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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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GPCR, G protein coupled receptor; K_{app}, apparent binding affinity (i.e., the affinity for the [^{3}H]NMS-occupied receptor in this study; k_0, true dissociation rate constant in the presence of atropine; k_{obs}, observed dissociation rate in the presence of atropine and allosteric modulator; NMS, N-methylscopolamine chloride; o2, the second outer (extracellular) loop of the receptor; o3, the third outer loop; mAChR, muscarinic acetylcholine receptor; PB, sodium-potassium phosphate buffer; THA, tetrahydroaminoacridine; TM, transmembrane domain; W84, hexamethylene-bis-[3-phthalimidopropyl] ammonium) dibromide.

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 about 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. Most interestingly, the strong positive homotropic interactions of THA at both M_2 and M_3 receptors were markedly reduced by the cysteine mutations. Additionally, 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 appears that these cysteine residues play a role in linking cooperating allosteric sites, although it is not possible at the present time 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, which 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, like the muscarinic acetylcholine receptors (mAChRs). The family of mAChRs is one 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 $[^3H]$N-methylscopolamine (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 because it slows the dissociation rate of $[^3H]$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), 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 been further investigated recently (Tränkle et al., 2003; 2005).

There are four extracellular cysteine residues that are highly conserved in the mAChR

family (Figure 1) and in most GPCRs. Two of these have been reported to form a disulfide bond

between top of the third transmembrane domain (TM III) 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 et al., 1999; Zeng and Wess 1999), as

well as many other GPCRs, for their roles in surface expression, orthosteric binding, and

receptor functions. Consistently, the disulfide cysteine residues (but not the o3 loop cysteine

residues) have been found to be involved in receptor folding, surface expression, and in

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, as mutations

at these residues eliminated M_3 homodimerization (Zeng and Wess 1999).

It has been suggested that the o2 loop might fold back, due to 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

appears to play a critical role in receptor activation, as 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 (Figure 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 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.

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Materials and Methods

Materials. Atropine sulfate, gallamine triethiodide, tacrine hydrochloride, and polyethyleneimine were purchased from Sigma (St. Louis, MO). W84 is from Tocris Cookson Inc. (Ellisville, MO). [^{3}H]N-methylscopolamine chloride ([^{3}H]NMS; 81 Ci/mmol) was ordered from PerkinElmer Life and Alalytical 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 (NIDDK, NIH). The modification to the rat M3 receptor includes a 1xHA 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 3rd intracellular loop (Zeng et al., 1999).

Site-directed Mutagenesis, Receptor Expression, and Membrane Preparation. All mutations and modifications to muscarinic receptors were carried out using the QuickChange method (Stratagene) 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. Qiagen plasmid purification kits were used for all plasmid purification. Deletion of potential N-terminal N-glycosylation sites (Asn to Asp) of the human M$_2$ (hM$_2$) receptor at positions of 2, 3, 6 and 9 and insertion of one hemagglutinin (HA) epitope (YPYDVPDYA) were designed into a pair of long primers. The direct primer is 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 (5' to 3', the same for others), while the complementary primer is 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. For the C96A mutation in the M_2 receptor, the primers are

GGA CCT GTG GTG GCT GAC CTT TGG CTA GC and GCT AGC CAA AGG TCA GCC

ACC ACA GGT CC. For the C176A mutation in the M_2 receptor, the primers are GGA GGA

TGG GGA GGC CTA CAT TCA GTT TTT TTC CAA TGC TGC and GCA GCA TTG GAA

AAA AAC TGA ATG TAG GCC TCC CCA TCC TCC. For the rM_3' C140A mutation, the

primers are GGA ACT TAG CCG CCG ACC TCT GGC and GCC AGA GGT CGG CGG CTA

AGT TCC. For the rM_3' C220A mutation, the primers are CCC CAG GAG AAG CTT TCA

TTC AGT TTC TG and CAG AAA CTG AAT GAA AGC TTC TCC TGG GG. For the hM_3

C141A mutation, the primers are GGA ACT TGG CCG CTG ACC TCT GGC TTG C and GCA

AGC CAG AGG TCA GCG GCC AAG TTC C. For the hM_3 C221A mutation, the primers are

CCT CCG GGA GAG GCC TTC ATT CAG TTC C and GGA ACT GAA TGA AGG CCT

CTC CCG GAG G. For the hM_2 C413A, C416A mutation, the primers are CAT TAA CAC CTT

TGC TGC ACC TGC CAT CCC CAA CAC TG and CAG TGT TGG GGA TGG CAG GTG

CAG CAA AGG TGT TAA TG. For the hM_3 C517A, C520A mutations, the primers are GTG

AAC ACC TTT GCT GAC AGC GCC ATA CCC AAA ACC and GGT TTT GGG TAT GGC

GCT GTC AGC AAA GGT GTT CAC. For the hM_3 F222A mutation, the primers are GAG

TGC GCC ATT CAG TTC CTC AGT G and CTG AAT GGC GCA CTC TCC CGG AGG. For

the hM_3 F222Y mutation, the primers are GGG AGA GTG CTA CAT TCA GTT CC and GGA

ACT GAA TGT AGC ACT CTC CC.

Receptor constructs were transiently transfected in COS-7 cells using PolyFect Reagent

(Qiagen). Approximately 48 hours 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 50,000g 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.

[³H]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). Briefly, membranes were incubated with 6 concentrations of [³H]NMS (ranging from 3 to 3000 pM), in duplicate, in a final volume of 1ml for 30 minutes.   In most assays, 2-5 µg proteins per assay tube were used; however, the low expression of the disulfide mutants necessitated much higher amounts (30-100 µg protein).   The incubation was terminated by filtration onto #32 glass-fiber filters (S & S, Keen, NH) pretreated with ice-cold 0.1% polyethyleneimine solution and followed with two quick washes with 5ml 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.

[³H]NMS Dissociation Assays. [³H]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 [³H]NMS for 30 minutes in 1 ml. Dissociation of [³H]NMS was then initiated by the addition of atropine (3 µM in a final volume

of 2ml) 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 must 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, 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 following equation:

$$\frac{k_{obs}}{k_0} = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC_{50} - X)^{n}H}}$$

Where X is 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 where the standard-slope model is appropriate, the potency (EC_{50}) is also the apparent binding affinity of the allosteric modulator at [^{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 (SE) 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 (Figure 1). Since the first two cysteine residues form a disulfide bond that is highly conserved in the majority of GPCRs (and therefore are referred to as the disulfide cysteine residues in this study) and the last two cysteine residues are only separated by 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 et al., 1999; Zeng and Wess, 1999). Anticipating future characterizations of some of the mutants, and to maintain similarity to the rM$_3$' constructs, we also inserted a 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 (Figure 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 to the parental control (i.e., hM$_2$'; see Table 1). Similarly, corresponding mutations in hM$_3$ (C141A, C221A) reduced surface expression by over 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 et al., 1999; Zeng and Wess 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 previously reported (Zeng et al., 1999), possibly due to 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 comparable expression levels and [^{3}H]NMS affinity to those of the hM_2 wild type receptors. Similarly, the hM_3 mutant with corresponding mutations in the o3 loop (C517A, C520A) also had comparable expression levels and [^{3}H]NMS affinity to 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 previous results from similar modifications in the rM_3 receptor (Zeng et al., 1999).

N-terminal modifications have no effect on allosteric binding. Since 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 Figure 2D, Tables 2 and 3, 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 1). This difference in

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[³H]NMS dissociation rates did not appear 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 [³H]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 2). 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 one; that is, in this mutated construct, there was no evidence for positive homotropic cooperativity in the interaction of THA. Additionally, the mutation caused a reduction in the potency of THA of about 6-fold (Figure 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 (Figures 2B and 2C, Table 2); 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 [³H]NMS, and would therefore be labeled at far below saturation with 1 to 2 nM [³H]NMS. To determine whether non-saturation labeling at these mutants might be responsible for the observed effects, we chose concentrations [³H]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 [³H]NMS

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dissociation. As indicated in Figure 2D and Table 2, 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 about twice as fast as that from the parental hM$_2$' receptor; hM$_3$ C141A, C221A was about 6 times faster than hM$_3$; and the rM$_3$' C140A, C220A mutant was about 13 times faster than its parent, rM$_3$' (see data in Table 1).

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 (about 2.5-fold) but reduced W84 potency by about 70-fold (Figure 3A and Table 3). 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; Figure 3B, 3C and Table 3). 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. Interestingly, 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 (Figure 3B, 3C and Table 3), 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 (Figure 3B and Table 3). The slope factors for W84 were not significantly different from unity at either hM$_3$ or rM$_3$' (Table 3); 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 Figure 4 and Table 3, 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 at the corresponding wild-type receptors. In addition, the dissociation rate of [^{3}H]NMS was not altered by the cysteine mutations in the o3 loop (Table 1).

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 (Figure 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 in fact 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 pmol/mg and 5.81 mol/mg, with [^{3}H]NMS binding affinities (pK$_d$) of 10.16 and 10.47, respectively. As indicated

in Figure 5 and Table 4, replacing Phe222 with Ala had similar effects on gallamine, THA, and

W84, reducing allosteric potencies by 2 – 4 fold. Replacing Phe222 with Tyr slightly increased

the potencies of gallamine and W84 by 2 – 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 (though not significantly), whereas the F222A mutant receptor

retained homotropic interactions in THA binding, but with a significantly reduced Hill slope

(Figure 5C, Table 4).   Both mutations decreased the slope factors for gallamine and W84 to

near unity, although statistical significance was only achieved for the F222A mutation, and only

for gallamine (Figure 5A, B, and Table 4).

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 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 about 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 about 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, since 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 following the elimination of the disulfide bond could be mainly attributed 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

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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 about 4-fold, slightly more than the reduction we have observed from the single Y177Q mutation in hM$_2$ (Huang and 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 one. It appears 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), which was abolished by the mutation (to Hill slopes not significantly different from one). 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 subtype in this type of assay. However, it has been known for some time that gallamine exhibits a biphasic regulation of the dissociation of quinuclidinylbenzilate 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 to 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 appear 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 paper were carried out using receptors that were prelabeled with [^{3}H]NMS (i.e., dissociation assays, see 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

appears 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 Figure 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), while others appear to bind at different site (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,

due to 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 (Tränkle et al., 2005). 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 (ibid.).

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).

Interestingly, Zeng and Wess (1999) have reported that Cys140 and Cys220 are 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 non-covalent complexes that were identified by immunoprecipitation, even though expression was greatly reduced (to a similar level as 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 (above), 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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Legends for figures.

Figure 1. Schematic presentations of the human M_2 muscarinic acetylcholine receptor and partial sequence alignment of the muscarinic acetylcholine receptor family around the 4 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).

Figure 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 same 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 in Methods and data are shown with the best-fit curves (see Table 2 for parameters and statistical data).   Points represent the means ± S.E.M. from 3 to 5 independent assays, each conducted in duplicate.

Figure 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 Figure 2 and best-fit parameters are presented in Table 3. Points represent the mean ± S.E.M. from 3 to 10 independent assays, each conducted in duplicate.

Figure 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 Figure 2 and best-fit parameters are presented in Tables 2 and 3. Points represent the mean ± S.E.M. from 3 to 6 independent assays, each in duplicate.

Figure 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 Figure 2 and Figure 3 are shown in broken lines in each panel. [^{3}H]NMS dissociation assays were conducted and data were analyzed as in Figure 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.

Table 1.   Binding properties of M$_2$ and M$_3$ receptors.

The hM$_2$' construct is the hM$_2$ receptor with 1xHA tagging after the initiating Met residue and deletion of potential N-glycosylation sites (see Methods).   The rM$_3$' construct is the rM$_3$ receptor with 1xHA 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 ± SEM from 3 or more assays and are the standard half times of [^{3}H]-NMS dissociation in the presence of atropine. Bmax and pKd values are the mean ± SEM from 2 or more assays.

Receptor	t$_{1/2}$ (min)	pKd	Bmax (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

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Table 2. Allosteric properties of THA at M_2 and M_3 receptors.

The parameters pKapp and Hill slope (n_H) represent best-fit values ± SE from global curve

fitting to the combined data of 3 or more assays. Statistical significance was determined by F-

test, either between or within data sets (see Methods). The between-set p values shown are

for comparisons with the immediate parental receptor; for example, hM_2' was compared to

hM_2, while hM_2'C96A, C176A was compared to hM_2'. Assays with reduced labeling were

compared to assays with typical (near-saturation) labeling at the same receptor. 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	pKapp and F-test		Hill slope (n_H) and F-tests		
	pKapp	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 3.   Allosteric properties of gallamine and W84 at M$_2$ and M$_3$ receptors.

The parameters pKapp and Hill slope (n$_H$) represent best-fit values ± SE from global curve fitting to the combined data of 3 or more assays. Statistical significance was determined as described in Table 2.

Receptor	pKapp and F-test		Hill slope (n$_H$) and F-tests		
	pKapp	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	

Table 4. Allosteric properties of hM$_3$ receptors with mutations at Phe222.

The parameters pKapp and Hill slope (n$_H$) represent best-fit values ± SE from global curve fitting to the combined data of 3 or more assays. Data for wildtype hM$_3$ receptors were included for comparison. Statistical significance was determined as in Table 2.

Receptor	pKapp and F-test		Hill slope (n$_H$) and F-tests		
	pKapp	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	

Figure 1

```
HM₂: V C96 .. V E D G E C Y177 ......... C A P C416
HM₃: A C141 . V P P G E C F222 ......... C D S C520
RM₃: A C140 . V P P G E C F221 ......... C D S C519
HM₁: A C .... V L A G Q C Y ............ C K D C
HM₄: V C .... V P D N H C F ............ C Q S C
HM₅: A C .... V P L D E C Q ............ C D K C
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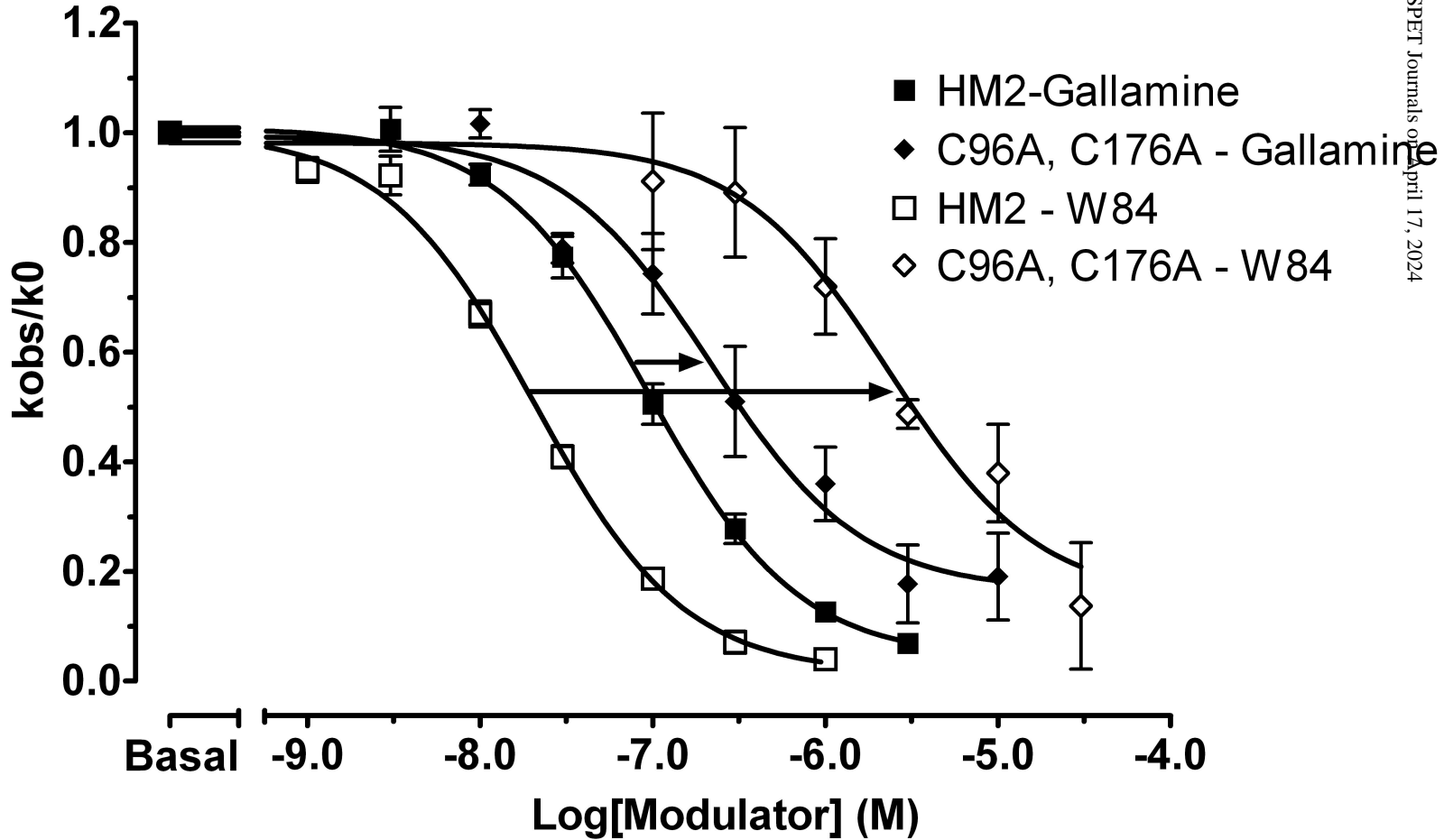
Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 3A

Figure 3A

Figure 3B

Figure 3C

Figure 4A

A

C413A, C416A + Gallamine
C413A, C416A + W84
C413A, C416A + THA

······HM2 + THA
—·—HM2 + W84
— — HM2 + Gallamine

Figure 4B

Figure 5A

Figure 5B