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Mutational Disruption of a Conserved Disulfide Bond in Muscarinic Acetylcholine Receptors Attenuates Positive Homotropic Cooperativity between Multiple Allosteric Sites and Has Subtype-Dependent Effects on the Affinities of Muscarinic Allosteric Ligands

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ABSTRACT

The 2nd outer loop (o2) of muscarinic acetylcholine receptors (mAChRs) contains a highly conserved cysteine residue that is believed to participate in a disulfide bond and is flanked on either side by epitopes that are critical to the binding of many muscarinic allosteric modulators. We determined the allosteric binding parameters of the modulators gallamine, W84, and tetrahydroaminoacridine (THA) at M_2 and M_3 mAChRs in which these cysteine residues had been mutated to alanines. THA is known to bind to mAChRs with a strong positive homotropic cooperativity (a Hill slope of approximately 2) that implies that it must interact with multiple allosteric sites. The disulfide cysteine mutations in M_2 receptors reduced the allosteric potencies of the tested modulators as if the critical adjacent residue (Tyr177) itself had been mutated. However, in M_3 receptors, the disulfide cysteine mutations had no effect on the potencies of gallamine or W84 and even increased the potency of THA. It was most interesting that the strong, positive, homotropic interactions of THA at both M_2 and M_3 receptors were markedly reduced by the cysteine mutations. In addition, gallamine also displayed positive homotropic cooperativity in its interactions with M_3 receptors (but not M_2 receptors), and this cooperativity was not evident in the cysteine mutants. Thus, it seems that these cysteine residues play a role in linking cooperating allosteric sites, although it is not currently possible to say whether these multiple sites lie within one receptor or on two linked receptors of a dimer or higher order oligomer.

A growing number of G protein-coupled receptors (GPCRs) are known to possess extracellular allosteric binding sites that are topographically distinct from, but conformationally linked to, the orthosteric binding site for the endogenous agonist and its competitive ligands (Christopoulos and Kenakin, 2002). Binding of the allosteric modulator triggers conformational changes in the receptor that in turn modify the orthosteric binding site and/or receptor function. Allosteric modulators can have several favorable advantages as therapeutic agents over traditional orthosteric ligands, such as better subtype selectivity and safety and the ability to preserve spatiotemporal patterning in the CNS (Christopoulos, 2002). Therefore, they represent a new trend for drug design and development for many GPCRs, especially those for which highly selective orthosteric agonists or antagonists are not available yet, such as the muscarinic acetylcholine receptors (mAChRs). The family of mAChRs is one of the most intensively studied model systems for allosteric modulation, especially at the molecular level. A dramatic demonstration of this modulation is the retardation of the dissociation rate of orthosteric ligands, such as [^{3}H]*N*-methyl scopolamine (NMS), by allosteric modulators, such as the prototypical mAChR allosteric modulators gallamine and W84 and the atypical modulator tetrahydroaminoacridine (THA/tacrine). THA is considered atypical among muscarinic allosteric modulators in that it slows the dissociation rate of [^{3}H]NMS with a steep concentration-response curve that has a Hill slope of approximately 2 at all five subtypes of mAChRs (Potter et al., 1989; Ellis and Seidenberg, 2000),

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ABBREVIATIONS: GPCR, G protein-coupled receptor; NMS, *N*-methyl scopolamine; W84, hexamethylene-bis-[3-phthalimidopropyl]ammonium) dibromide; THA, tetrahydroaminoacridine; o2, the second outer (extracellular) loop of the receptor; o3, the third outer (extracellular) loop of the receptor; mAChR, muscarinic acetylcholine receptor; HA, hemagglutinin; PB, sodium/potassium/phosphate buffer; h, human; r, rat; rM_3', N-terminal-modified rat M_3 receptor.

suggesting that it interacts with multiple allosteric sites that are cooperatively linked in a positive homotropic interaction. The molecular mechanism underlying this unusual allosteric interaction has recently been investigated further (Tränkle et al., 2003, 2005).

Four extracellular cysteine residues are highly conserved in the mAChR family (Fig. 1) and in most GPCRs. Two of these have been reported to form a disulfide bond between the top of the third transmembrane domain and the second outer (o2) loop of the M_1 receptor (Curtis et al., 1989; Kurtenbach et al., 1990), as do the corresponding cysteine residues in rhodopsin (Karnik and Khorana, 1990; Palczewski et al., 2000). The other two cysteines are located in the short third outer (o3) loop of the receptor. These extracellular cysteine residues have been systematically characterized in site-directed mutagenesis studies in rat M_1 (Savarese et al., 1992) and rat M_3 receptors (Zeng and Wess, 1999; Zeng et al., 1999), as well as in many other GPCRs, for their roles in surface expression, orthosteric binding, and receptor functions. The disulfide cysteine residues (but not the o3 loop cysteine residues) have consistently been found to be involved in receptor folding, surface expression, and maintaining the structure of the orthosteric binding site. In addition, the disulfide cysteine residues in M_3 receptors have been reported to be crucial to receptor dimerization, because mutations at these residues eliminated M_3 homodimerization (Zeng and Wess 1999).

It has been suggested that the o2 loop might fold back, because of constraints imposed by the conserved disulfide bond, to form a lid structure on top of the orthosteric binding pocket and might even participate in orthosteric ligand binding for many rhodopsin-like GPCRs (Palczewski et al., 2000; Shi and Javitch, 2004). Indeed, the o2 loop of the complement factor 5a receptor seems to play a critical role in receptor activation, because many mutations in the loop cause constitutive activity (Klco et al., 2005). The Cys176 residue in the o2 loop of the hM_2 receptor is right in the middle of two epitopes that are important for allosteric binding (Fig. 1). Previous chimeric and site-directed mutagenesis studies have indicated that the acidic EDGE motif (172–175) and residue Tyr177 are crucial for the high-affinity binding of a number of typical mAChR allosteric modulators (including gallamine and W84) to M_2 receptors (Leppik et al., 1994; Gnagey et al., 1999; Voigtländer et al., 2003; Huang et al., 2005). In addition, that region of the receptor is also important for THA binding (Ellis and Seidenberg, 2000; Tränkle et al., 2005; Huang and Ellis, manuscript in preparation). In the present study, we have replaced the four extracellular cysteine residues with alanines, in pairs, and we have examined the effects of these mutations on the allosteric binding of gallamine, THA, and W84 in M_2 and M_3 receptors. We found that mutation of the cysteine residues involved in the disulfide bond did alter the potencies of these allosteric ligands in some cases, but the most striking finding was that positive homotropic interactions of the allosteric ligands were markedly reduced or eliminated.

Materials and Methods

Materials. Atropine sulfate, gallamine triethiodide, tacrine hydrochloride, and polyethylenimine were purchased from Sigma (St. Louis, MO). W84 is from Tocris Cookson Inc. (Ellisville, MO). [^{3}H]NMS (81 Ci/mmol) was ordered from PerkinElmer Life and Analytical Sciences (Boston, MA). Glass fiber filters and all other inorganic chemicals are all from VWR International, Inc. (Bridgeport, NJ). The modified rat M_3' plasmid (rM_3') was kindly provided by Dr. J. Wess (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). The modification to the rat M_3 receptor includes a 1×HA tag at the N terminus, mutations of Asp to Gln at positions of 6, 15, 41, 48, and 52 to delete *N*-glycosylation sites, and a large deletion (Ala274 to Lys469) of the central portion of the third intracellular loop (Zeng et al., 1999).

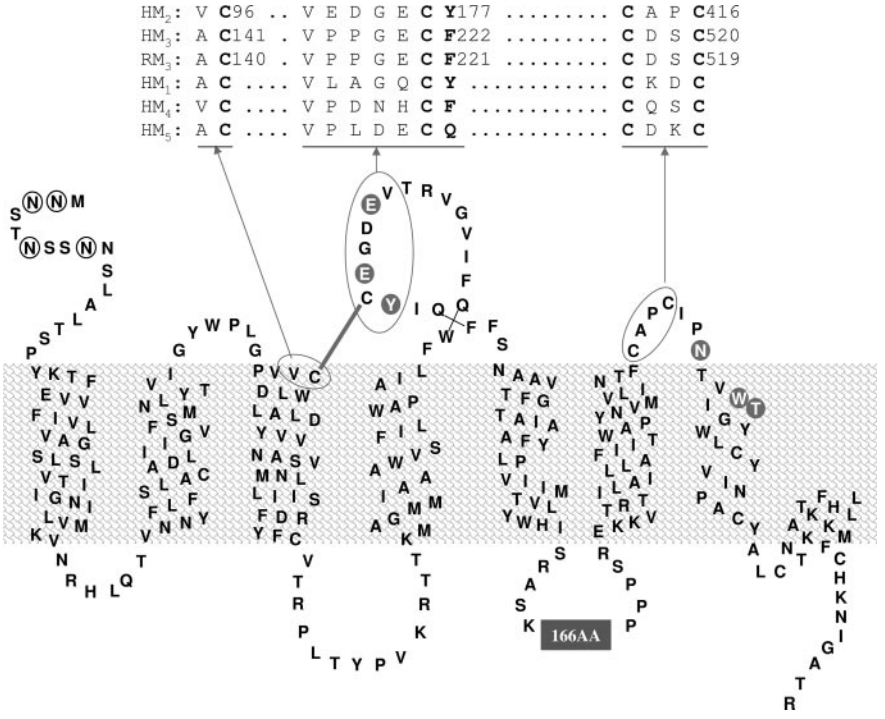

Fig. 1. Schematic presentations of the human M_2 muscarinic acetylcholine receptor and partial sequence alignment of the muscarinic acetylcholine receptor family around the four conserved extracellular cysteine residues. The transmembrane domain boundaries were set by manual alignment of the sequences to bovine rhodopsin, whose crystal structure has been solved (Palczewski et al., 2000).

Site-Directed Mutagenesis, Receptor Expression, and Membrane Preparation. All mutations and modifications to muscarinic receptors were carried out using the QuikChange method (Stratagene, La Jolla, CA) in the pcD vector (Okayama and Berg, 1983; Bonner et al., 1987). Primer synthesis and sequence confirmation were carried out in the Core Facility of Hershey Medical Center (Hershey, PA). Plasmid purification kits from QIAGEN (Valencia, CA) were used for all plasmid purification. Removal of potential N-terminal *N*-glycosylation sites (Asn to Asp) of the human M_2 (hM_2) receptor at positions 2, 3, 6, and 9 and insertion of one hemagglutinin (HA) epitope (YPYDVPDYA) were designed into a pair of long primers. Primers are shown in Table 1.

Receptor constructs were transiently transfected in COS-7 cells using PolyFect Reagent (QIAGEN). Approximately 48 h after transfection, cells were scraped into 5 mM sodium/potassium/phosphate buffer (PB, pH 7.4; 4 mM Na_2HPO_4 and 1 mM K_2HPO_4) and homogenized in the PB on ice with a mechanical Bio Homogenizer from Biospec Products, Inc. (Bartlesville, OK). After centrifugation at 50,000*g* for 30 min at 4°C, the membrane pellet was resuspended with a glass homogenizer in ice-cold 5 mM PB. The membrane suspension was either used immediately or stored in aliquots at −70°C.

[^{3}H]NMS Saturation Binding Assays. Saturation binding assays were carried out in 5 mM PB, pH 7.4, in a 25°C water bath as described previously (Huang et al., 2005). In brief, membranes were incubated with six concentrations of [^{3}H]NMS (ranging from 3 to 3000 pM), in duplicate, in a final volume of 1 ml for 30 min. In most assays, 2 to 5 μg of protein per assay tube were used; however, the low expression of the disulfide mutants necessitated much higher amounts (30–100 μg of protein). The incubation was terminated by filtration onto #32 glass-fiber filters (Whatman Schleicher and Schuell, Keene, NH) pretreated with ice-cold 0.1% polyethylenimine solution and followed with two quick washes with 5 ml of ice-cold 40 mM PB, pH 7.4. Radioactivity from membranes trapped on filter discs was determined by liquid scintillation counting. Data were fitted to a one-site hyperbolic binding curve, using Prism 4.03 (GraphPad Software, Inc., San Diego, CA). Nonspecific binding was determined in the presence of 3 μM atropine at each concentration point.

[^{3}H]NMS Dissociation Assays. [^{3}H]NMS dissociation assays were set up and conducted as described previously (Ellis and Seidenberg, 2000; Buller et al., 2002; Huang et al., 2005) in 5 mM PB, pH 7.4, in a 25°C water bath. In brief, membranes (similar amounts to those quoted in the saturation assays, above) were first labeled with [^{3}H]NMS for 30 min in 1 ml. Dissociation of [^{3}H]NMS was then initiated by the addition of atropine (3 μM in a final volume of 2 ml) with or without serial concentrations of allosteric modulators. The dissociation process was stopped by filtration after a period of time, which is typically set between 2 and 3 times the standard half-time of [^{3}H]NMS dissociation (determined in the presence of atropine but in the absence of any allosteric modulator). Radioactivity was counted as above.

Data Analysis. Dissociation assays were set up such that the delay of [^{3}H]NMS dissociation had to be mediated by the binding of the allosteric modulator to an allosteric site, distinct from the orthosteric site at which [^{3}H]NMS binds, because orthosteric binding sites were prelabeled and then blocked by the high concentration of atropine during the dissociation phase. Dissociation was assumed to follow a mono-exponential time course. The standard dissociation rate constant (k_0) was determined in the presence of atropine alone,

TABLE 1
Primers

Receptor and Mutation	Primer Sequences (5′ to 3′)
hM_2	
Insertion of HA tag and deletion of *N*-glycosylation sites	
Direct	GAG AAC GCA AAA TGT ACC CAT ACG ATG TTC CTG ACT ATG CGG ATG ACT CAA CAG ACT CCT CTG ACA ATA GCC TGG CTC
Complementary	GAG CCA GGC TAT TGT CAG AGG AGT CTG TTG AGT CAT CCG CAT AGT CAG GAA CAT CGT ATG GGT ACA TTT TGC GTT CTC
C96A	
Direct	GGA CCT GTG GTG GCT GAC CTT TGG CTA GC
Complementary	GCT AGC CAA AGG TCA GCC ACC ACA GGT CC.
C176A	
Direct	GGA GGA TGG GGA GGC CTA CAT TCA GTT TTT TTC CAA TGC TGC
Complementary	GCA GCA TTG GAA AAA AAC TGA ATG TAG GCC TCC CCA TCC TCC
C413A, C416A	
Direct	CAT TAA CAC CTT TGC TGC ACC TGC CAT CCC CAA CAC TG
Complementary	CAG TGT TGG GGA TGG CAG GTG CAG CAA AGG TGT TAA TG
rM_3'	
C140A	
Direct	GGA ACT TAG CCG CCG ACC TCT GGC
Complementary	GCC AGA GGT CGG CGG CTA AGT TCC
C220A	
Direct	CCC CAG GAG AAG CTT TCA TTC AGT TTC TG
Complementary	CAG AAA CTG AAT GAA AGC TTC TCC TGG GG
hM_3	
C141A	
Direct	GGA ACT TGG CCG CTG ACC TCT GGC TTG C
Complementary	GCA AGC CAG AGG TCA GCG GCC AAG TTC C
C221A	
Direct	CCT CCG GGA GAG GCC TTC ATT CAG TTC C
Complementary	GGA ACT GAA TGA AGG CCT CTC CCG GAG G
C517A, C520A	
Direct	GTG AAC ACC TTT GCT GAC AGC GCC ATA CCC AAA ACC
Complementary	GGT TTT GGG TAT GGC GCT GTC AGC AAA GGT GTT CAC
F222A	
Direct	GAG TGC GCC ATT CAG TTC CTC AGT G
Complementary	CTG AAT GGC GCA CTC TCC CGG AGG
F222Y	
Direct	GGG AGA GTG CTA CAT TCA GTT CC
Complementary	GGA ACT GAA TGT AGC ACT CTC CC

and the apparent dissociation rate constant (k_{obs}) was determined in the presence of both atropine and the allosteric modulator. The ratios of k_{obs}/k_0 were then plotted against the logarithms of the concentrations of allosteric modulator (including the basal value, obtained in the absence of allosteric ligand). The variable slope model uses the equation $k_{obs}/k_0 = Bottom + [(Top - Bottom)/\{1 + 10^{n_H(\log EC_{50} - X)}\}]$, where X is the logarithm of the concentration of allosteric modulator and *Top* and *Bottom* refer to the upper and lower plateaus of the sigmoidal curve, respectively. When the best-fit Hill slope (n_H) has an absolute value greater than 1, a positive homotropic allosteric interaction between two or more binding sites is indicated. In the "standard slope" model, n_H was constrained to unity. This concentration-response curve corresponds to the occupancy of the [^{3}H]NMS-bound receptor by the allosteric modulator; the concentration at which allosteric modulators reduce the k_{obs}/k_0 ratio to 50% of the maximal effect is defined as potency (EC_{50}). For cases in which the standard-slope model is appropriate, the potency (EC_{50}) is also the apparent binding affinity of the allosteric modulator at the [^{3}H]NMS-occupied receptor, K_{app} (often presented as pK_{app}; Ellis and Seidenberg, 1992; Lazareno and Birdsall, 1995). Statistical tests were carried out between data sets to determine whether changes in the Hill slope that were induced by mutation were statistically significant relative to the Hill slope of the relevant parental receptor; if the mutation did produce a significant change, then tests were carried out within data sets to determine whether the standard or variable slope model was the most appropriate fit. In all cases, statistical significance was set at the $p < 0.05$ level, based on an F test, using the built-in features of Prism 4.03. The program also estimates standard errors that give an indication of the precision with which the parameter values are known, but these estimates are not suitable for calculating P values. The F test, based on global curve-fitting, is the most robust and appropriate method for testing the boundaries of parameter values (Motulsky and Christopoulos, 2003).

Results

In this study, we targeted four extracellular cysteine residues, Cys96, Cys176, Cys413, and Cys416 of hM_2 receptors, and the corresponding cysteine residues in hM_3 and rM_3 receptors (Fig. 1). Because the first two cysteine residues form a disulfide bond that is highly conserved in the majority of GPCRs (and therefore referred to as the disulfide cysteine residues in this study) and the last two cysteine residues are separated by only two amino acids, we decided to mutate them in pairs rather than singly. This approach could eliminate the potential complication that remaining unpaired cysteine residues might form an abnormal disulfide bond that could compromise our interpretation. A double cysteine mutant in the rM_3' receptor was created to make the construct that was identical to the one reported previously by Dr. Wess and his colleagues (Zeng and Wess, 1999; Zeng et al., 1999). Anticipating future characterizations of some of the mutants, and to maintain similarity to the rM_3' constructs, we also inserted an HA epitope in the hM_2 receptor after the initiating Met residue and at the same time deleted potential *N*-glycosylation sites in the N terminus at position of 2, 3, 6, and 9 (Fig. 1). For consistency, this modified receptor was named hM_2'. Such modifications at the N termini of M_3 receptors (Zeng et al., 1999) and M_2 receptors (van Koppen and Nathanson, 1990) do not affect surface expression, ligand binding, or receptor functions.

Disulfide Cysteine Residues, but Not o3 Cysteine Residues, Are Important for Surface Expression and [^{3}H]NMS Binding. Mutations of the disulfide cysteine residues in the mutant hM_2' C96A, C176A dramatically reduced cell surface expression levels and also reduced the binding affinity for [^{3}H]NMS compared with the parental control (i.e., hM_2'; see Table 2). Likewise, corresponding mutations in hM_3 (C141A, C221A) reduced surface expression by more than 90% and substantially reduced [^{3}H]NMS binding affinity. In addition, we also constructed and tested the corresponding double-cysteine mutations in rM_3' receptors (C140A, C220A). Consistent with their counterparts in hM_2 and hM_3, as well as previously reported numbers (Zeng and Wess, 1999; Zeng et al., 1999), expression levels and [^{3}H]NMS binding affinity of rM_3' C140A, C220A were also greatly reduced. The degree of reduction of affinity for [^{3}H]NMS for the rM_3' C140A, C220A mutant was smaller in our study than reported previously (Zeng et al., 1999), possibly because of different assay conditions. In contrast to the dramatic effects produced by mutations of the disulfide cysteine residues, receptors with mutations at the two cysteine residues in the short o3 loop of hM_2 (C413A,C416A) retained expression levels and [^{3}H]NMS affinity comparable with those of the hM_2 wild-type receptors. Likewise, the hM_3 mutant with corresponding mutations in the o3 loop (C517A, C520A) also had expression levels and [^{3}H]NMS affinity comparable with those of the hM_3 wild type receptor. N-terminal modifications (HA tagging and deletion of *N*-glycosylation sites) of hM_2 receptors did not alter receptor expression or [^{3}H]NMS binding affinity very much, consistent with previ-

TABLE 2

Binding properties of M_2 and M_3 receptors

The hM_2' construct is the hM_2 receptor with 1× HA tagging after the initiating Met residue and deletion of potential *N*-glycosylation sites (see *Materials and Methods*). The rM_3' construct is the rM_3 receptor with 1× HA tagging after the initiating Met residue, deletion of potential *N*-glycosylation sites, and a large deletion from Ala274 to Lys469 in the center portion of the third intracellular loop (Zeng et al., 1999). The $t_{1/2}$ values represent the mean ± S.E.M. from three or more assays and are the standard half times of [^{3}H]NMS dissociation in the presence of atropine. B_{max} and pK_d values are the mean ± S.E.M. from two or more assays.

Receptor	$t_{1/2}$	pK_d	B_{max}
	min		*pmol/mg protein*
hM_2	4.0 ± 0.2	10.30 ± 0.07	4.81 ± 0.69
hM_2'	3.5 ± 0.2	9.99 ± 0.13	1.95 ± 0.39
hM_2', 6.5% labeling	3.1 ± 0.2		
hM_2' C96A, C176A	1.7 ± 0.2	9.43 ± 0.15	0.03 ± 0.01
hM_2 C413A, C416A	2.7 ± 0.1	9.82 ± 0.20	4.51 ± 1.16
hM_3	22.0 ± 1.4	10.35 ± 0.12	6.78 ± 2.79
hM_3 C141A, C221A	3.9 ± 0.2	9.16 ± 0.06	0.12 ± 0.02
hM_3 C517A, C520A	19.4 ± 1.1	9.97 ± 0.07	4.47 ± 1.33
rM_3'	56.5 ± 2.5	10.52 ± 0.11	4.30 ± 0.80
rM_3', 8.2% labeling	42.0 ± 6.4		
rM_3' C141A, C220A	4.2 ± 0.2	8.99 ± 0.08	0.27 ± 0.10

ous results from similar modifications in the rM_3 receptor (Zeng et al., 1999).

N-Terminal Modifications Have No Effect on Allosteric Binding. Because several mutant receptors in this study contain modifications (HA tagging and deletion of potential *N*-glycosylation sites) at the N terminus, we first performed control assays to determine whether such modifications themselves affected the allosteric binding of the tested modulators, THA, gallamine, and W84. As indicated in Fig. 2D and Tables 3 and 4, allosteric binding at hM_2 receptors was not significantly affected by HA tagging or by deletion of *N*-glycosylation sites. In addition, the N-terminal modified rM_3 receptor (rM_3'), had essentially the same allosteric binding profiles as hM_3 receptors without modifications, except for a somewhat slower dissociation rate with rM_3' receptors than with hM_3 receptors (Table 2). This difference in [^{3}H]NMS dissociation rates did not seem to be due to the N-terminal modifications, because unmodified rM_3 receptors also had slower rates ($t_{1/2}$ = 61 min) than hM_3 receptors, in agreement with previous studies (Ellis et al., 1993; Gnagey et al., 1999).

Subtype-Specific Effects of Disulfide Cysteine Mutations on Allosteric Binding Properties of THA. The atypical mAChR allosteric modulator THA showed steep dose-response curves in inhibiting [^{3}H]NMS dissociation with variable slopes of approximately 2, indicating that positive homotropic allosteric interaction was present at both M_2 and M_3 receptors (Table 3). The mutations C96A and C176A in the hM_2' receptor significantly reduced the slope factor for the THA curve to a value that was no longer significantly different from 1; that is, in this mutated construct, there was no evidence for positive homotropic cooperativity in the interaction of THA. In addition, the mutation caused a reduction in the potency of THA of approximately 6-fold (Fig. 2A). The corresponding mutations in hM_3 (C141A, C221A) and rM_3' (C140A, C220A) produced similar effects on the slope factors for the THA curves (Fig. 2, B and C, Table 3); for these M_3 mutants, the best-fit slope factors suggested *negative* cooperativities ($n_H < 1$), but were not significantly less than unity. In contrast to the result with the hM_2 mutant, THA potency was significantly *increased* by the double mutation in both hM_3 and rM_3'.

As we have noted above, these cysteine mutants showed reduced binding affinity for [^{3}H]NMS, and would therefore be labeled at far below saturation with 1 to 2 nM [^{3}H]NMS. To determine whether nonsaturation labeling at these mutants might be responsible for the observed effects, we chose concentrations [^{3}H]NMS that would label less than 10% of the total available binding sites at the hM_2 and rM_3' receptors and examined the effects of THA on [^{3}H]NMS dissociation. As indicated in Fig. 2D and Table 3, results obtained with this low percentage labeling did not differ significantly from those obtained with saturation labeling.

These cysteine mutations also enhanced the rate of dissociation of [^{3}H]NMS from the orthosteric binding site. Dissociation from the hM_2' C96A, C176A mutant was approxi-

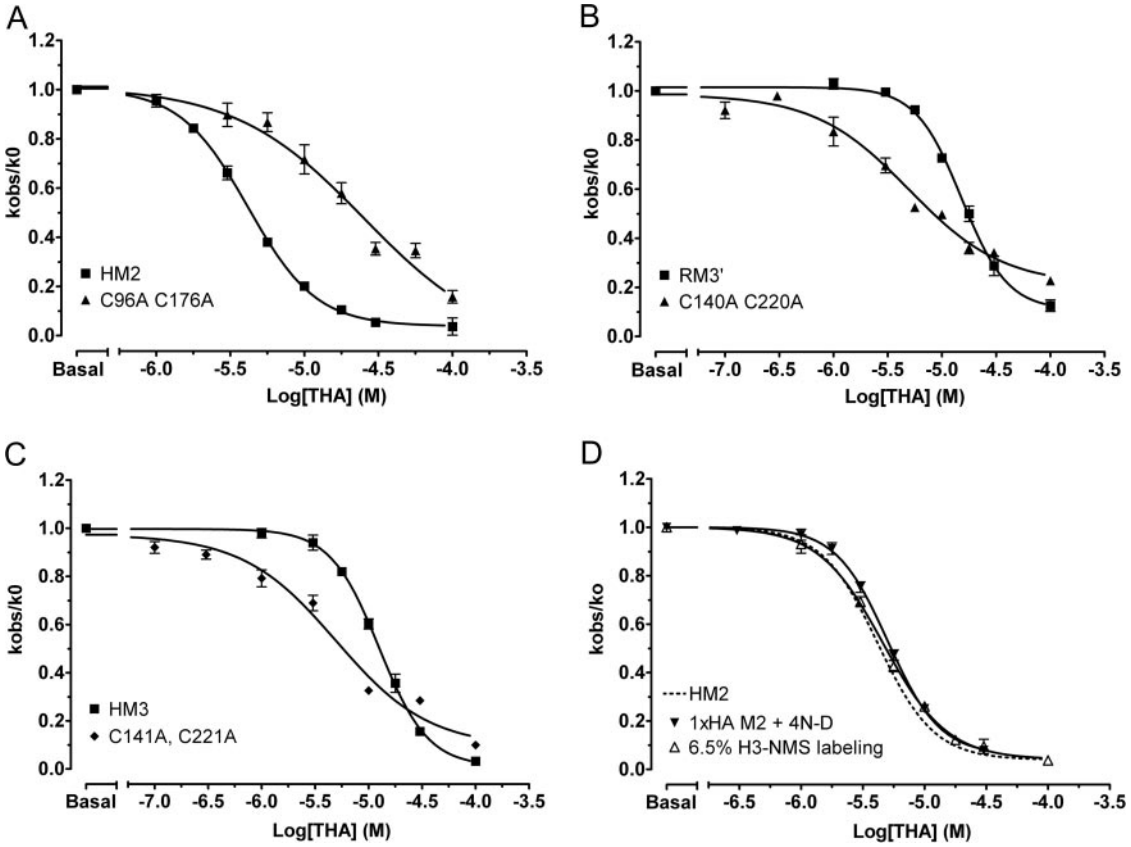

Fig. 2. Effects of disulfide cysteine mutations on allosteric binding of tetrahydroaminoacridine (THA) in hM_2 receptors (A), N-terminally modified rM_3 receptors (B), hM_3 receptors (C), and N-terminally modified hM_2 receptors, under typical conditions and low percentage labeling with a lower concentration of [^{3}H]NMS (D). The M_2 curve from A is shown as the broken line in D. Allosteric modulation of the [^{3}H]NMS dissociation rate was determined as described under *Materials and Methods* and data are shown with the best-fit curves (see Table 3 for parameters and statistical data). Points represent the means ± S.E.M. from three to five independent assays, each conducted in duplicate.

mately twice as fast as that from the parental hM_2' receptor; hM_3 C141A, C221A was approximately 6 times faster than hM_3; and the rM_3' C140A, C220A mutant was approximately 13 times faster than its parent, rM_3' (Table 2).

Subtype Specific Effects of Disulfide Cysteine Mutations on Allosteric Binding Properties of Gallamine and W84. The double-cysteine mutations in the hM_2' receptor (C96A and C176A) reduced the potency of gallamine somewhat (approximately 2.5-fold) but reduced W84 potency by approximately 70-fold (Fig. 3A and Table 4). In contrast, however, the potency of W84 was not changed by the double-cysteine mutation in the hM_3 receptor (C141A and C221A) or in the rM_3' receptor (C140A, C220A; Fig. 3B, 3C and Table 4). The data for both gallamine and W84 were better fitted to the standard slope model at the hM_2' receptor, and the mutations of the disulfide cysteines did not significantly alter that slope. It is noteworthy that the data for gallamine at both hM_3 and rM3′ receptors were significantly better fitted to the variable slope model, with Hill slopes of 1.21 and 1.34 (Fig. 3, B and C, and Table 4), respectively. That is, the Hill slopes for gallamine at both of these M_3 receptors indicated positive homotropic allosteric interactions. Double-cysteine mutations of C141A and C221A in hM_3 or C140A and C220A in rM_3' resulted in curves with significantly lower slopes; moreover, these slopes were not significantly different from unity (Fig. 3B and Table 4). The slope factors for W84 were not significantly different from unity at either hM_3 or rM_3' (Table 4); eliminating the disulfide cysteines did not significantly alter these slopes.

Mutations of o3 Cysteine Residues Do Not Alter Binding Parameters. For comparison, we also examined the allosteric effects of gallamine, W84, and THA in receptors with double-cysteine mutations in the o3 loop, hM_2 C413A, C416A, and hM_3 C517A, C520A. As indicated in Fig. 4 and Table 4, these mutations were quite benign. That is, there were no significant changes in potencies, and THA and gallamine displayed the same positive homotropic allosteric features at these mutant receptors as they did at the corresponding wild-type receptors. In addition, the dissociation

TABLE 3

Allosteric properties of THA at M_2 and M_3 receptors

The parameters pK_{app} and Hill slope (n_H) represent best-fit values ± S.E. from global curve fitting to the combined data of three or more assays. Statistical significance was determined by *F* test, either between or within data sets (see *Materials and Methods*). The between-set *P* values shown are for comparisons with the immediate parental receptor; for example, hM_2' was compared with hM_2, while hM_2'C96A, C176A was compared with hM_2'. When n_H was found to be significantly ($P < 0.05$) different from the parental receptor (or when there was no parental receptor), a global *F* test was also carried out to determine whether n_H differed from the standard (unit) slope.

Receptor	pK_{app} and F-test		Hill slope (n_H) and *F* tests		
	pK_{app}	vs Parental	n_H	vs Parental	vs Unity
hM_2	5.38 ± 0.02		1.89 ± 0.11		<0.0001
hM_2'	5.30 ± 0.02	0.0013	2.07 ± 0.13	0.3203	
hM_2, 6.5% labeling	5.34 ± 0.02	0.1441	1.67 ± 0.12	0.2686	
hM_2' C96A C176A	4.73 ± 0.08	<0.0001	1.31 ± 0.23	0.0129	0.1715
hM_2 C413A, C416A	5.41 ± 0.02	0.2778	1.74 ± 0.11	0.4021	
hM_3	4.90 ± 0.02		1.90 ± 0.13		<0.0001
hM_3 C141A, C221A	5.23 ± 0.09	0.0087	0.79 ± 0.10	<0.0001	0.1380
hM_3 C517A, C520A	5.01 ± 0.04	0.0461	1.84 ± 0.21	0.7913	
rM_3'	4.82 ± 0.02		2.05 ± 0.16		<0.0001
rM_3', 8.2% labeling	4.86 ± 0.06	0.5713	1.65 ± 0.32	0.3308	
rM_3' C140A, C220A	5.25 ± 0.09	0.0015	0.83 ± 0.11	<0.0001	0.1987

TABLE 4

Allosteric properties of gallamine and W84 at M_2 and M_3 receptors

The parameters pK_{app} and Hill slope (n_H) represent best-fit values ± S.E. from global curve fitting to the combined data of three or more assays. Statistical significance was determined by *F* test, either between or within data sets (see *Materials and Methods*). The between-set *P* values shown are for comparisons with the immediate parental receptor; for example, hM_2' was compared with hM_2, whereas hM_2'C96A, C176A was compared with hM_2'. When n_H was found to be significantly ($P < 0.05$) different from the parental receptor (or when there was no parental receptor), a global *F* test was also carried out to determine whether n_H differed from the standard (unit) slope.

Receptor	pK_{app} and *F* Test		Hill Slope (n_H) and *F* Tests		
	pK_{app}	vs Parental	n_H	vs Parental	vs Unity
Gallamine					
hM_2	7.03 ± 0.05		1.02 ± 0.09		0.7886
hM_2'	7.01 ± 0.08	0.8170	0.95 ± 0.14	0.6734	
hM_2' C96A, C176A	6.63 ± 0.16	0.0298	0.83 ± 0.23	0.6510	
hM_2 C413A, C416A	7.09 ± 0.06	0.4829	1.07 ± 0.14	0.8266	
hM_3	5.02 ± 0.03		1.21 ± 0.10		0.0107
hM_3 C141A, C221A	5.08 ± 0.07	0.5200	0.88 ± 0.10	0.0329	0.2471
hM_3 C517A, C520A	5.09 ± 0.03	0.2397	1.26 ± 0.08	0.7660	
rM_3'	4.95 ± 0.06		1.34 ± 0.08		<0.0001
rM_3' C140A, C220A	4.66 ± 0.06	<0.0001	0.99 ± 0.11	0.0286	0.9610
W84					
hM_2	7.70 ± 0.03		1.05 ± 0.07		0.4945
hM_2'	7.62 ± 0.03	0.1863	1.12 ± 0.07	0.6270	
hM_2' C96A, C176A	5.22 ± 0.73	<0.0001	0.62 ± 0.28	0.1118	
hM_2 C413A, C416A	7.77 ± 0.05	0.3098	0.97 ± 0.09	0.5220	
hM_3	5.79 ± 0.03		1.10 ± 0.07		0.1391
hM_3 C141A, C221A	5.79 ± 0.12	0.9929	0.93 ± 0.20	0.4291	
hM_3 C517A, C520A	5.88 ± 0.05	0.1589	1.11 ± 0.11	0.9166	
rM_3'	5.64 ± 0.03		1.04 ± 0.07		0.5946
rM_3' C140A, C220A	5.78 ± 0.07	0.1060	1.20 ± 0.23	0.5295	

rate of [^{3}H]NMS was not altered by the cysteine mutations in the o3 loop (Table 2).

Phe222 in the o2 Loop of the hM$_3$ Receptor Is Important for the Binding of Allosteric Modulators. Tyr177 in the hM$_2$ receptor is known to be more important for the allosteric binding of W84 than for that of gallamine, participating in π-π interactions with W84 (Voigtländer et al., 2003; Huang et al., 2005; Prilla et al., 2006). This residue is immediately adjacent to Cys176. Therefore, mutations of C176A and C96A that eliminate the conserved disulfide bond could potentially change the orientation of Tyr 177 in hM$_2$ receptors. An aromatic residue is conserved at the corresponding position of every mAChR except hM$_5$, which has the lowest binding affinities for gallamine or W84 of any muscarinic subtype. In M$_3$ receptors, the corresponding residue is Phe222 (Fig. 1). Mutations of C141A and C221A in the hM$_3$ receptor, however, had no effect on the potency of either gallamine or W84. This observation suggested that Phe222 might not be important for allosteric binding. To directly examine the role of hM$_3$ Phe222 in allosteric binding, we replaced it with either Tyr (conserving the aromatic ring) or Ala. HM$_3$ F222A and hM$_3$ F222Y were expressed at 1.87 and 5.81 mol/mg, with [^{3}H]NMS binding affinities (pK_d) of 10.16 and 10.47, respectively. As indicated in Fig. 5 and Table 5, replacing Phe222 with Ala had similar effects on gallamine, THA, and W84, reducing allosteric potencies by 2- to 4-fold. Replacing Phe222 with Tyr slightly increased the potencies of gallamine and W84 by 2- to 4-fold but had no effect on the potency of THA. In addition to these modest effects on the potencies of allosteric binding, mutations at Phe222 also changed the Hill slopes associated with THA binding. The slope factor for THA at the F222Y mutant was increased somewhat (although not significantly), whereas the F222A mutant receptor retained homotropic interactions in THA binding but with a significantly reduced Hill slope (Fig. 5C, Table 5). Both mutations decreased the slope factors for gallamine to near unity, although statistical significance was achieved only for the F222A mutation and only for gallamine (Fig. 5A and Table 5).

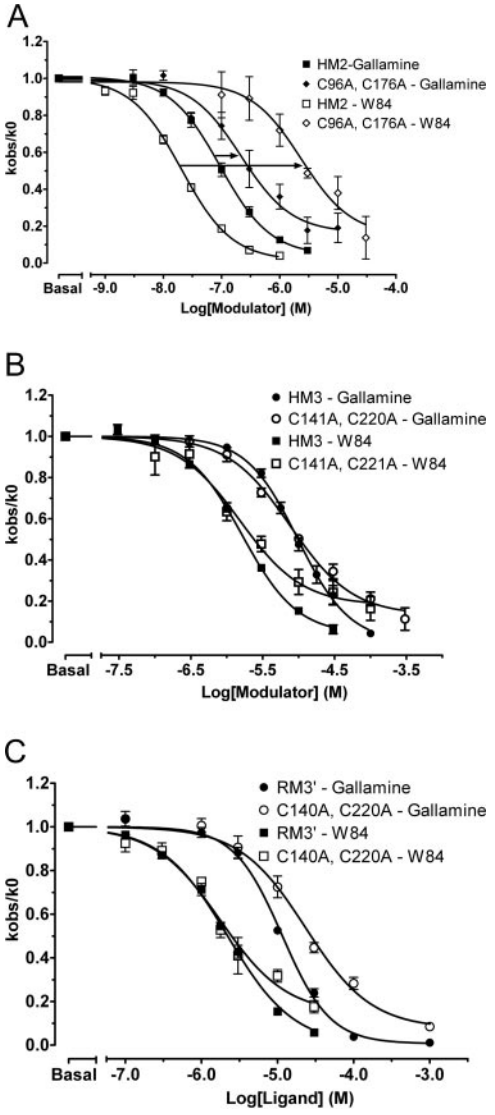

Fig. 3. Effects of disulfide cysteine mutations on allosteric binding of gallamine and W84 in hM$_2$ receptors (A), hM$_3$ receptors (B), and N-terminally modified rM$_3$ receptors (C). [^{3}H]NMS dissociation assays were conducted and data were analyzed as in Fig. 2 and best-fit parameters are presented in Table 4. Points represent the mean ± S.E.M. from 3 to 10 independent assays, each conducted in duplicate.

Discussion

The hM$_2$ receptor contains two epitopes in the o2 loop (the EDGE motif and Tyr177) that are important for allosteric binding. Each is adjacent to the Cys176 residue that links to Cys96 at the top of TM III. In this study, we examined

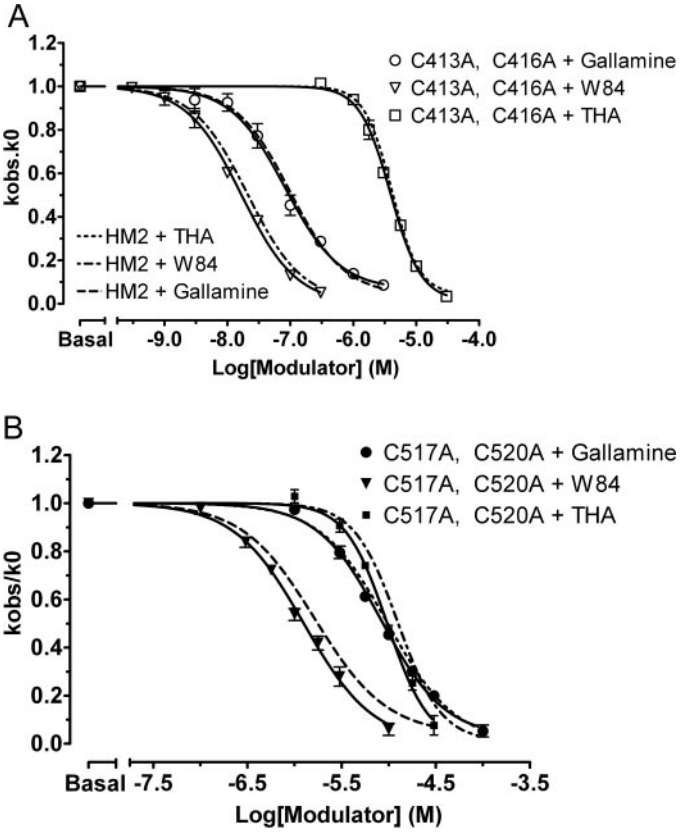

Fig. 4. Effects of cysteine mutations in the o3 loop on allosteric binding of gallamine, W84, and THA in the hM$_2$ receptor (A) and hM$_3$ receptor (B). [^{3}H]NMS dissociation assays were conducted and data were analyzed as in Fig. 2 and best-fit parameters are presented in Tables 3 and 4. Points represent the mean ± S.E.M. from three to six independent assays, each in duplicate.

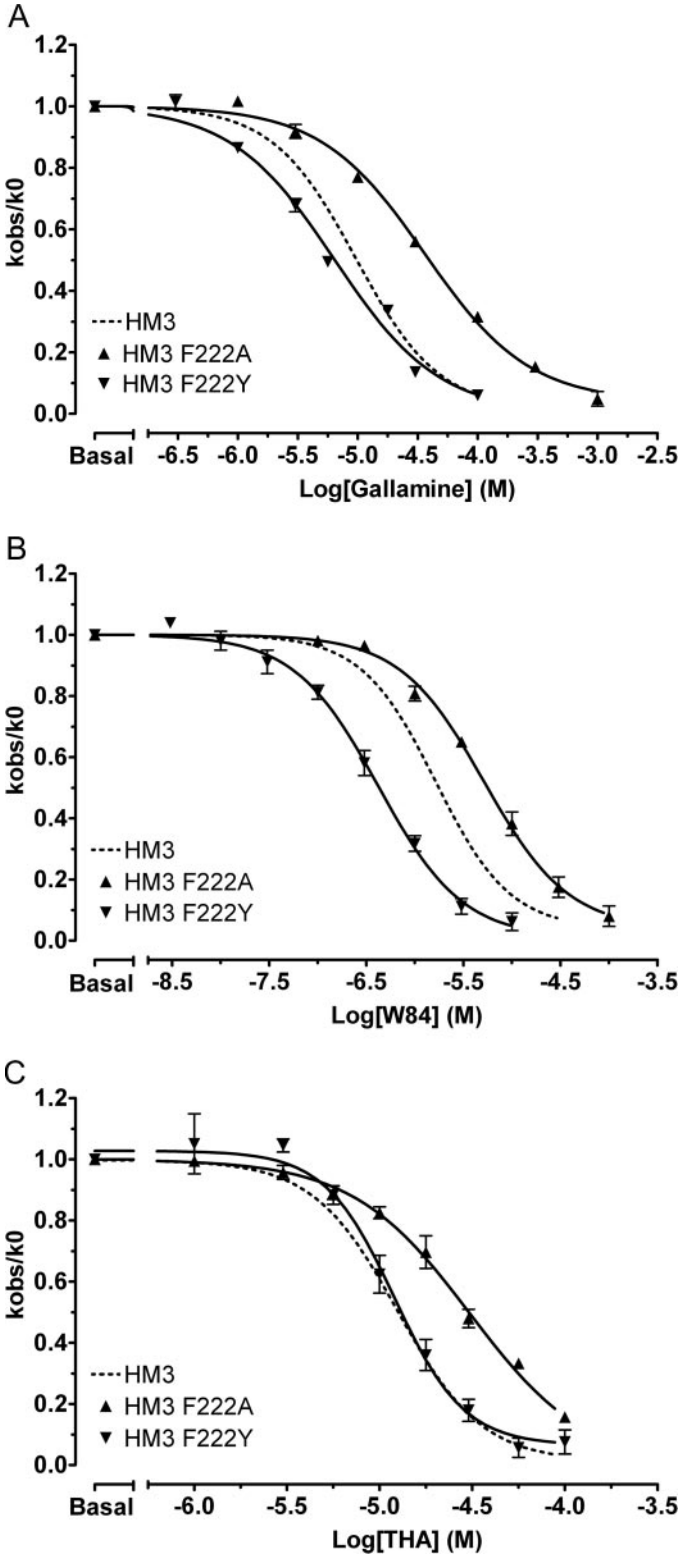

Fig. 5. Effect of Phe222 mutations in hM_3 receptors on allosteric binding of gallamine (A), W84 (B), and THA (C). Binding curves for each allosteric modulator at the hM_3 wild type receptor from Fig. 2 and Fig. 3 are shown in broken lines in each panel. [^{3}H]NMS dissociation assays were conducted and data were analyzed as in Fig. 2 and best-fit parameters are presented in Table 5. Points represent the mean ± S.E.M. from 3 to 10 independent assays, each conducted in duplicate.

allosteric interactions in mutants of M_2 and M_3 receptors that lacked this disulfide link.

The disulfide cysteine mutations in hM_2' receptors reduced affinity for W84 by approximately 70-fold, slightly more than the 50-fold reduction we observed previously with the single Y177Q mutation. The same mutations also reduced gallamine potency by approximately 2-fold, equivalent to that of the Y177Q mutation (Huang et al., 2005). This big difference between W84 and gallamine is consistent with the fact that Tyr177 is much more important for W84 binding than for gallamine binding, because Tyr177 participates in a unique π-π interaction with W84 (Voigtländer et al., 2003; Huang et al., 2005; Prilla et al., 2006). Thus, it seems that the degrees of reduction in potency of these two modulators after the elimination of the disulfide bond could be attributed mainly to changes in the contributions of the Tyr177 residue. This suggests that the disulfide bond helps to constrain the o2 loop into a proper conformation for allosteric binding, especially for the binding of W84. Recent studies have suggested that the aromatic ring of W84 is sandwiched between Tyr177 and Trp422 (Prilla et al., 2006), which would require a very specific orientation of these two residues. On the other hand, the binding of gallamine is much more sensitive to acidic residues in the EDGE region on the other side of Cys176 (Huang et al., 2005); it seems reasonable to assume that this ionic interaction with gallamine would still be available, given the probable increase in the flexibility of the o2 loop that would result from the elimination of the disulfide attachment. Indeed, an increase in the flexibility of the o2 loop may be inferred from the doubling of the rate of dissociation of [^{3}H]NMS from the disulfide cysteine mutant, relative to its parent receptor, hM_2'. Elimination of the corresponding disulfide bond by cysteine mutation in bovine rhodopsin also confers greater conformational flexibility (Davidson et al., 1994).

The elimination of the disulfide bond in hM_2' reduced the potency (EC_{50}) toward THA by approximately 4-fold, slightly more than the reduction we have observed from the single Y177Q mutation in hM_2 (X.-P. Huang and J. Ellis, manuscript in preparation). More interesting than the change in potency, however, is the finding that the Hill slope for the interaction of THA with the mutant receptor is not significantly different from 1. It seems that this mutation either interferes with the communication between the THA binding sites or occludes one of the binding sites. Elimination of the disulfide bond in M_3 receptors produced results that were quite similar to those seen with the hM_2' receptor. That is, both hM_3 and rM_3' exhibited robust positive cooperativity (Hill slope of approximately 2) that was abolished by the mutation (to Hill slopes not significantly different from 1). These mutations accelerated the rate of dissociation of [^{3}H]NMS even more so than those in hM_2', again suggesting that enhanced flexibility in the o2 loop relieved constraints on the access of NMS to the orthosteric site. However, there were also several differences between the M_2 and M_3 receptors. For one, gallamine showed significant positive cooperativity at both hM_3 and rM_3', although it was not as robust (Hill slope estimated at 1.21 at hM_3 and 1.34 at rM_3') as that seen with THA. As with THA, M_3 mutants that lacked the disulfide cysteines did not exhibit positive cooperativity in the action of gallamine. This is the first report of homotropic cooperativity for gallamine at any muscarinic receptor sub-

type in this type of assay. However, it has been known for some time that gallamine exhibits a biphasic regulation of the dissociation of quinuclidinyl benzilate from M_2 receptors. Low concentrations of gallamine accelerate the dissociation of this orthosteric antagonist, whereas higher concentrations slow the dissociation, implying actions at two different allosteric sites (Ellis and Seidenberg, 1989). These complex interactions seen with gallamine suggest that the differentiation between typical and atypical ligands may be quantitative rather than qualitative and that it may take a particular assay or condition to reveal the "atypical" characteristics of a given ligand.

In contrast to the 70-fold reduction in potency toward W84 that was caused by the elimination of the disulfide bond in the hM_2' mutant, both the hM_3 mutant and the rM_3' mutant had essentially the same potencies for W84 as their respective parent receptors. This difference led us to investigate the contribution of the Phe222 residue in hM_3 to the potencies of the allosteric ligands. Our results with hM_3F222A and hM_3F222Y mutants can be compared with the corresponding hM_2 mutants that we have reported previously (Huang et al., 2005). Gallamine and W84 show similar qualitative relative potencies in the two subtypes, Tyr > Phe > Ala, but the magnitudes are significantly different. The loss of the aromatic quality (F222A) produces a significantly greater loss in affinity toward W84 in the M_2 background than in the M_3 background. This suggests that features of the M_3 receptor structure may prevent the participation of the Phe residue in the π-π sandwich that has been suggested for hM_2 (Prilla et al., 2006). However, this Phe residue does seem to play a role in modulating the cooperativity between allosteric sites. Eliminating the aromatic quality altogether (hM_3F222A) eliminates the cooperativity toward gallamine and reduces it partially, but significantly, in the case of THA.

It should be noted that all of the allosteric assays in the present article were carried out using receptors that were prelabeled with [^{3}H]NMS (i.e., dissociation assays, see *Materials and Methods*). Therefore, we cannot know how much the mutations may have altered the potencies of the interactions between these allosteric ligands and the free receptor. However, this approach has the advantage that we can be certain that we are measuring cooperativity (and changes in cooperativity) between sites that are necessarily allosteric to the NMS binding site. These assays also support the suggestion that mutations at the disulfide cysteines enhance the flexibility of the o2 region of the receptor. That is, at wild-type receptors, the dissociation of [^{3}H]NMS seems to be completely prevented by high concentrations of the allosteric ligands, but, at receptors lacking the disulfide cysteines, a significant rate of dissociation remains even at saturating concentrations (for example, see Fig. 3B).

The cooperativity shown by THA raises the question of where the multiple sites are located and how they relate to the sites for other muscarinic allosteric modulators. One way to approach this question is to determine whether two modulators with different efficacies interact competitively (Ellis and Seidenberg, 1992; Waelbroeck, 1994). Using this technique, a number of modulators have been shown to act at a common site (Ellis and Seidenberg, 1992, 2000; Tränkle and Mohr, 1997), whereas others seem to bind at different sites (Lazareno et al., 2000, 2002). Therefore, there seem to be at least two different allosteric binding sites per muscarinic receptor. If THA interacts with two cooperative sites, are they on the same receptor, or are they the result of dimerization of receptors? Recent molecular modeling studies have suggested that, because of its small size, two molecules of THA can fit simultaneously into the allosteric space of the M_2 receptor, one in the more typical space between o2 and o3, and the other closer to o1 and the N terminus. However, the experimental data that accompany that model revealed additional complexities that make it impossible to exclude the explanation that two sites are linked via the interface between two receptors. Indeed, at this point, the two explanations are not incompatible; there could be four sites involved, two on each receptor (Tränkle et al., 2005). Mechanistic, molecular, and biophysical studies have demonstrated that GPCRs, including M_2 and M_3 muscarinic receptors, can form dimers and even higher order oligomers (Park et al., 2004).

It is noteworthy that Zeng and Wess (1999) have reported Cys140 and Cys220 to be involved in covalent homodimerization of the same rM_3' receptor construct that we employed in the present studies; the mutations C140A and C220A eliminated the covalent link between the receptors. However, the mutant receptors were still able to form noncovalent complexes that were identified by immunoprecipitation, even though expression was greatly reduced (to a similar level as

TABLE 5

Allosteric properties of hM_3 receptors with mutations at Phe222

The parameters pK_{app} and Hill slope (n_H) represent best-fit values ± S.E. from global curve fitting to the combined data of three or more assays. Data for wild-type hM_3 receptors were included for comparison. Statistical significance was determined by *F* test, either between or within data sets (see *Materials and Methods*). The between-set *P* values shown are for comparisons with the immediate parental receptor, hM_3. When n_H was found to be significantly ($P < 0.05$) different from the parental receptor (or when there was no parental receptor), a global *F* test was also carried out to determine whether n_H differed from the standard (unit) slope.

Receptor	pK_{app} and *F* Test		Hill slope (n_H) and *F* Tests		
	pK_{app}	vs Parental	n_H	vs Parental	vs Unity
THA					
hM_3	4.90 ± 0.02		1.89 ± 0.13		<0.0001
hM_3 F222A	4.52 ± 0.06	<0.0001	1.37 ± 0.16	0.0220	0.0225
hM_3 F222Y	4.91 ± 0.03	0.7285	2.36 ± 0.27	0.0802	
Gallamine					
hM_3	5.02 ± 0.03		1.21 ± 0.09		0.0107
hM_3 F222A	4.42 ± 0.04	<0.0001	0.89 ± 0.06	0.0406	0.0705
hM_3 F222Y	5.21 ± 0.05	0.0233	0.98 ± 0.09	0.1068	
W84					
hM_3	5.79 ± 0.03		1.10 ± 0.07		0.1391
hM_3 F222A	5.26 ± 0.06	<0.0001	0.92 ± 0.09	0.0929	
hM_3 F222Y	6.37 ± 0.06	<0.0001	0.96 ± 0.10	0.2620	

in our studies; Zeng and Wess, 1999; Zeng et al., 1999). This finding is consistent with other studies that have detected dimerization of muscarinic receptors at low expression levels in live cells, using bioluminescence resonance energy transfer (Goin and Nathanson, 2006). Indeed, it is likely that many GPCRs dimerize within the endoplasmic reticulum, even before transport to the plasma membrane (Bulenger et al., 2005). In any event, these disulfide cysteine residues are located approximately at the junction of the two allosteric pockets predicted by the molecular model (Tränkle et al., 2005), making it difficult to choose between the possibilities that the cysteine mutations disrupted an intrareceptor communication between the two pockets or that they disrupted communication between binding sites on two linked receptors. It is expected that future studies will determine which mechanism applies.

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