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Critical amino acid residues of the common allosteric site on the M_2 muscarinic acetylcholine receptor: more similarities than differences between the structurally divergent agents gallamine and bis(ammonio)alkane-type W84

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Running title: key determinants of the common muscarinic allosteric site

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[1]Abbreviations
CR, chimeric receptor construct; GPCRs, G-protein coupled receptors; Kapp, apparent binding affinity which is the affinity for [^{3}H]NMS-occupied receptors in this study; k_o, true dissociation rate constant; k_{obs}, observed (apparent) dissociation rate constant; mAChRs, muscarinic acetylcholine receptors; NMS, N-methylscopolamine chloride; o2, the second outer (extracellular) loop; o3, the third outer loop; PB, sodium-potassium phosphate buffer, pH 7.4; TM, transmembrane domain.

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Abstract

The structurally divergent agents gallamine and W84 are known to interact competitively at a common allosteric site on muscarinic receptors. Previous studies reported that the M_2 selectivity of gallamine depended largely on the EDGE (172-175) sequence in the second outer loop (o2) and on ^{419}Asn near the junction of o3 and the seventh transmembrane domain (TM7), while the selectivity of W84 depended on nearby residues ^{177}Tyr and ^{423}Thr. However, it has so far proven difficult to confer the high sensitivity for allosteric modulation of the M_2 subtype onto the weakly sensitive M_5 subtype by substituting these key residues. We have now found that M_2^{423}Thr, not ^{419}Asn, is the dominant residue in the o3/TM7 region for gallamine's high potency, although ^{419}Asn can substitute for ^{423}Thr in some contexts; in contrast, the presence of ^{419}Asn reduces the potency of W84 in every context we have studied. Also, the orientation of ^{177}Tyr is crucial to high sensitivity toward W84 and it appears that the proline residue at position 179 in M_5 (corresponding to M_2^{172}Glu) may interfere with that orientation. Consistent with these observations, a mutant M_5 receptor with these three key mutations, M_5P179E, Q184Y, and H478T, showed dramatically increased sensitivity for W84 (>100 fold), compared to the wild-type M_5 receptor. This same mutant receptor approached M_2 sensitivity toward gallamine. Thus, gallamine and W84 derive high potency from the same receptor domains (epitopes in o2 and near the junction between o3 and TM7), even though these allosteric agents have quite different structures.

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Introduction

Muscarinic acetylcholine receptors (mAChRs)[1] belong to the super family of G protein-coupled receptors (GPCRs) and possess a highly conserved binding site (orthosteric site) for the endogenous agonist acetylcholine and for other traditional agonists or competitive antagonists (orthosteric ligands); this site is formed by the transmembrane domains (TM). The mAChRs are among the best known of a growing number of GPCRs that possess another site (allosteric site), at which a second small ligand can bind, allowing ligand-ligand allosteric interactions at the external surface of the receptor (Christopoulos and Kenakin, 2002; Ellis, 2002). For ligands with significant selectivity, the M_2 subtype is the most sensitive of the five subtypes of mAChRs to allosteric modulation (Ellis *et al*., 1991; Lee and El-Fakahany, 1991; Trankle *et al*., 1998; Ellis and Seidenberg, 2000). Although there is evidence for the existence of multiple muscarinic allosteric sites (Ellis and Seidenberg, 1989; Potter *et al*., 1989; Trankle and Mohr, 1997; Birdsall *et al*., 2001, Lazareno *et al*., 2002; Trankle *et al*., 2003), many muscarinic allosteric modulators appear to act at a "common allosteric site" (Ellis and Seidenberg, 1992; Trankle and Mohr, 1997).

A number of investigations have attempted to identify the residues that comprise this common allosteric site. Initial studies in this area mutated either conserved residues or subtype-specific residues and both approaches suggested that the binding site for allosteric ligands lies extracellular to the orthosteric site (Ellis *et al*., 1993; Leppik *et al*., 1994; Matsui *et al*., 1995). Most subsequent mutagenic studies have continued the subtype-specific approach, following initial findings based on M_2/M_1 and, especially, M_2/M_5 chimeric receptors. The structures of two allosteric ligands that have been investigated intensively, gallamine and W84, are shown in

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Figure 1. The saturable type of antagonism seen with these agents in isolated organ preparations was the first evidence for a sensitivity of mAChRs for allosteric modulation (Lüllmann et al., 1969; Clark and Mitchelson, 1976). Schematic diagrams of some M_2/M_5 chimeras are shown in the top part of Figure 2. The first chimeric studies investigated gallamine and implicated only a short segment of sequence in TM6 or o3 (i.e., CR4 in Figure 2). However, subsequent studies with a number of other ligands (including W84) have implicated epitopes within a broad region containing o2 (CR3) and within TM7 (CR6), but not the TM6/o3 region (CR4) found for gallamine (see Figure 2). Furthermore, despite the lack of influence of the o2 loop on gallamine's potency in M_2/M_5 chimeras, studies in which M_1 and M_2 sequences were interchanged did identify a role for an acidic region of o2 in gallamine's preference for M_2 (the "EDGE motif" at $M_2^{172\text{-}175}$); it appeared that the essential acidic epitope in this region was shared by M_5 (Leppik *et al*., 1994; Gnagey *et al*., 1999). Mutations of individual amino acids have suggested that the essential residue for gallamine in the CR4 chimera is M_2^{419}Asn in o3, while the essential residues for W84 are M_2^{177}Tyr in o2 and M_2^{423}Thr in TM7 (Gnagey *et al*., 1999; Buller *et al*., 2002; Voigtlander *et al*., 2003). Thus, the binding selectivities of these two ligands are dependent on different but nearby residues, consistent with the finding that they seem to bind to a common site (Trankle and Mohr 1997). However, in spite of this rather detailed knowledge concerning the residues that appear to be responsible for the subtype selectivities of these allosteric ligands, it has so far not been possible to replicate their high M_2-like potency by the substitution of a few amino acids into the low-potency M_5 structure.

Because the known essential residues are in such close proximity in the receptor structure, we felt that it would be useful to compare the effects of a detailed series of mutations in these regions on the allosteric affinities of gallamine and W84. We have found that the

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substitution of just three residues of M_2 sequence into the M_5 receptor (M_5P179E,Q184Y,H478T) is sufficient to raise its sensitivity toward W84 by more than 100-fold, slightly exceeding the sensitivity of the M_2 receptor for this ligand. This triply mutated M_5 receptor also has greatly increased sensitivity for gallamine, although in this case it did not reach the level of the wildtype M_2 receptor.

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Experimental Procedures

Materials. Atropine sulfate, gallamine triethiodide, and polyethyleneimine were purchased from Sigma Chemical Co. (St. Louis, MO). W84 is commercially available from Tocris Cookson Inc. (Ellisville, MO). The orthosteric radioligand [^{3}H]NMS (N-methylscopolamine chloride, 81 Ci/mmol) was obtained from NEN-DuPont (Boston, MA). Glass fiber filters and all other inorganic chemicals were from VWR International, Inc. (Bridgeport, NJ).

Mutagenesis, Receptor Expression, and Membrane Preparation. Human mAChRs were used throughout. Some receptor constructs have been described previously: chimera CR3 (Ellis *et al*., 1993); mutants M_2T423H, M_5H478T, and CR3H478T (Buller *et al*., 2002); and mutants M_2EDGE-LAGQ, M_2N419V, M_2N419K, and M_5V474N (Gnagey *et al*., 1999). All mutation primers and sequencing primers used in this study were synthesized in the Core Facility at Hershey Medical Center. Site-directed mutagenesis was carried out using the QuickChange kit from Stratagene (La Jolla, CA), as reported previously (Buller *et al*., 2002). Mutations were confirmed by sequencing in the Core Facility at Hershey Medical Center. Plasmids containing wild-type or mutant receptor genes were purified using the QIAGEN plasmid purification kit (Valencia, CA). Purified plasmid was transiently transfected into COS-7 cells using the PolyFect Transfection Reagent from QIAGEN. About 48 hours after transfection, cells were scraped into 5 mM Na,K,Pi buffer, pH 7.4 (4mM Na_2HPO_4, 1mM KH_2PO_4; PB) and homogenized in the PB buffer on ice. After centrifugation at 50,000g for 30 minutes, membrane pellets were resuspended with a glass homogenizer in 5 mM PB and stored in aliquots at –70^{o}C.

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Protein concentrations were determined using the Advanced Protein Assay Reagent from Cytoskeleton Inc. (Denver, CO).

[^{3}H]NMS Saturation Binding Assays. All binding assays, including dissociation assays (below), were carried out in 5 mM PB, pH 7.4, at 25^{o}C. To determine receptor expression levels and binding affinities for [^{3}H]NMS, membranes were incubated with 6 concentrations of [^{3}H]NMS (ranging from 3 pM to 1000 pM) in duplicate in a final volume of 1 ml for 30 min. The reactions were terminated by filtration onto S&S 32 glass fiber filters pretreated with cold 0.1% polyethyleneimine solution, and followed with two washes with cold 40 mM PB. Radioactivity from membranes trapped on filter discs was determined by liquid scintillation counting. Data were fitted to a one site hyperbolic binding curve in the Prism 4.0 from GraphPad Software (San Diego, CA). Nonspecific binding was determined in the presence of 3 μM atropine at each concentration point.

[^{3}H]NMS Dissociation Assays. Dissociation assays were set up and conducted as previously described (Ellis and Seidenberg, 2000; Buller *et al*., 2003). Briefly, receptors (quantity of membrane protein estimated to produce approximately 2000 dpm in total binding) were first labeled with a saturating concentration of [^{3}H]NMS (1 nM) for 30 min in 1 ml. Dissociation of [^{3}H]NMS was initiated by addition of atropine (3 μM, in a final volume of 2 ml), with or without the indicated concentrations of allosteric modulators in duplicate, and terminated by filtration after a period of time, which is typically set between 2-3 times the standard half-time of [^{3}H]NMS dissociation (determined in the presence of atropine but in the absence of any allosteric modulator), up to a maximum of 120 min for M_5 receptor and some of its mutants.

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Dissociation assays were set up such that the delay of [^{3}H]NMS dissociation was mediated by the binding of the allosteric modulator to an allosteric site, separate from the orthosteric site at which for [^{3}H]NMS binds. The true dissociation rate constant (k_0) was determined in the presence of 3 μM atropine without the allosteric modulator 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 (X) and the resulting curve was fitted (using the program Prism 4.0 from GraphPad Software Inc.) to a three parameter logistic function

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

where Top (constrained to 1.0 in curving-fitting) and Bottom refer to the upper and lower plateaus of the sigmoidal curve. The curve represents the effect of allosteric delay of [^{3}H]NMS dissociation and corresponds to the occupancy curve of the tested allosteric modulator at the [^{3}H]NMS-occupied receptor (Ellis and Seidenberg, 1992; Lazareno and Birdsall, 1995). Curve fitting yielded EC_{50} values, which correspond to equilibrium dissociation constants of the allosteric modulator on the [^{3}H]NMS-occupied receptor, Kapp. For convenient comparison of effects of a given mutation on the binding of gallamine versus W84 on the same scale, we have transformed pKapp values (negative logarithm of Kapp values) into a percentage degree of the difference between M_2 and M_5 receptors:

$$relative\ \ pKapp\ (\%) = 100\% \frac{pKapp - pKapp_{M5}}{pKapp_{M2} - pKapp_{M5}}$$

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On this scale, M_2 would score 100% and M_5 would score 0%; a mutation that reduced the pKapp of M_2 by one third of the M_5/M_2 span would score 67%, and a mutation that raised the pKapp of M_5 by one third of that span would score 33% (see Table 1).

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Results

In this study, we systematically mutated a series of amino acid residues in o2 and o3/TM7 regions of M_2 or M_5 receptors, carried out [^{3}H]NMS dissociation assays to measure allosteric modulator potencies (apparent binding affinity, Kapp) and compared their individual roles in the binding and subtype selectivities of gallamine and W84. We employed dissociation assays for reasons that have been presented previously (Ellis, 1997; Ellis and Seidenberg, 2000; Buller *et al*., 2002). Briefly, a change in the dissociation rate of the labeled ligand is a purely allosteric phenomenon that is not confounded by any concomitant competitive interaction. Additionally, the marked slowing of the kinetics of the labeled ligand that is caused by these allosteric modulators does not alter the rapid equilibration of the allosteric modulator, whereas the slow kinetics of the labeled orthosteric ligand may be problematic in equilibrium (or pseudo-equilibrium) experiments (Seidenberg and Ellis, unpublished data).

The half times of [^{3}H]NMS dissociation from each receptor construct are reported in Tables 1 to 3 and are consistent with previous data (Ellis *et al*., 1993) that M_2 receptors have short half times (fast dissociation) and M_5 receptors have long half times (slow dissociation). Buller *et al*. (2002) initially reported that the M_2 T423H mutant exhibits a significantly slower rate of [^{3}H]NMS dissociation, compared to the wild-type M_2 receptor. We have also observed that our M_2 mutants that included histidine at that position (and only these M_2 mutants) were more than 3-fold slower than the wild-type receptor. Furthermore, the converse mutation in any of the M_5 backgrounds, or in the CR3 chimera, induced significantly faster dissociation, compared to the parent receptor.

Residue M_2^{423}Thr is equally important for both gallamine and W84 binding.

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As illustrated above (Figure 2), gallamine has been found to be unique among many tested allosteric ligands in that its potency is sensitive to epitopes found in both CR4 and CR6, but not CR3; W84, like many other ligands, is sensitive to epitopes in CR3 and CR6, but not CR4 (Ellis and Seidenberg, 2000; Buller *et al*., 2002). The residue in CR4 that confers specificity toward gallamine appears to be ^{419}Asn, whereas ^{423}Thr, which is included in CR6 but not in CR4, has been identified as the critical residue for the selectivity of W84 (Gnagey *et al*., 1999; Buller *et al*., 2002). The proximity of residues 419 and 423 (Figure 3a) led us to examine gallamine's potency at the M_2T423H mutant. This substitution of the M_5 residue reduced gallamine's potency significantly; additionally, the converse mutation, M_5H478T, significantly enhanced gallamine's potency (Figure 4). To facilitate comparison of the changes in the affinities of gallamine and W84 that are caused by receptor modification, we have expressed these changes as percentages of the M_2-M_5 span of potency for each ligand, on a logarithmic scale (as described in Methods). On this scale, the M_2T423H mutation reduces gallamine's potency by 31% and reduces W84's potency by 45%; the converse mutation in M_5 enhances gallamine's potency by 41% and enhances W84's potency by 31% (see Table 1). Similar degrees of enhancement and reduction in affinities have been reported previously for W84, dimethyl-W84, and diallylcaracurine V (Buller *et al*., 2002), indicating the importance of residue ^{423}Thr to these structurally different muscarinic allosteric modulators.

The presence of Asn at M_2^{419} or M_5^{474} leads to complex effects on gallamine's potency, but always reduces the potency of W84.

As stated above, residue ^{419}Asn in the o3 loop has been found to be important for gallamine's subtype selectivity (Gnagey *et al*., 1999). Briefly, residues ^{523}Lys in M_3 receptors and ^{474}Val in M_5 receptors are associated with significantly lower potency ofgallamine, and

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acidic residues in M_1 and M_4 receptors are associated with slightly higher potency of gallamine. Furthermore, the K523N mutation in M_3 increased both the pKapp toward gallamine and also the negative cooperativity between gallamine and NMS (Krejci and Tucek, 2001). All of these data have led to the conclusion that residue ^{419}Asn is very important in the binding of gallamine and in its ability to modulate the orthosteric site.

In light of the impact and proximity of ^{419}Asn and ^{423}Thr in M_2, we examined the effects of mutating each of these residues to their M_5 counterparts. The reverse mutations were also studied in the M_5 receptor, both individually and in concert. In Figure 5a, it can be seen that the mutations M_5H478T and M_5V474N produce similar effects, as each construct exhibits significantly higher sensitivity for gallamine than does the wild-type M_5 receptor. However, the enhancements are clearly not additive; indeed, the sensitivity of the double mutant is slightly lower than that of the M_5H478T single mutant. Figure 5b shows a different pattern of effects toward W84. As previously reported (Voigtlander *et al*., 2003), the M_5H478T mutation does significantly increase sensitivity toward this ligand; however, the M_5V474N mutation actually reduces sensitivity toward W84. These results are summarized in Table 1, along with data from additional single mutations at the $M_2{}^{419}$Asn site. Inserting a positively charged lysine residue produces almost as much decrement in sensitivity toward W84 as toward gallamine. However, W84 has significantly higher potency at the M_2N419V mutant than it has at the wild-type M_2 receptor, in agreement with the converse result in M_5. Thus, the potency of W84 is attenuated by the presence of Asn, whether at $M_2{}^{419}$ or at $M_5{}^{474}$. Perhaps more surprising is the finding that gallamine also has moderately higher potency at the M_2N419V mutant; nonetheless, this effect is consistent with the attenuation by asparagine of the beneficial effect of the threonine mutation in the M_5 receptor (compare M_5V474N, H478T to M_5H478T in Table 1).

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Residue M_2^{177}Tyr is more important for W84 than for gallamine

The mutation M_2Y177Q (Figure 3b) has been found to reduce the affinities for the NMS occupied receptor of bis(ammonio)alkane-type (such as W84) and caracurine V-type allosteric modulators; affinities are enhanced by the reverse mutation M_5Q184Y (Voigtlander *et al.*, 2003). Our experiments with W84 in receptors with mutations at this residue were in good agreement with this previous data. The mutation in M_2 reduced potency of W84 precipitously, falling more than 80% on our M_2/M_5 scale; the converse mutation in M_5 was less dramatic, but still raised the potency of W84 by 28% (see Table 2). In contrast, gallamine's potency is reduced much less (15%) by the M_2Y177Q mutation and is raised even less (7%) by the M_5Q184Y mutation (Figure 6, Table 2).

It was noted previously that the potency of W84 is greatly enhanced in a chimeric receptor that includes M_2 sequence in the o2 region (CR3, see Figure 2), compared to its potency at the wild-type M_5 receptor. Figure 7 shows that this greatly increased potency is predominantly due to the presence of the tyrosine residue in that chimeric receptor, as the construct that contains the other 144 residues of M_2 sequence but lacks the tyrosine actually has *lower* sensitivity for W84 than the wild-type M_5 receptor. In studies with W84, the substitution of glutamine for tyrosine at this position, whether in the wild-type M_2 receptor or in the CR3 chimeric receptor, produces a greater decline in potency than any other single-residue mutation in the present study (see Table 2).

The impact of M_2^{177}Tyr on W84 binding is due to the residue's aromatic nature

Molecular modeling studies have suggested that aromatic rings in diallylcaracurine V or W84 interact with M_2^{177}Tyr via π-π interactions (Voigtlander *et al.*, 2003). To test this

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suggestion experimentally, we systematically replaced M_2[177]Tyr with residues containing different functional side chains. Thus, in the M_2 receptor, we replaced the tyrosine with phenylalanine to retain the aromatic ring, or serine to retain the hydroxyl group, or alanine to retain neither characteristic. Figure 8 shows that the M_2Y177A and M_2Y177S mutations reduced the receptor's sensitivity toward W84 almost as much as the M_2Y177Q mutation did (Table 2). However, the M_2Y177F mutation caused a much smaller reduction in sensitivity toward W84, as would be expected if a π-π interaction is the important feature at that residue. Interestingly, all of the mAChR subtypes except M_5 have an aromatic residue at this position, tyrosine in M_1 and M_2 and phenylalanine in M_3 and M_4 (Figure 3b).

Glutamic acid residues within the EDGE motif in M_2-o2 exert differential and complex effects on the potencies of gallamine and W84

Leppik *et al*. (1994) first reported that mutation of EDGE to LAGQ (the corresponding M_1 sequence) significantly reduced gallamine's potency at the M_2 receptor. This was somewhat surprising, because the chimera CR3 includes the EDGE motif from the M_2 receptor but did not increase gallamine's potency, relative to M_5 (Ellis *et al*., 1993). The explanation for these divergent results appeared to be that the M_5 receptor also has acidic residues in the corresponding motif (PLDE) that were also important (perhaps equally important) for gallamine binding (Gnagey *et al*., 1999; sequence alignments are shown in Figure 3b). However, the roles of individual residues in the EDGE motif have not been investigated; therefore, in this study, we have investigated single amino acid mutations at strategic sites in this region. Specifically, we mutated residues within the EDGE motif to the corresponding M_1 residues and/or M_5 residues to evaluate their roles in the binding and subtype selectivities of gallamine (and W84). The rationale for using M_1 as well as M_5 residues is that the M_1 receptor is the only subtype with no

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acidic residues in the corresponding motif (LAGQ); the M_5 receptor maintains two acidic residues in the corresponding motif (PLDE), even though M_5 has the lowest sensitivity toward either gallamine or W84 among the mAChR subtypes.

As expected from previous studies (Gnagey *et al.*, 1999), the mutation EDGE-LAGQ, which removed all three acidic residues, reduced gallamine's pKapp by 42%; the same mutation also reduced W84's potency, albeit to a lesser extent (26%; Figure 9 and Table 2). In the EDGE-LAGQ (M_2/M_1) mutation, residue M_2^{174}Gly is retained, but the other three individual mutations remove three negatively charged residues. Previous studies have found that gallamine's potency at the M_4 receptor is unaffected by the PDNQ-LAGQ mutation, suggesting that the aspartate residue (corresponding to M_2^{173}Asp) does not play a major role (Gnagey *et al.*, 1999), leaving the glutamic acid residues (M_2^{172}Glu and ^{175}Glu) for further investigation. The mutations M_2E172L and M_2E175Q reduced gallamine's pKapp by 10% and 17% and W84's pKapp by 7% and 12%, respectively. Furthermore, the combined mutations (M_2E172L,E175Q), produced reductions in potency of gallamine and W84 that were similar to the EDGE-LAGQ mutation (Figure 9, Table 2). In the M_5 receptor, the residue corresponding to M_2^{175}E is conserved (as M_5^{182}E), but the residue corresponding to M_2^{172}E is a proline (M_5^{179}P). The mutation M_2E172P reduced the potencies of gallamine and W84 to similar extents, about 17% (Table 2).

M_2E172P mutation alters the conformation of M_2^{177}Tyr

One mechanism by which the M_2E172P mutation could exert a reduction in the potency of W84 would be via a change in the conformation of nearby residue ^{177}Tyr that might disrupt the π-π interaction between ^{177}Tyr and W84. This mechanism would also explain why the introduction of the tyrosine residue in M_5Q184Y might be under an unfavorable conformation

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for W84 binding, which in turn would explain why the enhancement due to this mutation is so much less than the decrement observed with the M_2Y177Q mutation (Table 2). If this explanation is correct, then additional mutations upstream of the inserted tyrosine might facilitate the interaction with W84. To test this possibility, we introduced the M_2 glutamate into M_5, in place of the proline. The M_5P179E mutation had virtually no effect by itself on W84 binding, but it significantly potentiated the ability of Q184Y to increase the potency of W84, from 28% to 60% above its potency at M_5 (Figure 10, Table 2). This mechanism is unlikely to apply to gallamine, however, because gallamine's potency is so much less sensitive to M_2^{177}Tyr. Indeed, although the M_5P179E mutation did significantly enhance potency ofgallamine, there was no potentiation when the Q184Y mutation was added (compare gallamine at M_5 P179E verse M_5 P179E, Q184Y in Table 2).

Effects of combining mutations in the o2 and o3/TM7 regions

The preceding studies have identified four epitopes that distinguish the M_2 receptor from the M_5 receptor and are involved in the binding and/or subtype selectivities of gallamine and W84. These are the M_2 residues ^{419}Asn in the o3 loop, ^{423}Thr at the top of TM 7, and, within the o2 loop, ^{172}Glu of the EDGE motif and ^{177}Tyr. The chimera CR3 possesses the entire o2 loop from the M_2 receptor (Figure 2), but does not contain the ^{419}Asn or ^{423}Thr of M_2. Therefore, we examined the allosteric effects of gallamine and W84 on CR3 receptors with the single or combined mutations V474N and H478T. For W84, the effects of the additional mutations were similar in CR3 and M_5. The histidine to threonine mutation enhanced potency, though in a somewhat less than additive manner, and resulted in a potency that was close to that of W84 at M_2 (compare CR3 H478T in Table 3 with M_5 H478T and CR3 in Table 1 and Table 3, respectively). This result is similar to a previous study (Buller *et al*., 2002). The valine to

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asparagine mutation had a negative effect in every case and, again, the effect was similar to the results obtained in M_5; the reductions in potency of W84 were approximately additive, in that the presence of the asparagine reduced the gains in potency caused by the CR3 or H478T manipulations by about the same amount that it reduced potency of W84 in M_5 itself (Table 1, Table 3). The effects of these mutations on the potency of gallamine were more complex. The M_2 sequence in CR3 did not enhance gallamine's potency very much, but it acted synergistically with the histidine to threonine mutation to yield a supra-additive gain in potency (compare Figure 5a to Figure 11, and Table 1 to Table 3). The valine to asparagine mutation, by contrast, was *less* than additive with the effect of CR3; however, the inclusion of this asparagine did not reduce the CR3-threonine synergy.

Compared to the M_5V474N, H478T mutant (Table 1) , the CR3 V474N, H478T mutant (Table 3) increased gallamine's pKapp by 0.66 log unit and that of W84 by 0.95 log unit. A major difference between these two mutants is the o2 loop of the M_2 receptor. Based on the studies presented above, we expected that the P179E and Q184Y mutations were dominant in producing the effects of the o2 loop in the CR3 chimeric constructs. Therefore, we created two quadruple mutants, the M_5P179E, Q184Y, V474N, H478T mutant and the converse construct in M_2, in which the corresponding four residues were replaced by their M_5 counterparts. These mutations in the M_2 receptor reduced its sensitivities toward gallamine and W84 dramatically, nearly to M_5 levels (Figure 12, Table 3). Confirming the importance of these residues, the M_5 quadruple mutant showed significantly higher sensitivity toward gallamine than M_5 (Figure 13a, Table 3). Remembering the frequently deleterious effects of the o3 ^{419}Asn, we also tested the triple mutant M_5P179E, Q184Y, H478T; it showed slightly higher sensitivity toward gallamine than the quadruple mutant, even higher than the CR3H478T mutant, rising 74% on the relative

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scale between M_5 and M_2 pKapp. The potency of W84 was enhanced to an even greater extent than that of gallamine in these triple and quadruple M_5 mutants; indeed, the sensitivity of the triple mutant for W84 actually surpassed that of M_2 (Figure 13b, Table 3).

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Discussion

The aim of this investigation was to examine the differences between the epitopes involved in the subtype selectivity of gallamine and W84, which represent structurally divergent muscarinic allosteric agents. Prior to this study, the existing data suggested that, although these ligands interacted at a common allosteric site (Trankle and Mohr 1997), the residues responsible for their subtype-selectivities were distinct. W84 has recently been thoroughly characterized and shown to require ^{177}Tyr in the o2 loop and ^{423}Thr at the top of TM7 for its high potency at the M_2 receptor, relative to M_5 (Voigtlander *et al.*, 2003). The M_2/M_5 selectivity of gallamine has been attributed to $M_2{}^{419}$Asn in the o3 loop and to a negatively charged sequence (EDGE) in the M_2-o2 loop (Leppik *et al*., 1994; Gnagey *et al*., 1999), but individual residues in this region of the o2 loop had not been thoroughly characterized. Here, we have found that gallamine and W84 are approximately equally dependent on $M_2{}^{423}$Thr for high potency, that the orientation of $M_2{}^{177}$Tyr is crucial to the selectivity of W84 and can be modulated by nearby upstream residues, that gallamine's high potency apparently relies more on the negative charge in o2 than on the aromatic nature of $M_2{}^{177}$Tyr, and that the role of $M_2{}^{419}$Asn is more complex than earlier studies had suggested.

The evidence pointing to $M_2{}^{419}$Asn in gallamine's subtype selectivity originated from studies in which a smaller or larger segment of the M_5 receptor was replaced with M_2 sequence (see CR4 and CR6 in Figure 2). The chimera CR6 includes both $M_2{}^{419}$Asn and $M_2{}^{423}$Thr, whereas CR4 contains only $M_2{}^{419}$Asn. Because the two chimeric constructs showed similar sensitivity toward gallamine and the larger segment included the smaller one completely, it was assumed that the essential residue resided within the smaller segment. Subsequent investigations

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found that the sensitivity of the M_5 receptor (and M_3 as well) toward gallamine was significantly enhanced when they contained an asparagine residue at the position corresponding to M_2^{419} (Gnagey *et al.*, 1999). However, inspection of Figure 2 reveals that the larger substitution of CR6 leads to higher sensitivity toward gallamine than does the smaller substitution of CR4, in much the same way that the double mutant M_5V474N, H478T compares to M_5V474N (Figure 5a), probably indicating that the slightly higher gallamine potency at CR6 (compared to CR4) is significant and that it represents the non-additive effects of V474N and H478T on gallamine's potency. Overall, it appears that this asparagine residue is beneficial to sensitivity toward gallamine when the threonine is absent, but not when that threonine is present. In other words, M_2^{419}Asn plays a minor role or even a negative role in M_2 wildtype receptors; however, when M_2^{423}Thr is removed or replaced with histidine, then M_2^{419}Asn becomes important for gallamine binding. These data indicate that the role of ^{419}Asn is more complicated and less important in the binding of gallamine to the M_2 subtype than earlier studies had suggested. In the case of W84, the data are not so complex, as the presence of that asparagine residue reduces W84's potency whether it is in the M_2 or M_5 context.

Similar to the situation at M_2^{419}Asn, the residues at the position corresponding to M_2^{423}Thr are unique in each subtype of mAChRs. As noted above and in previous studies, the histidine in the M_5 subtype is associated with low sensitivity for caracurine derivatives and dimethyl-W84 (as well as gallamine and W84), while the serine in the M_4 subtype appears to be functionally equivalent to the threonine residue (Buller *et al*., 2002). Thus, it appears that both M_2^{419}Asn and M_2^{423}Thr (and their corresponding residues in other subtypes) are important in generating subtype-selectivity, but the threonine residue is the dominant feature of the o3/TM7 region for the subtype-selectivity of both gallamine and W84. The importance of these two

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positions in the receptor may be related to the fact that, if the region containing these residues is helical, their side-groups will be adjacent on the same face of the helix. In addition, both ^{419}Asn and ^{423}Thr seem to be involved in mechanisms of cooperativity between NMS and several allosteric ligands, especially when comparisons are drawn between the M_2 and M_3 receptor subtypes (Krejci and Tucek, 2001; Jakubik *et al*, 2005).

In the o2 region, there seems to be no doubt that $M_2{}^{177}$Tyr is the most important residue in the subtype-selective binding of W84, as reported previously (Voigtlander *et al*., 2003). In the present study, we have shown that the dramatic increase in sensitivity toward W84 that is seen in the chimeric receptor named CR3, relative to M_5 sensitivity, is completely lost if just that tyrosine is excluded from the 145 amino acid exchange (Figure 7, Table 2). Subsequent mutations at position 177 also supported the suggestion that there is a π-π interaction between W84 and the tyrosine phenol ring, as phenylalanine was a much better substitute for that tyrosine than serine or alanine (or glutamine).

With regard to the potency of W84, we have previously suggested that the proline at position 179 in M_5 might interfere with the proper alignment of the tyrosine residue in M_5 mutants that include the Q184Y mutation (Voigtlander *et al*., 2003), and that now appears to be the case. That is, whereas the P179E mutation leaves the sensitivity toward W84 almost unaffected, relative to M_5, it greatly potentiates the effect of the tyrosine insertion at position 184 (Figure 10, Table 2). For gallamine, it appears that the negative charge within the EDGE motif is the most important factor in o2, and that the glutamates at positions 172 and 175 are the most important residues. However, the chimera CR3 contains the glutamate corresponding to $M_2{}^{172}$Glu instead of proline, yet does not have much better sensitivity toward gallamine than M_5 itself, suggesting that there must also be features within the M_2 sequence of CR3 that neutralize

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the expected gain in sensitivity; indeed, gallamine has significantly higher potency for M_5P179E, Q184Y than it has for CR3.

When mutations in the o2 and o3/TM7 regions are combined, some additional small synergies may be noted. For gallamine, the substitution of threonine for histidine within the CR3 context produces a greater gain in potency than the corresponding mutation in the M_5 receptor itself (i.e., H478T). On the other hand, insertion of that threonine into the M_5P179E, Q184Y produces just an additive effect (see Table 3). It is as if the threonine residue relieves the unidentified negative influence in CR3 (referred to above). For W84, the situation is reversed, with the histidine to threonine mutation and the CR3 substitution being essentially additive, but the insertion of the threonine into M_5P179E, Q184Y yielding a greater than additive effect; indeed, this triple mutant has slightly higher sensitivity than M_2 itself toward W84. The source of these small inter-regional synergies is not immediately apparent. In any event, this is the first report of high potency of a muscarinic allosteric ligand (i.e., W84) being so fully recreated by three discrete mutations in the low potency background of the M_5 subtype.

A number of studies have suggested that the o2 loop and the o3 loop (plus several residues predicted to lie in the extracellular ends of TM6 and TM7) can modulate the conformation of mAChR structure. Liu *et al.* (1995) found that a threonine at the position corresponding to M_2^{423} interfered with proper helix-helix packing in some recombinant receptor constructs (see discussion in Buller *et al*., 2002). We have noted that the presence or absence of a threonine at this same position modulates the kinetics with which NMS binds to the receptor (see Results and half times in Tables 1 and 3). Extending the observations of Spalding *et al*. (1995, 1997) at the M_5 receptor, Ford *et al.* (2002) have found that homologous mutations near the o3/TM6 junction constitutively activate all of the five mAChR subtypes. In o2, endogenous

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antibodies that bind to this loop region can activate the M_2 receptor (Goin *et al*, 1999; Baba *et al*., 2004) and a monoclonal antibody fragment generated against a smaller peptide that still includes the EDGE motif displays inverse agonist activity (Peter *et al*., 2004).

In summary, we now have a more detailed explanation of the source of the relative affinities of the allosteric agents W84 and gallamine for the NMS-bound forms of the M_2 and M_5 mAChRs. The affinities of these two ligands benefit from a common feature, namely the threonine residue found near the junction of the o3 and TM7 in the M_2 receptor. Additionally, W84 interacts with a tyrosine residue in the o2 loop; the orientation of this residue is crucial to allow for an optimal π-π interaction with the ligand and may be modulated by nearby residues. The potency of gallamine appears to be influenced more by nearby negative charges than by the aromatic tyrosine residue. Thus, the subtype-selective features of the common allosteric site appear to reside in these two extracellular regions of the receptor.

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Figure legends

Figure 1. Chemical structures of muscarinic allosteric modulators, gallamine (upper) and W84 (lower), used in this study.

Note that although both agents are positively charged and interact competitively at an allosteric site on mAChRs, they have quite different structures. W84 is an elongated di-cationic molecule, whereas the tri-cationic gallamine is rather small and compact.

Figure 2. Schematic presentations of chimeric receptors used or mentioned in this study (upper) and their sensitivity profiles for gallamine and W84 (table).

Details of the constructions and the experimental data are given in Ellis *et al*. (1993) and Buller *et al*. (2002). Potencies of the allosteric agents are expressed as pKapp. To facilitate comparisons, these potency data have also been transformed to a scale of relative difference between the pKapp values of the M_2 and M_5 mAChR (see Methods). The diagrams are intended to illustrate the regions of the receptor in which human M_5 sequence has been replaced by the homologous human M_2 sequence. Briefly, the M_2 residues that have been inserted are (M_2 numbering): CR3, 156-300; CR4, 391-421; CR5, 1-155; CR6, 1-69 and 391-466.

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Figure 3. Schematic presentations of the 2nd and 3rd outer loops (o2 and o3) and their adjacent transmembrane (TM) domains of the M_2 mAChR, shown with partial sequences of the outer loops and the adjacent TM domains of the mAChR family.

The boundaries of transmembrane domains of the human mAChR sequences were estimated by manually aligning the sequences to bovine rhodopsin, for which a high resolution crystal structure has been obtained (Palczewski *et al*., 2000). Panel A, o3; panel B, o2.

Figure 4. Gallamine's potency is reduced by the T423H mutation in the o3/TM7 region of the M_2 receptor and increased by the reverse mutation H478T in the M_5 receptor.

The modulation of the rate of dissociation of [^{3}H]NMS from the receptors was determined as described in Methods and the data were fitted to the model given in Methods. Points represent the mean ± SEM from 4-8 experiments.

Figure 5. Effects of mutations near the junction of o3/TM7 in the M_5 receptor on the potencies of gallamine (A) and W84 (B).

The M_2 (far left) and M_5 (far right) curves from Figure 4 are shown as dashed lines in panel A and in subsequent figures for convenient comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-5 experiments.

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Figure 6. Gallamine's potency is somewhat reduced by the Y177Q mutation in the M_2 receptor and is not increased by the reverse mutation Q184Y in the o2 loop of the M_5 receptor.

M_2 and M_5 curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 6-9 experiments.

Figure 7. The ^{177}Tyr residue is critical to the enhanced action of W84 in the chimera CR3, relative to the M_5 receptor.

M_2 and M_5 curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-4 experiments.

Figure 8. Non-aromatic substitutions at residue ^{177}Tyr of the M_2 receptor strongly reduce potency of W84.

The wild-type tyrosine was replaced by phenylalanine, serine, or alanine. The pKapp values for these mutant curves were 7.13, 6.39, and 6.25, respectively. M_2 (pKapp 7.66) and M_5 (pKapp 5.66) curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-7 experiments.

Figure 9. Effects of mutations at the glutamate residues of the EDGE motif on potencies of gallamine (A) and W84 (B).

The EDGE motif in the M_2-o2 region was replaced by M_1 sequence in whole (EDGE-LAGQ) or in part, as indicated. M_2 and M_5 curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-9 experiments.

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Figure 10. The mutations P179E and Q184Y in the o2 loop of the M_5 receptor synergistically enhance potency of W84.

M_2 and M_5 curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-4 experiments.

Figure 11. Effects of mutations in the o3/TM7 region of the CR3 chimera on potency of gallamine.

M_2 and M_5 curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-4 experiments.

Figure 12. Combined mutations in the o2 loop and the o3/TM7 region of the M_2 receptor dramatically reduce potencies of gallamine (A) and W84 (B).

M_2 and M_5 curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-6 experiments.

Figure 13. Combined mutations in o2 loop and the o3/TM7 region of the M_5 receptor dramatically enhance potencies toward gallamine and W84.

The quadruple mutation M_5 P179E, Q184Y, V474N, H478T (and the triple mutation without V474N) were constructed and assessed for allosteric actions of gallamine (A) and W84 (B). M_2 and M_5 curves are included for comparison. Experiments were conducted and analyzed as in Figure 4. Points represent the mean ± SEM from 3-4 experiments.

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Table 1. Effects of mutations in o3/TM 7 region on allosteric interactions.

Affinities (expressed as pKapp) for gallamine and W84 are shown, along with half times of dissociation of [^{3}H]NMS in the absence of allosteric modulators. To facilitate comparisons, the affinity data have also been transformed to a scale of relative difference between the pKapp values of the human M_2 and M_5 receptors (see Methods). Data shown represent mean ± S.E.M. from three or more assays for pKapp values and six or more assays for half times. Receptors were expressed in COS-7 cell membranes.

Receptors	Half times ($t_{1/2}$, Min.)	pKapp values, mean ± S.E.M.		Relative pKapp (%)	
		Gallamine	W84	Gallamine	W84
M_2	4.3 ± 0.4	6.99 ± 0.06	7.66 ± 0.10	100	100
M_5	95.2 ± 0.6	5.02 ± 0.04	5.66 ± 0.07	0	0
M_2 N419K	7.4 ± 0.3	5.89 ± 0.09	6.76 ± 0.10	44.2	55.0
M_2 N419V	3.7 ± 0.1	7.20 ± 0.04	8.11 ± 0.11	110.7	122.5
M_2 T423H	14.1 ± 0.9	6.38 ± 0.03	6.76 ± 0.11	69.0	55.0
M_5 V474N	85.5 ± 4.7	5.55 ± 0.03	5.29 ± 0.09	26.9	-18.5
M_5 H478T	37.6 ± 1.6	5.83 ± 0.10	6.27 ± 0.11	41.1	30.5
M_5 V474N, H478T	26.7 ± 0.6	5.65 ± 0.08	6.13 ± 0.09	32.0	23.5

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Table 2. Mutations in o2 region.

Data are presented as in Table 1.

Receptors	Half times ($t_{1/2}$, Min.)	pKapp values, mean ± S.E.M.		Relative pKapp (%)	
		Gallamine	W84	Gallamine	W84
M_2	4.3 ± 0.4	6.99 ± 0.06	7.66 ± 0.10	100	100
M_5	95.2 ± 0.6	5.02 ± 0.04	5.66 ± 0.07	0	0
M_2 E172L	4.2 ± 0.2	6.79 ± 0.09	7.53 ± 0.10	89.8	93.5
M_2 E172P	3.5 ± 0.2	6.64 ± 0.04	7.32 ± 0.09	82.2	83.0
M_2 E175Q	4.4 ± 0.1	6.65 ± 0.09	7.43 ± 0.11	82.7	88.5
M_2 Y177Q	4.2 ± 0.4	6.70 ± 0.07	5.99 ± 0.08	85.3	16.5
M_2 E172L, E175Q	5.7 ± 0.5	6.36 ± 0.10	6.89 ± 0.09	68.0	61.5
M_2 EDGE-LAGQ	5.0 ± 0.2	6.17 ± 0.07	7.14 ± 0.08	58.4	74.0
M_5 P179E	52.2 ± 1.8	5.49 ± 0.02	5.57 ± 0.12	23.9	-4.5
M_5 Q184Y	127.5 ± 5.7	5.16 ± 0.11	6.22 ± 0.11	7.1	28.0
M_5 P179E, Q184Y	95.7 ± 3.9	5.69 ± 0.04	6.86 ± 0.08	34.0	60.0
CR3	81.5 ± 4.0	5.26 ± 0.08	6.97 ± 0.12	12.2	69.5
CR3 Y177Q	88.0 ± 3.0	5.23 ± 0.03	5.36 ± 0.08	10.7	-15.0

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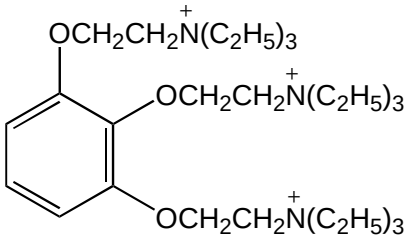
Table 3. Receptor constructs with combined mutations in o2 and o3/TM7 regions.

Data are presented as in Table 1.

Receptors	Half times ($t_{1/2}$, min.)	pKapp values, mean ± S.E.M.		Relative pKapp (%)	
		Gallamine	W84	Gallamine	W84
M_2	4.3 ± 0.4	6.99 ± 0.06	7.66 ± 0.10	100	100
M_5	95.2 ± 0.6	5.02 ± 0.04	5.66 ± 0.07	0	0
M_2 E172P, Y177Q, N419V, T423H	24.3 ± 1.8	5.24 ± 0.10	5.74 ± 0.11	11.2	4.0
M_5 P179E, Q184Y, H478T	32.3 ± 0.9	6.47 ± 0.08	7.86 ± 0.07	73.6	110.0
M_5 P179E, Q184Y, V474N, H478T	31.8 ± 1.0	6.36 ± 0.05	7.12 ± 0.14	68.0	73.0
CR3	81.5 ± 4.0	5.26 ± 0.08	6.97 ± 0.12	12.2	69.5
CR3 Y177Q, H478T	32.0 ± 0.7	5.98 ± 0.04	6.05 ± 0.08	48.7	19.5
CR3 V474N	76.7 ± 2.3	5.48 ± 0.06	6.61 ± 0.14	23.4	47.5
CR3 H478T	42.1 ± 3.3	6.34 ± 0.05	7.36 ± 0.06	67.0	85.0
CR3 V474N, H478T	32.0 ± 0.7	6.31 ± 0.06	7.08 ± 0.12	65.5	71.0

Figure 1



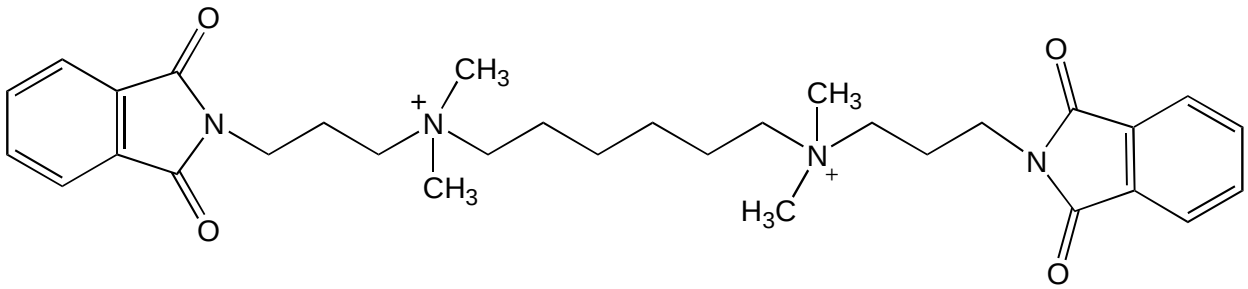
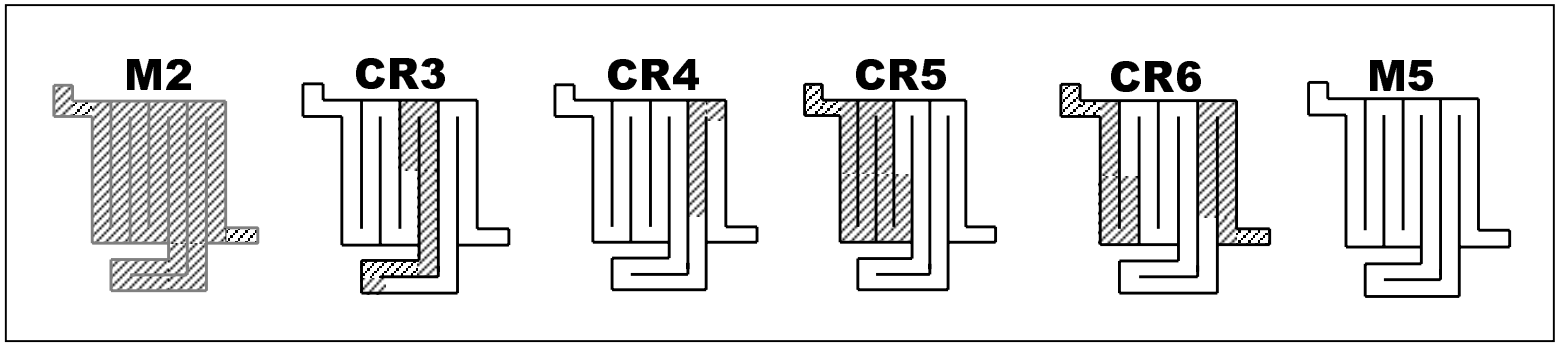
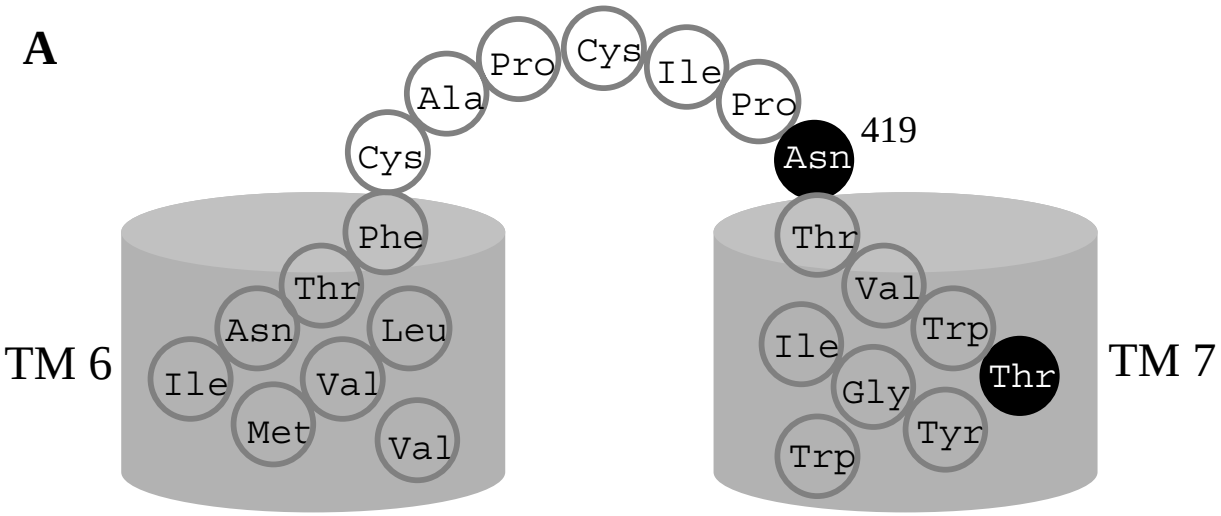


Figure 2

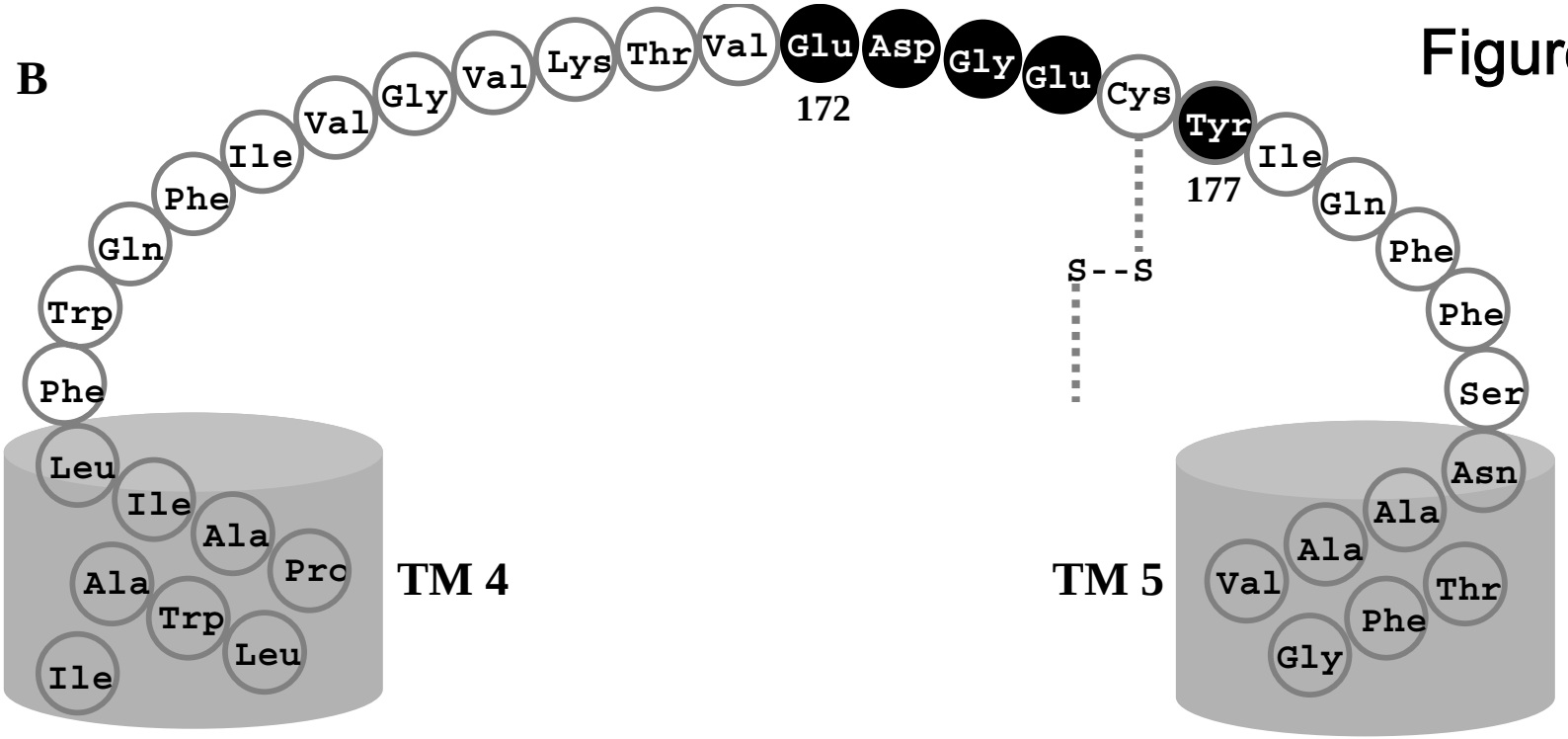


Receptors/chimeras	pKapp values, mean ± S.E.M.		Relative pKapp (%)	
	Gallamine	W84	Gallamine	W84
M_2	6.77 ± 0.13	7.62 ± 0.03	100	100
M_5	5.04 ± 0.09	5.79 ± 0.06	0	0
CR3	5.05 ± 0.21	7.00 ± 0.07	0.6	66.1
CR4	5.66 ± 0.10	5.66 ± 0.03	35.8	-7.1
CR5	5.17 ± 0.07	5.90 ± 0.03	7.5	6.0
CR6	5.90 ± 0.09	6.43 ± 0.06	49.7	35.0

Figure 3A



	<-TM 6--	- o3 --	-TM 7-->
M_4	VMVLVNTF	CQSCIP**D**	TVW**S**IGYW
M_1	IMVLVSTF	CKDCVP**E**	TLW**E**LGYW
		419	423
M_2	VMVLINTF	CAPCIP**N**	TVW**T**IGYW
		474	478
M_5	IMVLVSTF	CDKCVP**V**	TLW**H**LGYW
M_3	IMVLVNTF	CDSCIP**K**	TFW**N**LGYW




```
     <-TM 4-- ---------- o2 -------- -TM 5->
M4   VLWAPAIL FWQFVVGKRTVPDNHCFIQFLS NPAVTFG
M1   VLWAPAIL FWQYLVGERTVLAGQCYIQFLS QPIITFG

                          172     177
M2   ILWAPAIL FWQFIVGVRTVEDGECYIQFFS NAAVTFG

                          179     184
M5   ILWAPAIL CWQYLVGKRTVPLDECQIQFLS EPTITFG
M3   VLWAPAIL FWQYFVGKRTVPPGECFIQFLS EPTITFG
```

Figure 4

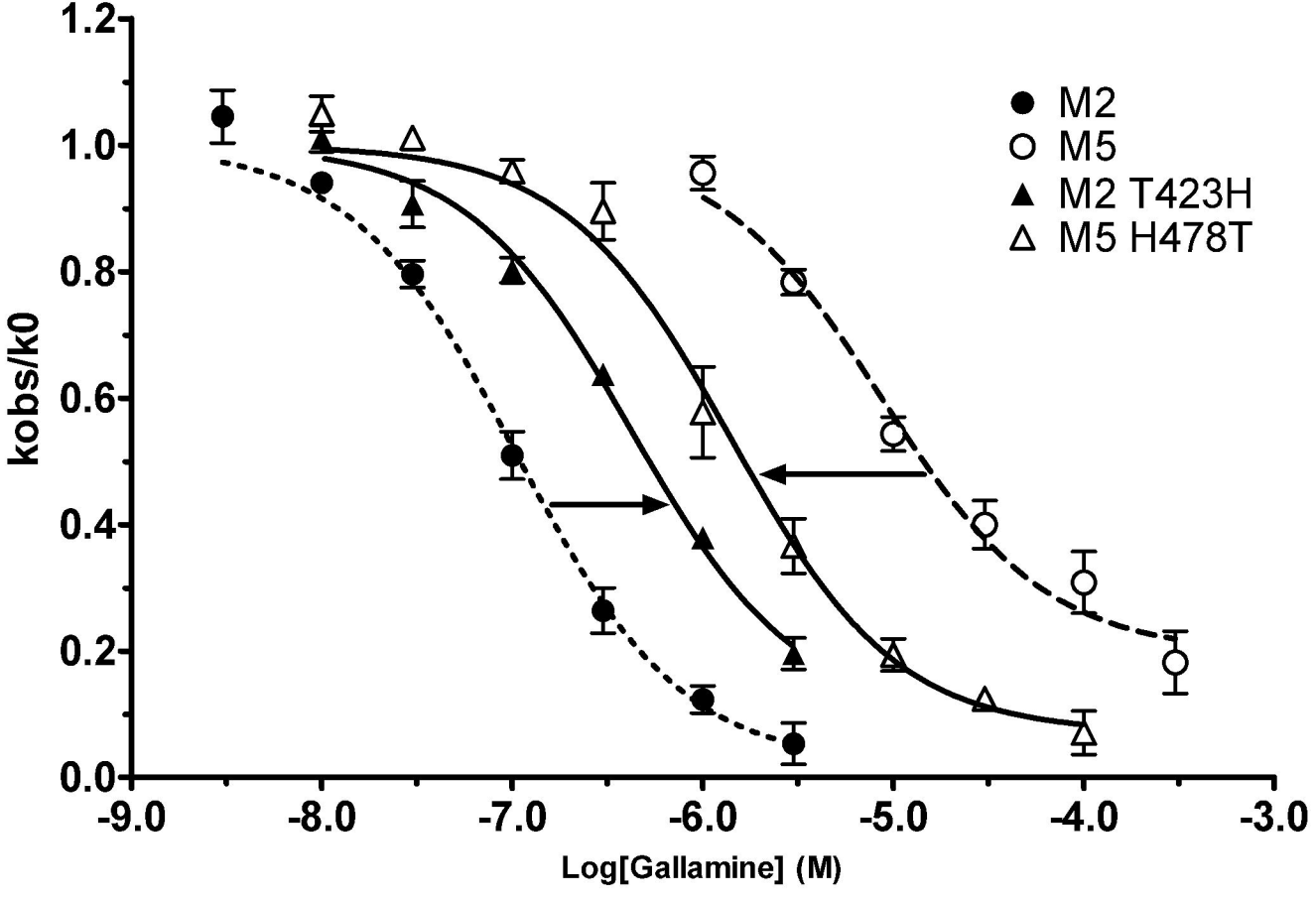

1.2
1.0
0.8
0.6
0.4
0.2
0.0
kobs/k0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)
M2
M5
M2 T423H
M5 H478T

Figure 5A

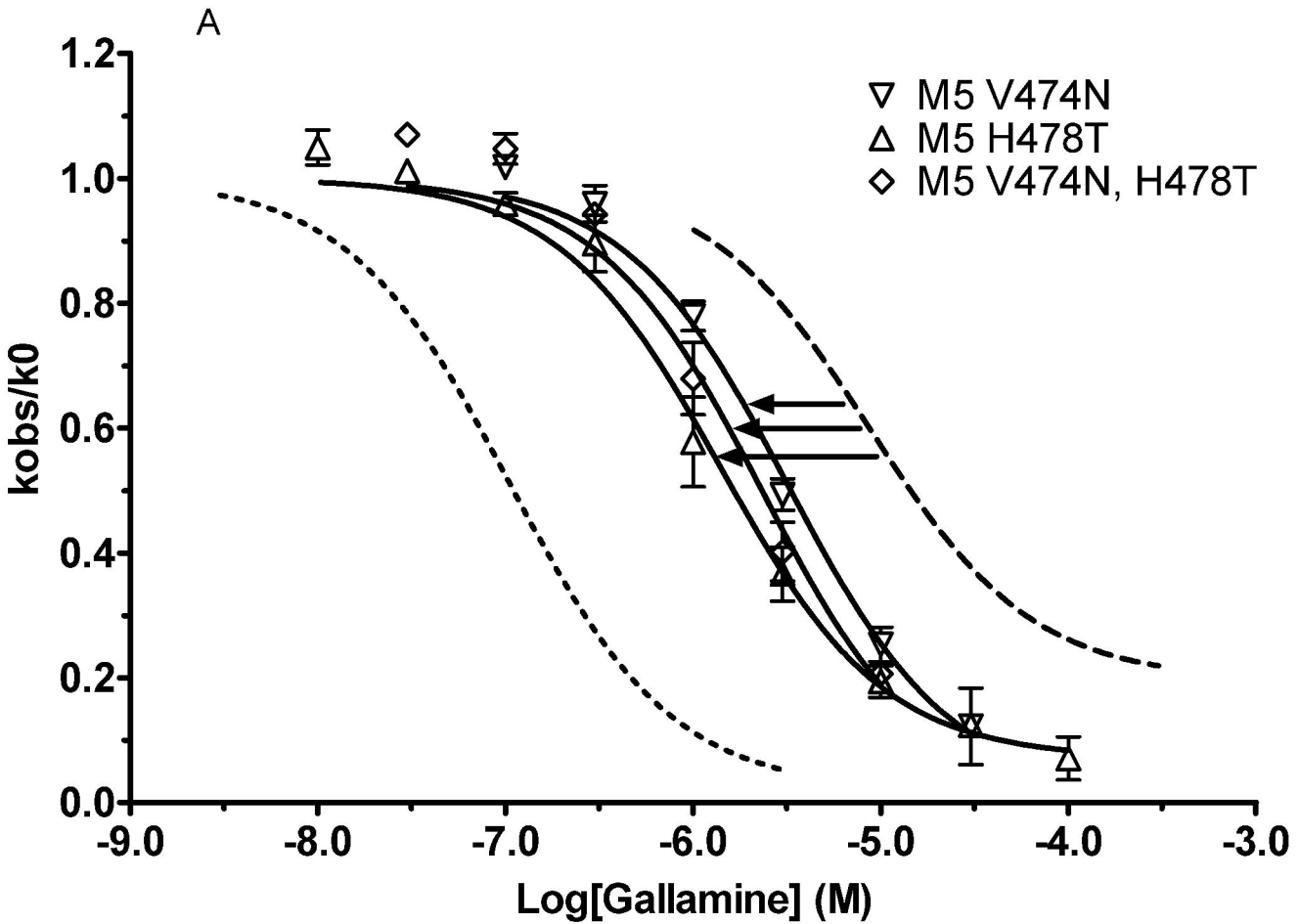

A
M5 V474N
M5 H478T
M5 V474N, H478T
kobs/k0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)

Figure 5B

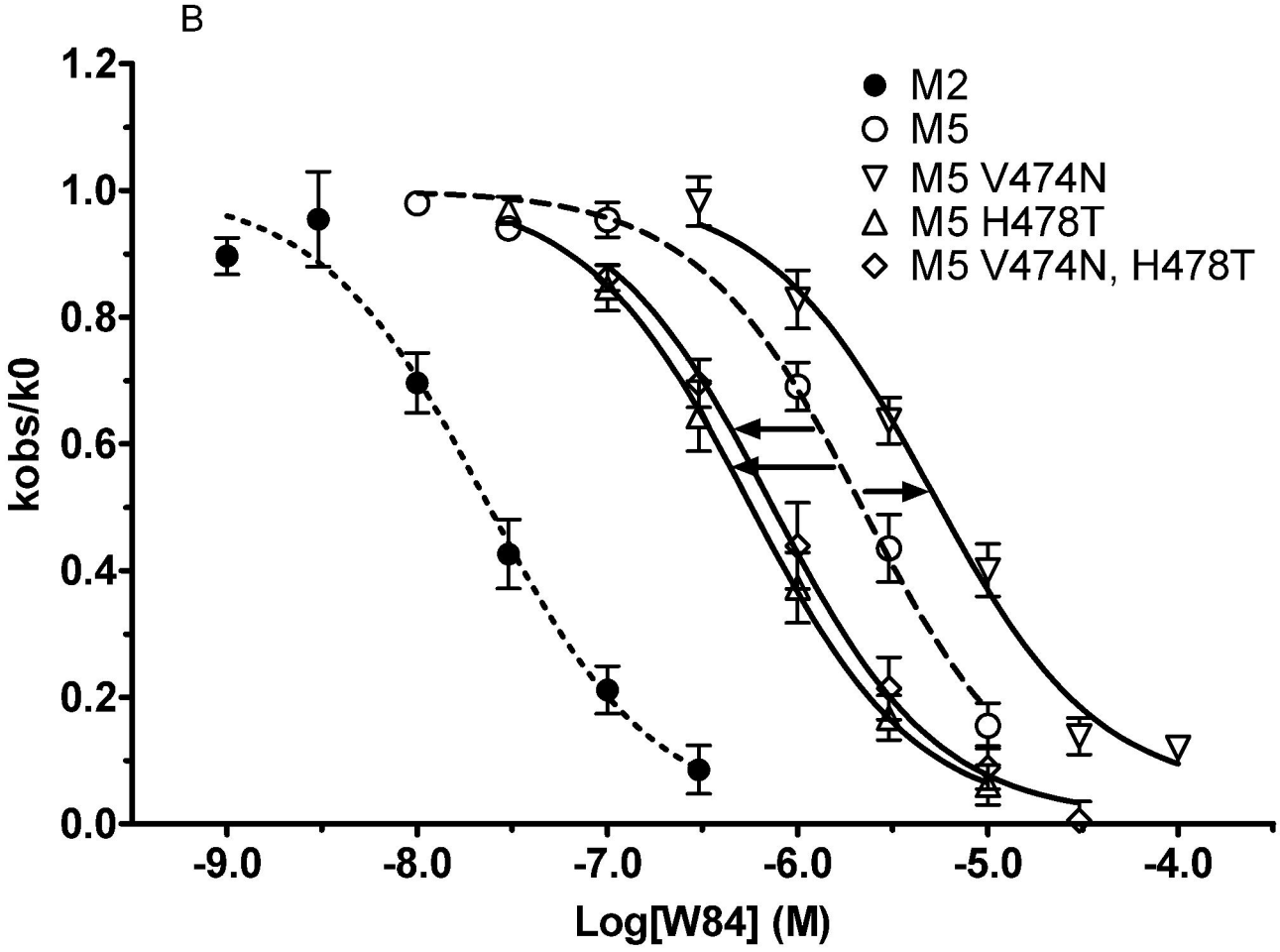

B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
kobs/k0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
Log[W84] (M)
M2
M5
M5 V474N
M5 H478T
M5 V474N, H478T

Figure 6

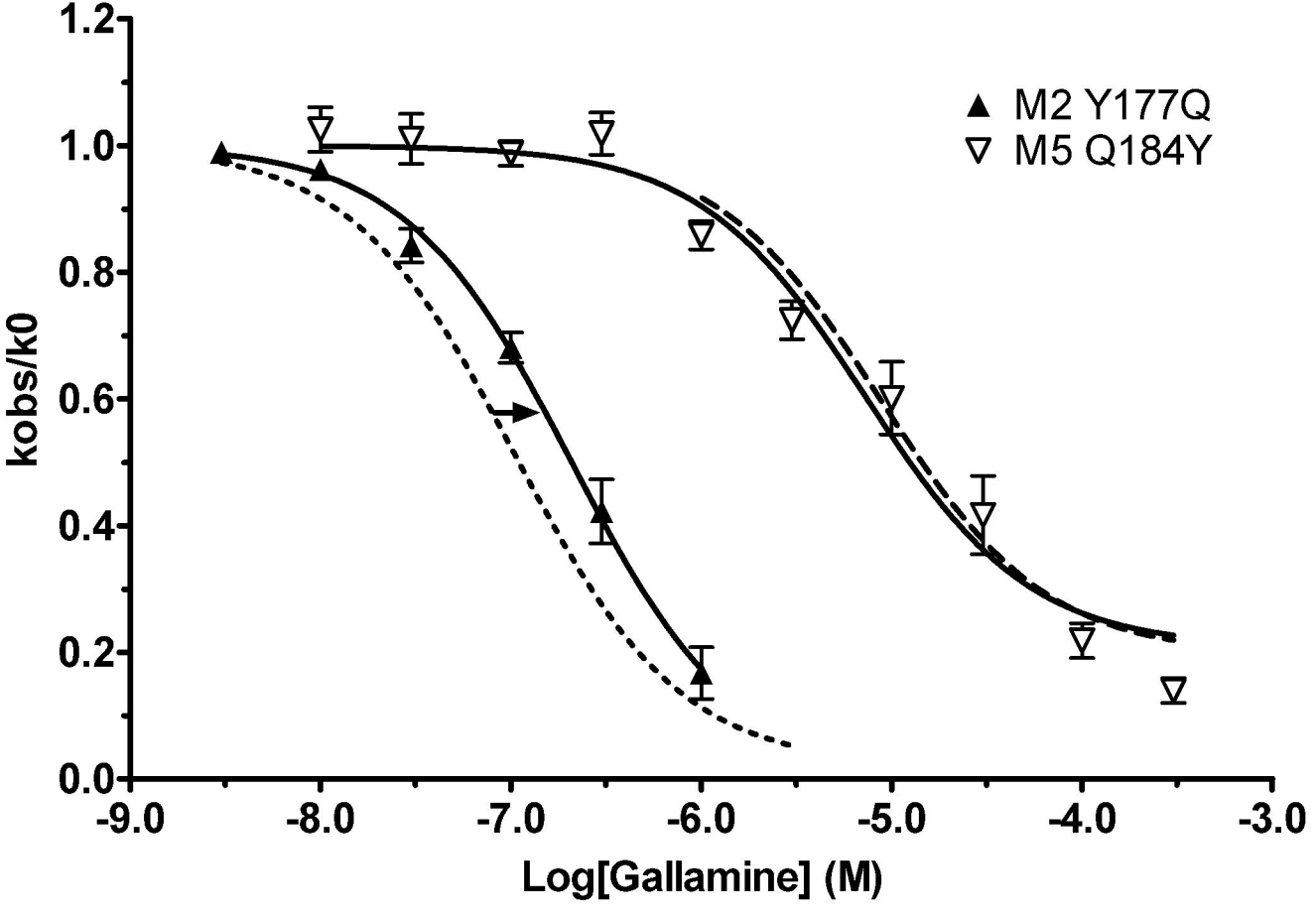

1.2
1.0
0.8
0.6
0.4
0.2
0.0
kobs/k0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)
M2 Y177Q
M5 Q184Y

Figure 7

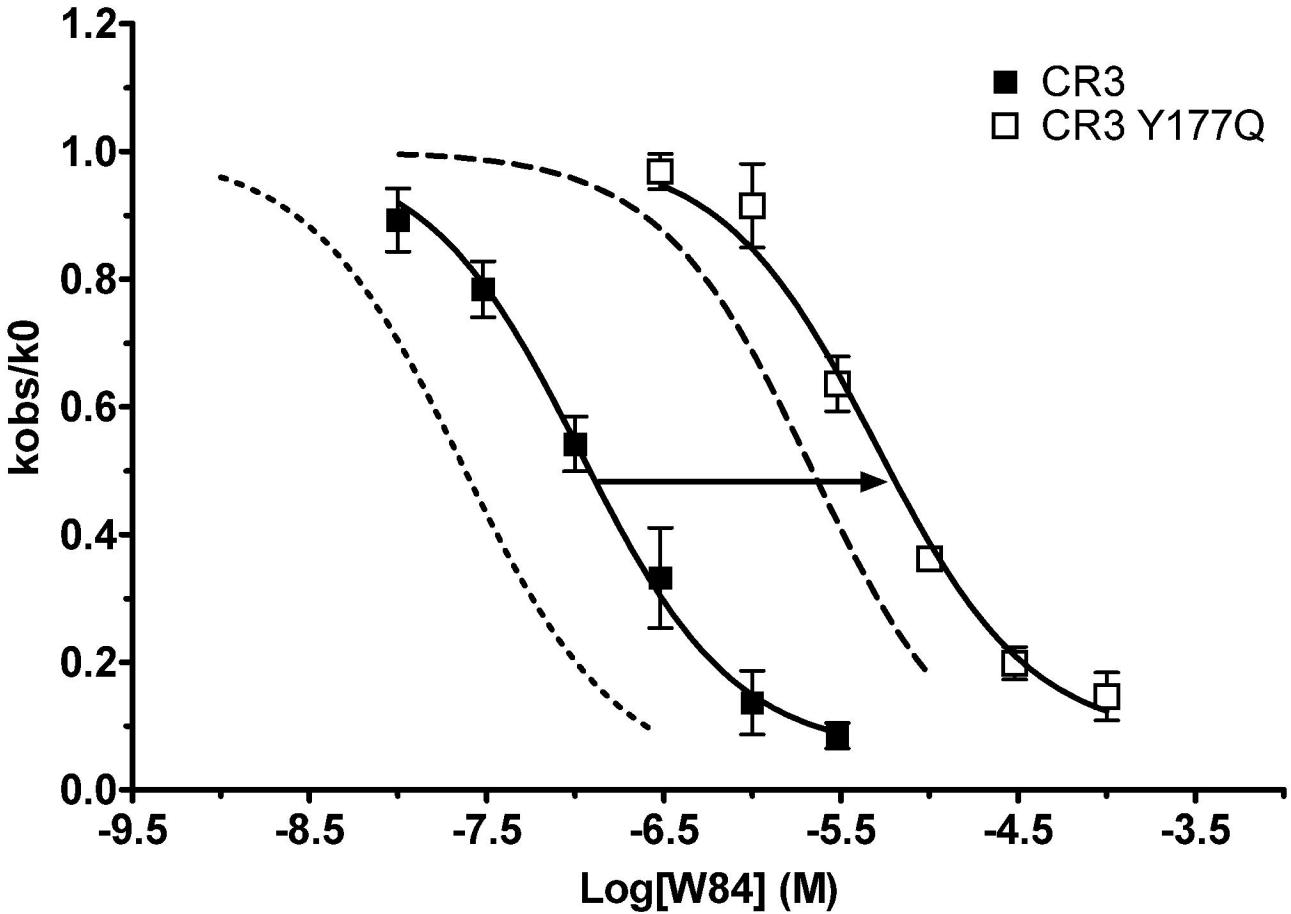

1.2
1.0
0.8
0.6
0.4
0.2
0.0
kobs/k0
-9.5
-8.5
-7.5
-6.5
-5.5
-4.5
-3.5
Log[W84] (M)
CR3
CR3 Y177Q

Figure 8A

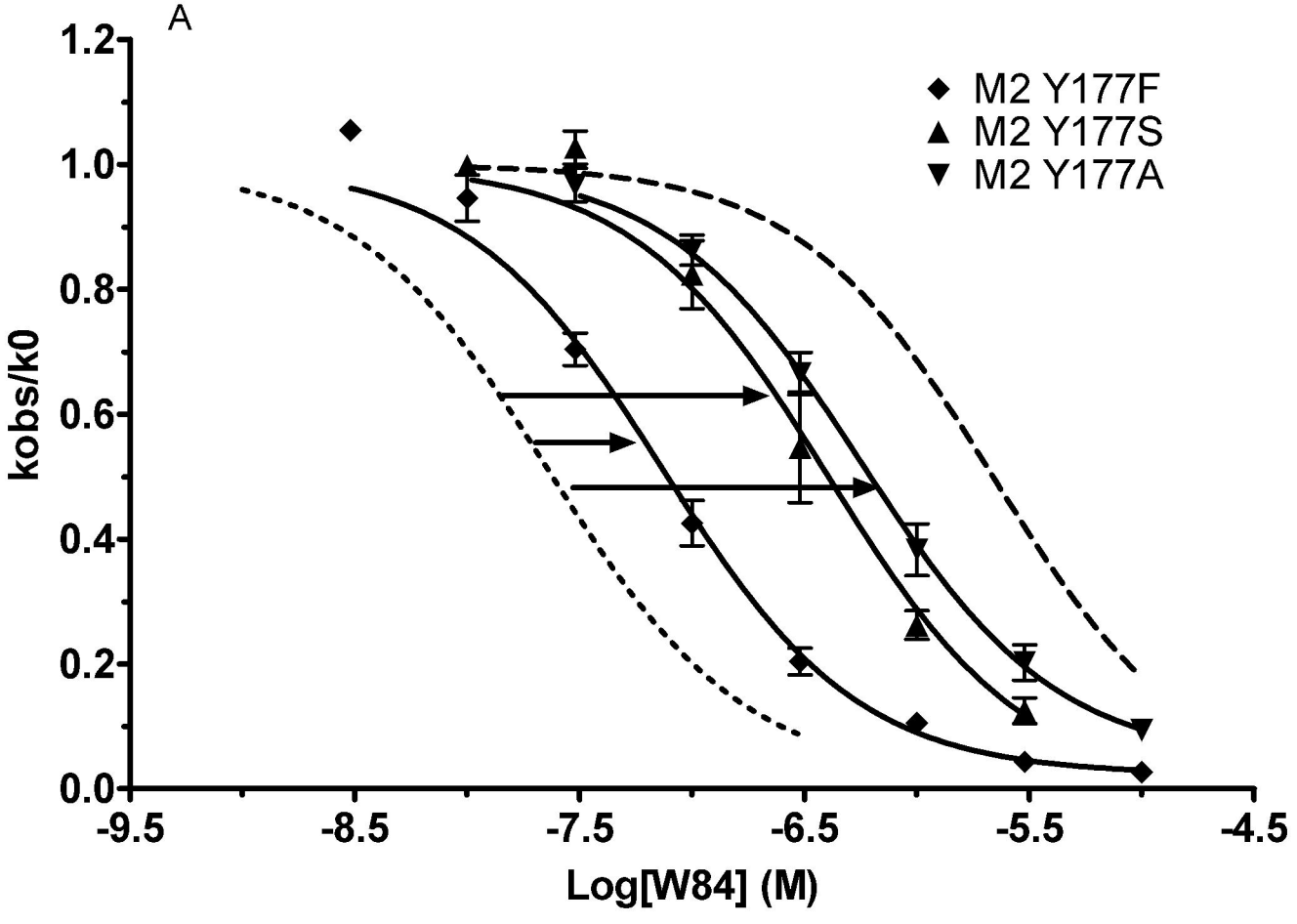

A
M2 Y177F
M2 Y177S
M2 Y177A
kobs/k0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9.5
-8.5
-7.5
-6.5
-5.5
-4.5
Log[W84] (M)

Figure 8B

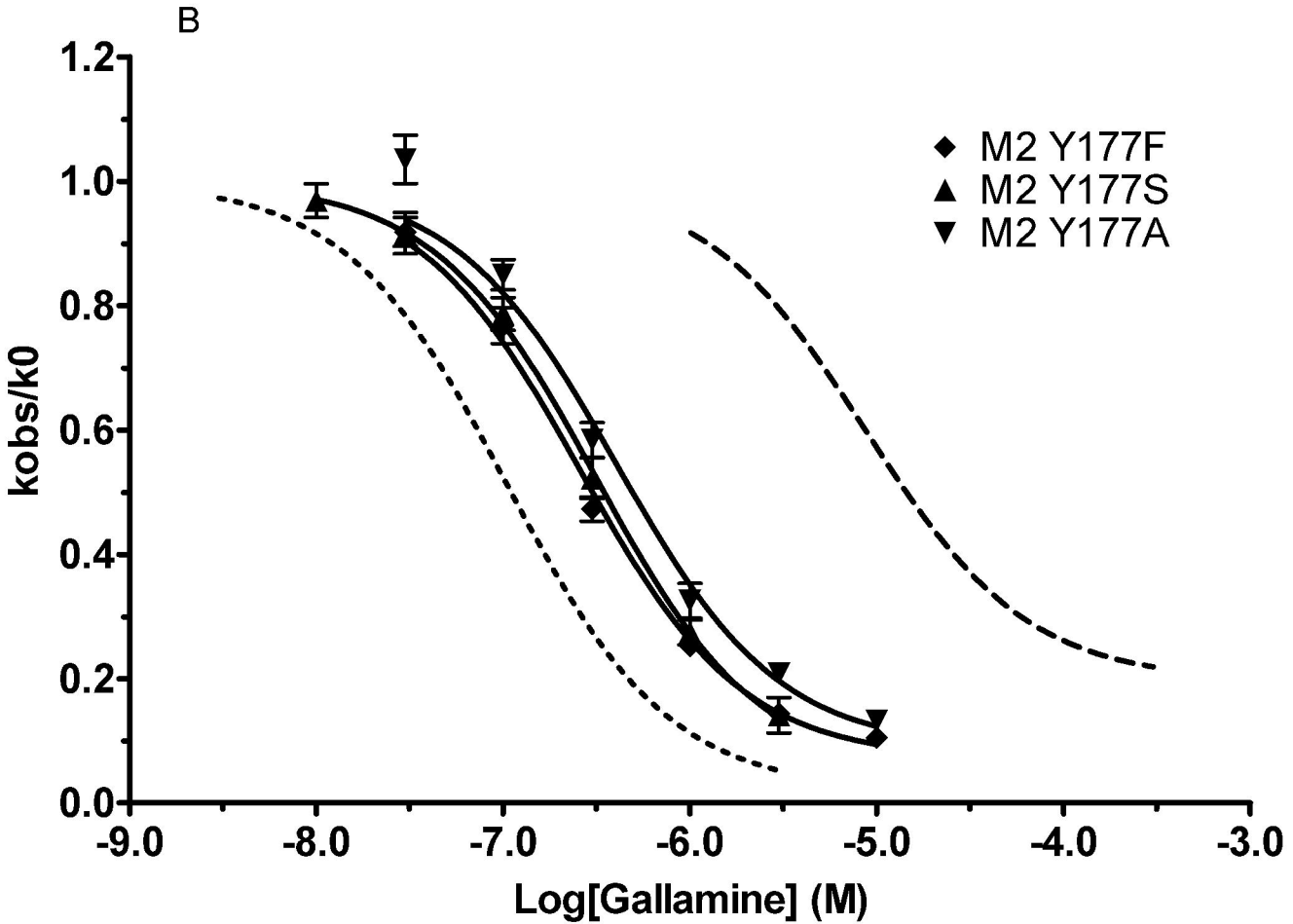

B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
kobs/k0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)
M2 Y177F
M2 Y177S
M2 Y177A

Figure 9A

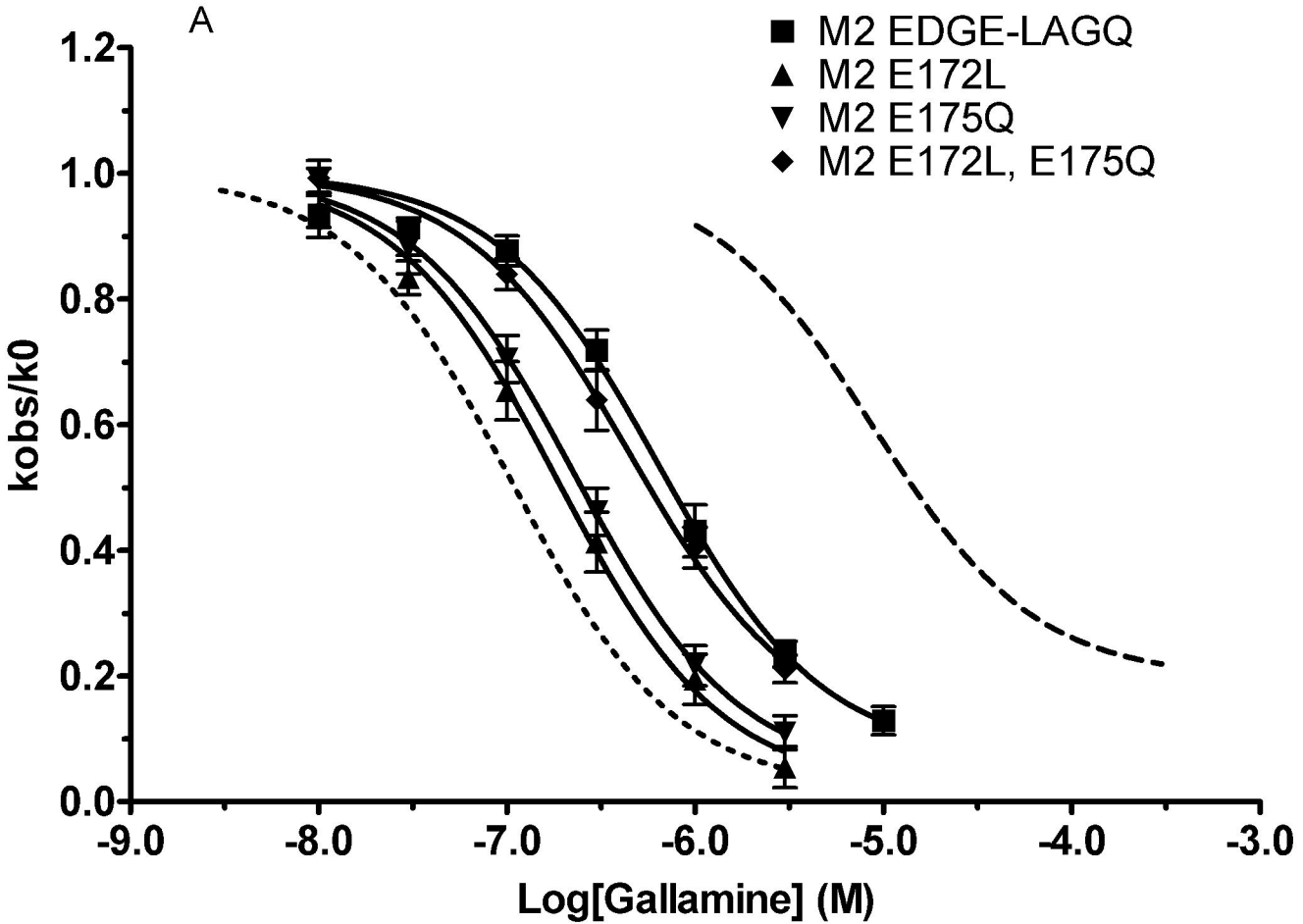

A
M2 EDGE-LAGQ
M2 E172L
M2 E175Q
M2 E172L, E175Q
kobs/k0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)

Figure 9B

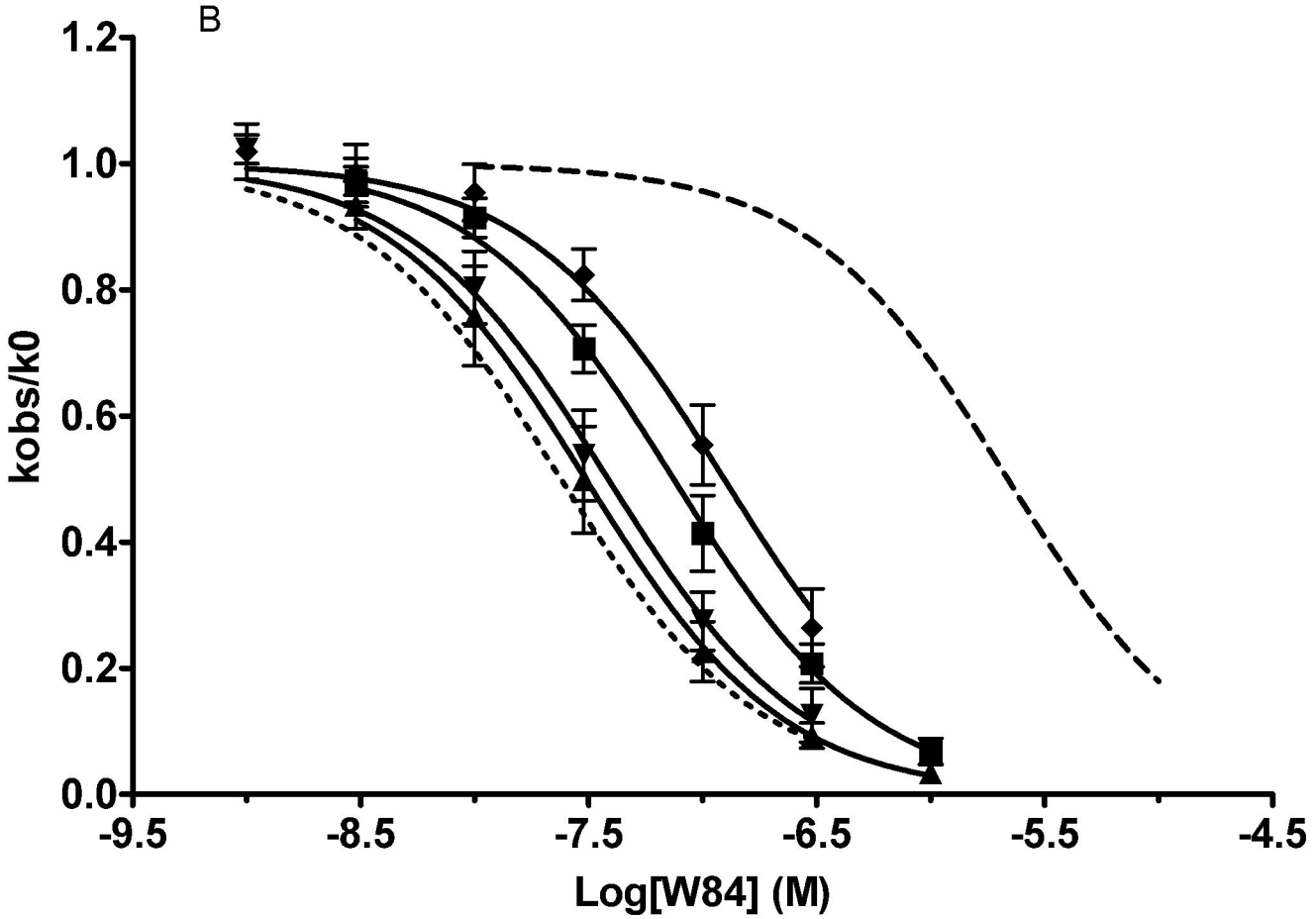


Figure 10

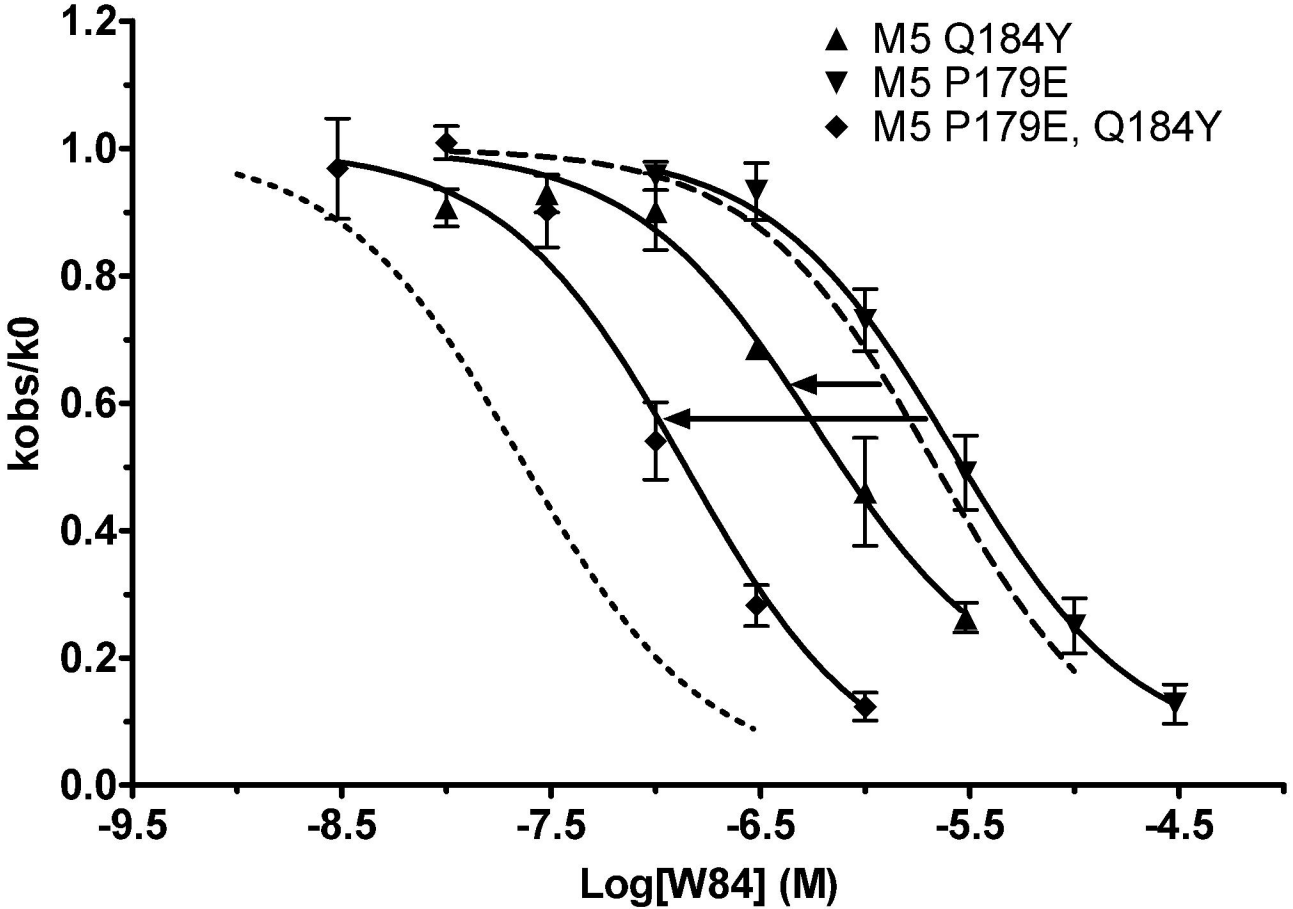

M5 Q184Y
M5 P179E
M5 P179E, Q184Y
kobs/k0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9.5
-8.5
-7.5
-6.5
-5.5
-4.5
Log[W84] (M)

Figure 11

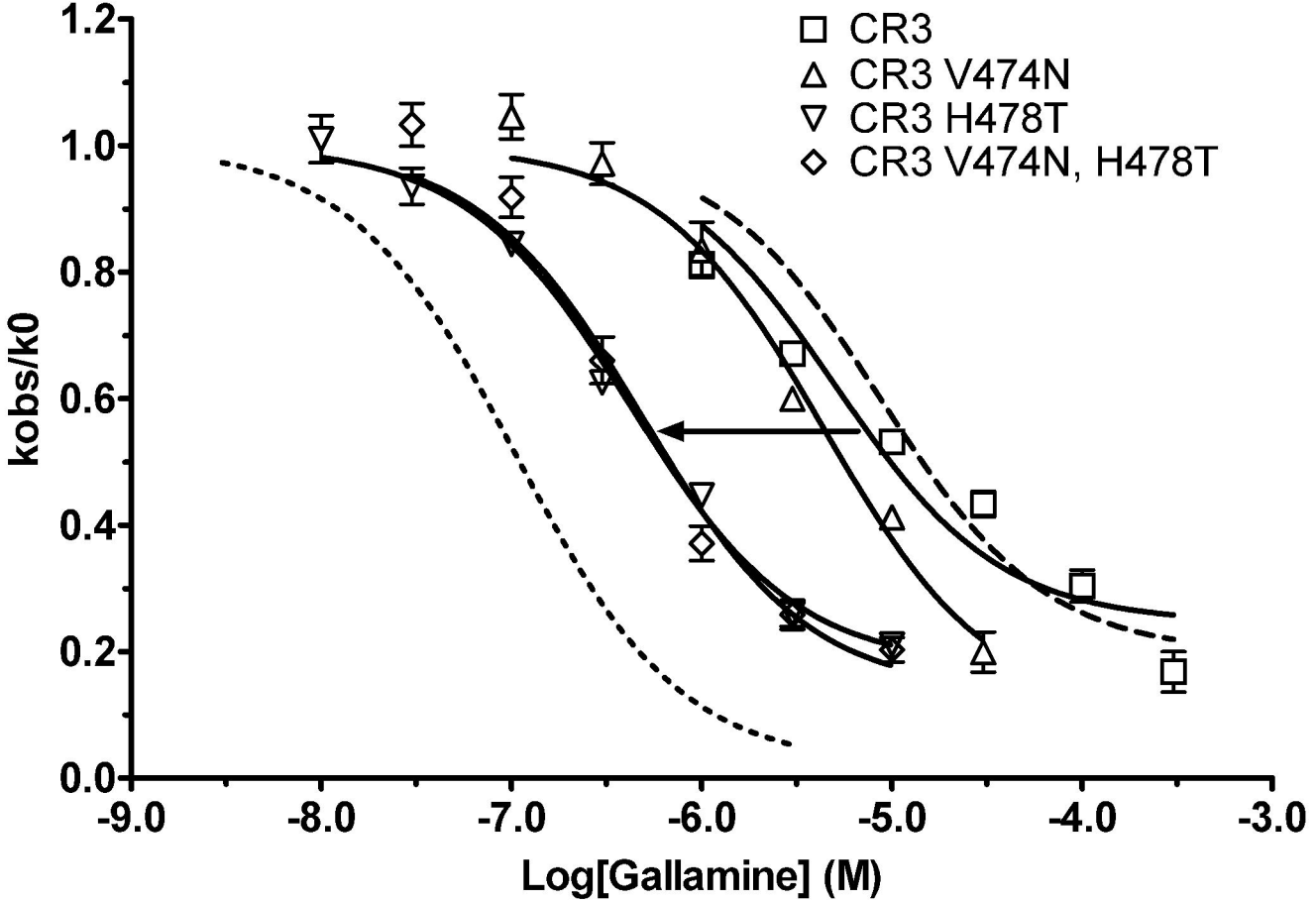

CR3
CR3 V474N
CR3 H478T
CR3 V474N, H478T
kobs/k0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)

Figure 12A

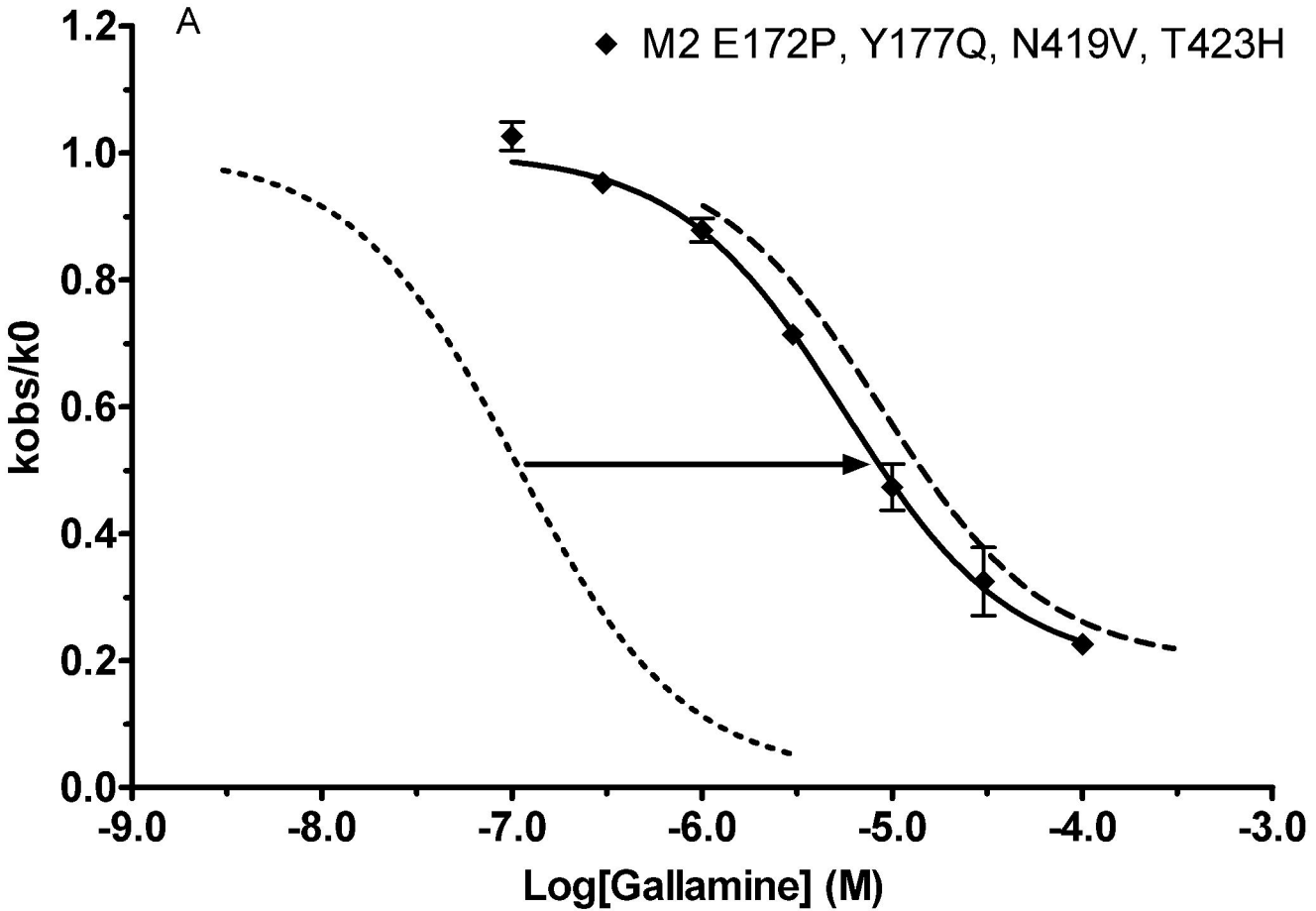

A
M2 E172P, Y177Q, N419V, T423H
kobs/k0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)

Figure 12B

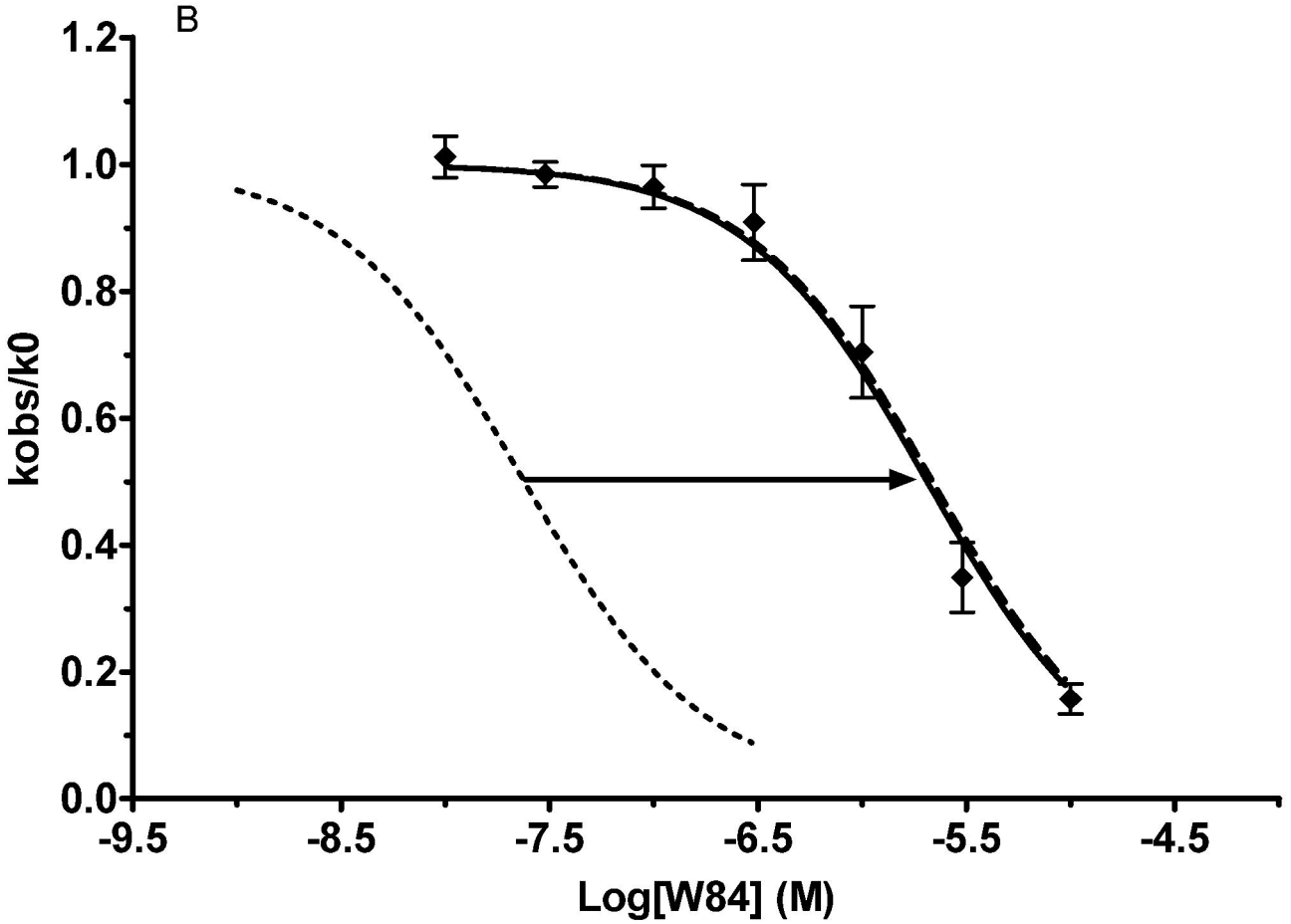


Figure 13A

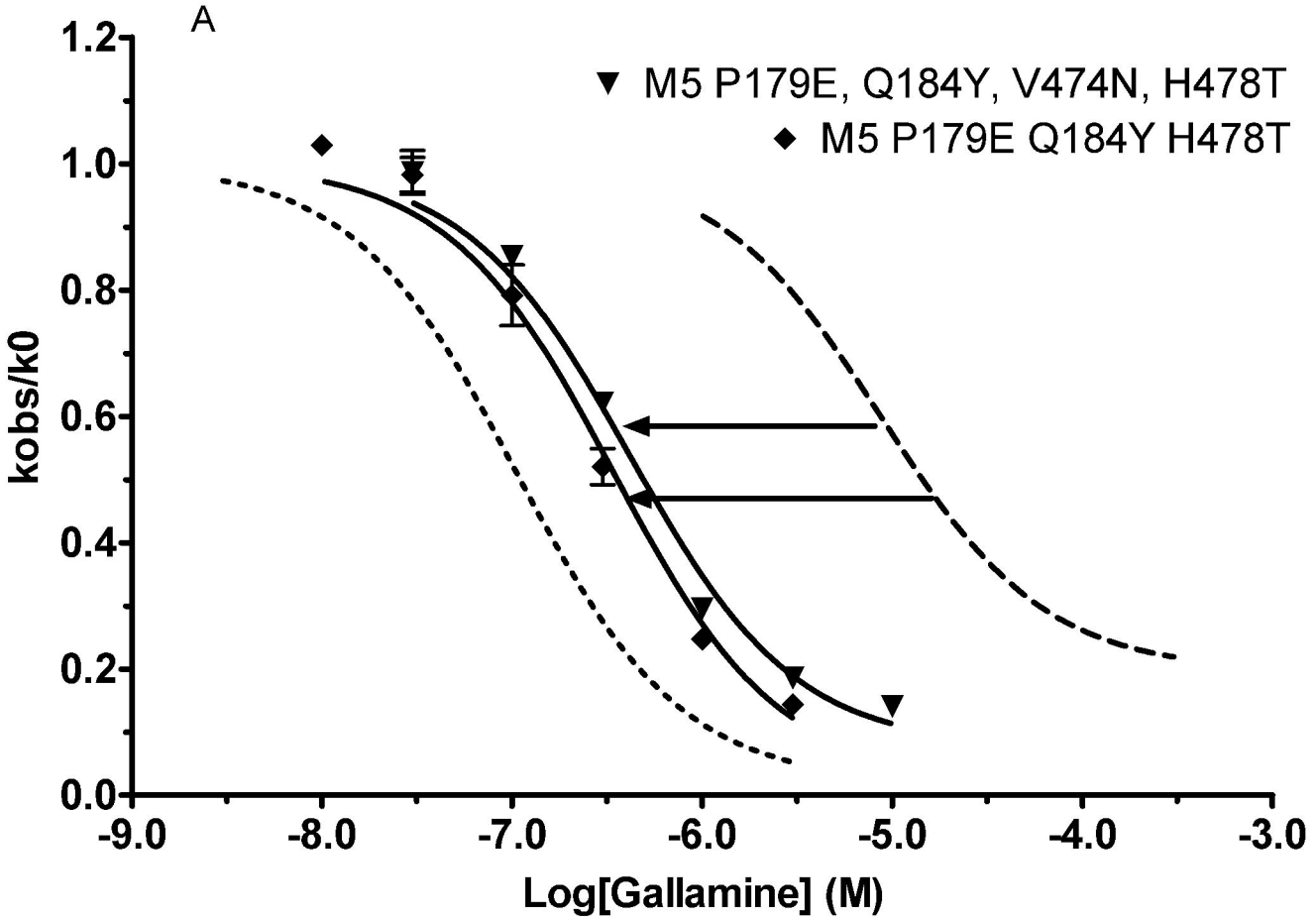

A
M5 P179E, Q184Y, V474N, H478T
M5 P179E Q184Y H478T
kobs/k0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9.0
-8.0
-7.0
-6.0
-5.0
-4.0
-3.0
Log[Gallamine] (M)

Figure 13B