4914.

Kobilka B, Gether U, Seifert R, Lin S and Ghanouni P (1998) Examination of ligand-induced conformational changes in the β_2 adrenergic receptor. *Life Sci* **62:**1509–1512.

Kremer H, Kraaij R, Toledo SP, Post M, Fridman JB, Hayashida CY, van Reen M, Milgrom E, Ropers HH and Mariman E (1995) Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene. *Nat Genet* **9:**160–164.

Kristiansen K, Dahl SG and Edvardsen O (1996) A database of mutants and effects of site-directed mutagenesis experiments on G protein-coupled receptors. *Proteins* **26:**81–94.

Kyle DJ, Chakravarty S, Sinsko JA and Stormann TM (1994) A proposed model of bradykinin bound to the rat B2 receptor and its utility for drug design. *J Med Chem* **37:**1347–1354.

Matsui H, Lazareno S and Birdsall NJ (1995) Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. *Mol Pharmacol* **47:**88–98.

Nardone J and Hogan PG (1994) Delineation of a region in the B2 bradykinin receptor that is essential for high-affinity agonist binding. *Proc Natl Acad Sci USA* **91:**4417–4421.

Nordvall G and Hacksell U (1993) Binding-site modeling of the muscarinic m1 receptor: A combination of homology-based and indirect approaches. *J Med Chem* **36:**967–976.

Novotny EA, Bednar DL, Connolly MA, Connor JR and Stormann TM (1994) Mutation of aspartate residues in the third extracellular loop of the rat B2 bradykinin receptor decreases affinity for bradykinin. *Biochem Biophys Res Commun* **201:**523–530.

Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor: Extending the ternary complex model. *J Biol Chem* **268:**4625–4636.

Sautel M, Martinez R, Munoz M, Peitsch MC, Beck-Sickinger AG and Walker P (1995) Role of a hydrophobic pocket of the human Y1 neuropeptide Y receptor in ligand binding. *Mol Cell Endocrinol* **112:**215–222.

Spalding TA, Burstein ES, Brauner-Osborne H, Hill-Eubanks D and Brann MR (1995) Pharmacology of a constitutively active muscarinic receptor generated by random mutagenesis. *J Pharmacol Exp Ther* **275:**1274–1279.

Spalding TA, Burstein ES, Henderson SC, Ducote KR and Brann MR (1998) Identification of a ligand-dependent switch within a muscarinic receptor. *J Biol Chem* **273:**21563–21568.

Spalding TA, Burstein ES, Wells JW and Brann MR (1997) Constitutive activation of the m5 muscarinic receptor by a series of mutations at the extracellular end of transmembrane 6. *Biochemistry* **36:**10109–10116.

Valiquette M, Vu HK, Yue SY, Wahlestedt C and Walker P (1996) Involvement of Trp-284, Val-296, and Val-297 of the human delta-opioid receptor in binding of delta-selective ligands. *J Biol Chem* **271:**18789–18796.

Walker P, Munoz M, Martinez R and Peitsch MC (1994) Acidic residues in extracellular loops of the human Y1 neuropeptide Y receptors are essential for ligand binding. *J Biol Chem* **269:**2863–2869.

Ward JS, Merritt L, Klimkowski VJ, Lamb ML, Mitch CH, Bymaster FP, Sawyer B, Shannon HE, Olesen PH and Honore T (1992) Novel functional M1 selective muscarinic agonists: 2. Synthesis and structure-activity relationships of 3-pyrazinyl-1,2,5,6-tetrahydro-1-methylpyridines: Construction of a molecular model for the M1 pharmacophore. *J Med Chem* **35:**4011–4019.

Ward SDJ and Hulme EC (1997) Alanine scanning mutagenesis in transmembrane domain six of the rat m1-muscarinic acetylcholine receptor (rat m1-receptor). *Br J Pharmacol* **120:**284P.

Wess J (1993) Mutational analysis of muscarinic acetylcholine receptors: Structural basis of ligand/receptor/G protein interactions. *Life Sci* **53:**1447–1463.

Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. *Crit Rev Neurobiol* **10:**69–99.

Wess J, Gdula D and Brann MR (1991) Site-directed mutagenesis of the m3 muscarinic receptor: Identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. *EMBO J* **10:**3729–3734.

Wess J, Maggio R, Palmer JR and Vogel Z (1992) Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation: A study with m3 muscarinic receptor point mutants. *J Biol Chem* **267:**19313–19319.

Zhao MM, Gaivin RJ and Perez DM (1998) The third extracellular loop of the β_2-adrenergic receptor can modulate receptor/G protein affinity. *Mol Pharmacol* **53:**524–529.

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