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Competitive and Allosteric Interactions of 6-Chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11*H*-dibenzo[*b*,*e*][1, 4]diazepine-11-one hydrochloride (UH-AH 37) at Muscarinic Receptors, via Distinct Epitopes

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ABSTRACT. 6-Chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11*H*-dibenzo[*b*,*e*][1,4]diazepine-11-one hydrochloride (UH-AH 37) is an analog of pirenzepine that has previously been reported to interact with classical muscarinic antagonists in a competitive manner, yet its binding has also been found to be sensitive to the same epitope as is that of the allosteric ligand gallamine. The present study was carried out with wild-type and chimeric muscarinic receptors to determine whether UH-AH 37 might also have an allosteric mode of action. In assays that detect only allosteric interactions, UH-AH 37 slowed the rate of dissociation of [^{3}H]*N*-methylscopolamine (NMS) from all five muscarinic receptor subtypes, with the highest apparent affinity at m2. By contrast, studies carried out under equilibrium conditions have found UH-AH 37 to have the lowest affinity for the m2 subtype. Studies with m2/m5 chimeric receptors found the allosteric potency of UH-AH 37 to be sensitive to an epitope in the seventh transmembrane domain (TM). Again, this contrasts with equilibrium studies, wherein an epitope in the sixth TM has been implicated. Simultaneous analysis of the interactions between UH-AH 37 and [^{3}H]NMS at the m2 receptor under equilibrium and non-equilibrium conditions found that a simple allosteric model could not accommodate both sets of data. On the other hand, the model did accommodate such data for gallamine; gallamine also displays concordance in order-of-potency and epitope sensitivity between equilibrium and non-equilibrium assays. Based on these results, we conclude that UH-AH 37 interacts at the classical muscarinic binding site with high affinity and at a second (allosteric) site with lower affinity. BIOCHEM PHARMACOL **57**;2:181–186, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. muscarinic receptors; UH-AH 37; gallamine; allosteric site; chimeric proteins; epitopes

Acetylcholine interacts with five subtypes of muscarinic receptors. Many agonists and antagonists compete for the acetylcholine binding site, but none with great selectivity [1]. Another group of ligands has been found to interact allosterically with acetylcholine, and it has been suggested that the allosteric site may allow for greater and more diverse selectivities, since it lies somewhat distant from the highly conserved site at which the endogenous ligand binds [2–4]. However, this has so far not proven to be true. Although many allosteric ligands do express good selectivity, it is an m2-selectivity in almost every case (see Discussion).

Studies of the residues and/or epitopes involved in the binding and selectivity of muscarinic allosteric ligands have been carried out, usually with the focus on gallamine. One region of the receptor structure has been implicated in the selectivity of gallamine for the m2 receptor over the m5 and m3 subtypes. This region consists of the sixth TM† and the third outer loop of the receptor [5]. The same region has been implicated in the subtype-selectivity of the pirenzepine analog, 6-chloro-5,10-dihydro-5-[(1-methyl-4-piperidinyl)acetyl]-11*H*-dibenzo[*b*,*e*][1,4]diazepine-11-one hydrochloride (UH-AH 37), which expresses similar high affinity for every subtype except m2 [6]. Although previous studies have found UH-AH 37 to interact competitively with muscarinic receptors [7], we felt it would be worthwhile to re-investigate this ligand, in view of the lack of allosteric ligands that are selective for subtypes other than m2. We have found that UH-AH 37 does, in fact, exert allosteric effects at all five muscarinic receptor subtypes. However, further investigation of these allosteric interactions revealed that UH-AH 37 appears to interact with two different sites on the receptor, thereby affecting the binding

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†*Abbreviations:* NMS, *N*-methylscopolamine; PB, sodium-potassium phosphate buffer, pH 7.4; and TM, transmembrane domain.

of the classical antagonist [^{3}H]NMS both competitively and allosterically.

MATERIALS AND METHODS

Materials

Atropine sulfate and gallamine triethiodide were obtained from Sigma, and UH-AH 37 was a gift of Karl Thomae. Labeled *N*-methylscopolamine chloride ([^{3}H]NMS; 84.5 Ci/mmol) was from NEN DuPont.

Receptors

Wild-type receptors. Except when compared directly with chimeric receptors, human wild-type receptors were stably expressed in CHO cells. Cells were grown to 80% confluency, and membranes were prepared as described below for chimeric receptors.

Chimeric receptors. The construction and verification of the m2/m5 chimeric muscarinic receptor genes used in this study have been described previously [6]. Plasmids containing the various gene constructs were transfected into COS-7 cells by calcium phosphate precipitation. Cells were harvested 72 hr after transfection by scraping into 5 mM PB (pH 7.4), homogenizing, and collecting the membranes at 50,000 *g* for 20 min. The membranes were resuspended in 5 mM PB and stored as aliquots at −70°.

Binding Assays

Dissociation assays. Binding assays were conducted in 5 mM PB at 25°. Membranes (approx. 30 μg of protein in 1 mL) were prelabeled with 1 nM [^{3}H]NMS for 30 min. Dissociation of the labeled ligand was initiated by the addition of 3 μM atropine, with or without modulator, and the incubation was allowed to continue for the appropriate length of time. The incubation was terminated by filtration through S&S No. 32 glass fiber filters (Schleicher & Schuell), followed by two rinses with 40 mM PB (0°). Nonspecific binding was determined by the inclusion of 3 μM atropine during the prelabeling period.

The data from the dissociation assays that included gallamine or UH-AH 37 were treated in the following manner. The apparent rate constant for the dissociation of [^{3}H]NMS was determined in the presence of each concentration of modulator (k_{obs}) and divided by the true rate constant (k_0), determined in the presence of 3 μM atropine only; thus, the resulting number indicates a dissociation of [^{3}H]NMS slower than the control rate when it is less than one. The concentrations of modulators that are used in these studies are expected to lead to rapid equilibration with the allosteric site. Under these conditions, the concentration-dependent effects of an allosteric ligand on the dissociation of [^{3}H]NMS should be proportional to the occupancy of the allosteric site, as previous studies have confirmed [2]. Therefore, data from these experiments were fitted to the following equation:

$$\frac{k_{obs}}{k_0} = 1 - \frac{mA}{A + K_{app}} \quad (1)$$

where A is the concentration of the allosteric modulator, m is the maximal reduction in the rate constant that can be exerted by the modulator, and K_{app} is the apparent equilibrium dissociation constant (for the interaction between the allosteric ligand and the *NMS-bound* form of the receptor). The rate constants for the dissociation of [^{3}H]NMS from the receptors in the absence of allosteric ligands (k_0) correspond to half-times that range from less than 5 min (m2) to more than 1 hr (m5); k_0 values for the wild-type and chimeric receptors have been reported previously [5, 8].

Equilibrium assays. Membranes (approx. 30 μg protein) were incubated with [^{3}H]NMS and the indicated concentrations of UH-AH 37 or gallamine at 25° in 5 mM PB for 3 hr. Over the range of concentrations used in these studies, the binding of [^{3}H]NMS had reached equilibrium under these conditions. The incubation was terminated by filtration, which was conducted as described above for dissociation assays. Data from these assays were expressed as the fractional inhibition of specific [^{3}H]NMS binding and then fitted to the following equation of allosteric inhibition (adapted from [9]):

$$Inh = \frac{A - \frac{A}{\alpha}}{K_A + A + \frac{X}{K_X}\left(K_A + \frac{A}{\alpha}\right)} \quad (2)$$

where A is as defined above, X is the concentration of [^{3}H]NMS, K_A and K_X are the equilibrium dissociation constants for the interactions of the allosteric ligand and [^{3}H]NMS, respectively, with the free receptor, and α is the degree of cooperativity between the allosteric ligand and [^{3}H]NMS ($\alpha > 1$ denotes negative cooperativity). The affinity of [^{3}H]NMS (K_X) for the m2 receptor was found to be 0.1 nM, and was fixed at that value. Where indicated, some equilibrium and dissociation data were fitted simultaneously, with and without the constraint that $K_{app} = \alpha K_A$. Curve-fitting was carried out with the Scientist (MicroMath) and MLAB (Civilized Software) programs.

RESULTS

UH-AH 37 (Fig. 1) was able to slow the dissociation of [^{3}H]NMS from all five subtypes of muscarinic receptors, with the order of potency m2 ≈ m4 > m1 > m3 > m5 (Fig. 2). This differs from the order of potency that we (data not shown) and others [7] have found when UH-AH 37 inhibits the binding of [^{3}H]NMS under equilibrium condi-

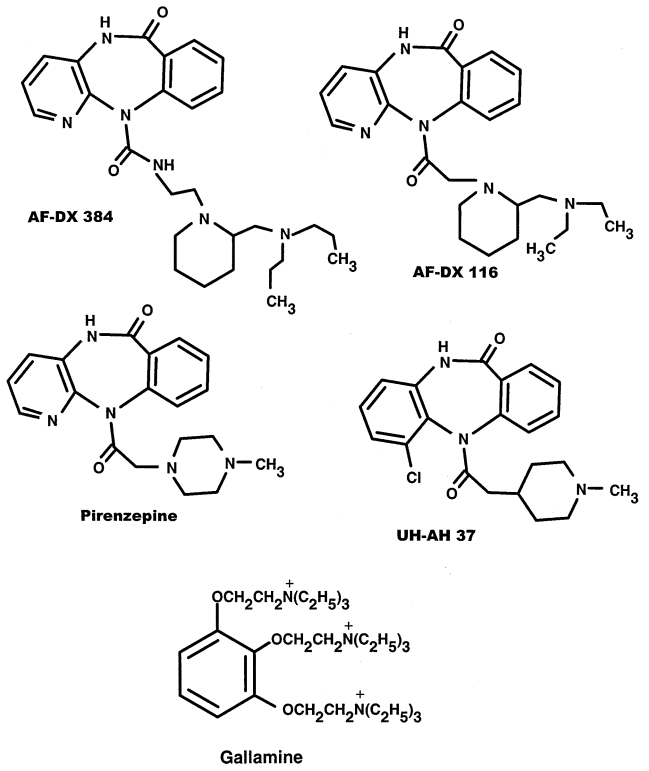

FIG. 1. Structures of AF-DX 384, AF-DX 116, pirenzepine, UH-AH 37, and gallamine.

tions, wherein the m2 subtype displays the lowest affinity. Different orders of potency in these two assays are not incompatible with an allosteric mechanism. However, if both sets of data are due to a single allosteric effect (at each subtype), one would expect the interaction between UH-AH 37 and [^{3}H]NMS to be the least negatively cooperative at the m2 subtype (see Discussion). To examine this issue further, and to specifically test the allosteric model at the m2 subtype, we simultaneously analyzed data from equilibrium and dissociation experiments utilizing UH-AH 37; for comparison, we applied the same analysis to the interaction between gallamine and the m2 receptor. When the two sets of data for UH-AH 37 were fitted with the constraint that the apparent affinity of UH-AH 37 in the dissociation assay, K_{app}, must be equal to the product of α and K_A in the equilibrium experiments, the best fit could not accommodate the points of highest inhibition in the equilibrium experiments (Fig. 3A). Under this constraint, the best fit parameters were: K_A = 15 nM, α = 732, m = 1.0. When the parameters were not so constrained (Fig. 3B), the fit was significantly better ($P < 0.0001$), and the best-fit parameters were: K_A = 21 nM, $\alpha = 1.4 \times 10^{23}$, m = 0.89, K_{app} = 6.9 μM. It can be readily seen that the difference between the two models is that the equilibrium data are better fit by a very large α. To set a lower limit on α, we mapped the parameter to find the value at which the sum of squares from the equilibrium data would be significantly ($P < 0.05$) increased (the other parameters were allowed to vary while α was kept constant); by this criterion, α must be greater than 2000. A similar approach to the simultaneous fit of the equilibrium and dissociation data (i.e. Fig. 3A) found the 95% confidence limit for α to be below 1000.

In contrast to UH-AH 37, when fits of gallamine data were carried out, the constrained model gave an adequate fit to the combined data (Fig. 3C). In this case the best-fit parameters were: K_A = 5.1 nM, α = 136, m = 0.94. The parameters for the best fit without the constraint were: K_A = 5.6 nM, α = 142, m = 0.93, K_{app} = 0.66 μM; these values did not result in a statistically better fit ($P > 0.25$).

The ability of UH-AH 37 to slow the dissociation of [^{3}H]NMS was also examined in chimeric receptors composed of segments of m2 or m5 sequences (Fig. 4). Only one chimeric receptor, CR6, exhibited significantly greater apparent affinity toward UH-AH 37 than did m5 or the other chimeric receptors. As indicated in the schematic receptors shown in Fig. 4, CR6 is composed of an m2 sequence from the amino terminal to the middle of TM2 and from the beginning of TM6 to the carboxyl terminal; elsewhere, it is an m5 sequence. The chimeric receptors CR1 and CR5 both include the amino terminal portion of the m2 sequence that is present in CR6, but do not exhibit significantly greater apparent affinity toward UH-AH 37 than does the m5 subtype. Likewise, the CR4 construct includes a portion of the m2 sequence present in CR6, without significant gain in affinity.

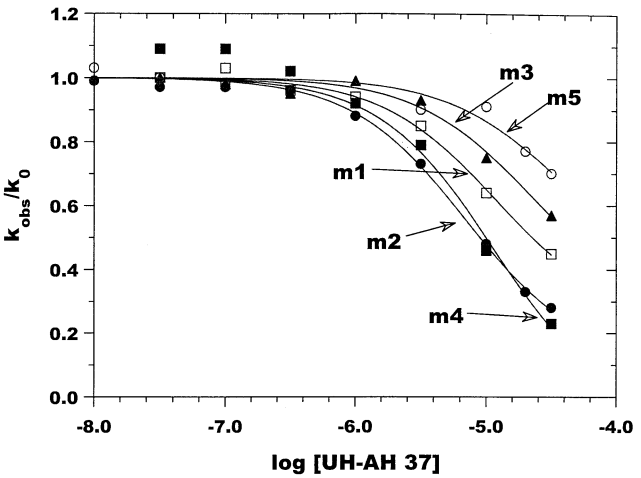

FIG. 2. Allosteric effects of UH-AH 37 on m1–m5 muscarinic receptor subtypes. Membranes derived from CHO cells containing the indicated muscarinic receptor subtype were prelabeled with [^{3}H]NMS. The labeled ligand was then allowed to dissociate in the presence or absence of the indicated concentrations of UH-AH 37. Results shown are the means of two experiments. The data points are the ratios of the dissociation rate constants obtained in the presence (k_{obs}) and absence (k_o) of UH-AH 37, and the curves represent the best fit to the model described in Materials and Methods. The best-fit parameters are given as the apparent affinities, K_{app}(μM), and the maximal effect on the rate constant, m [in brackets]: m1, 11.5 [0.76]; m2, 7.1 [0.88]; m3, 21.4 [0.73]; m4, 9.8 [1.0]; and m5, 40 [0.66].

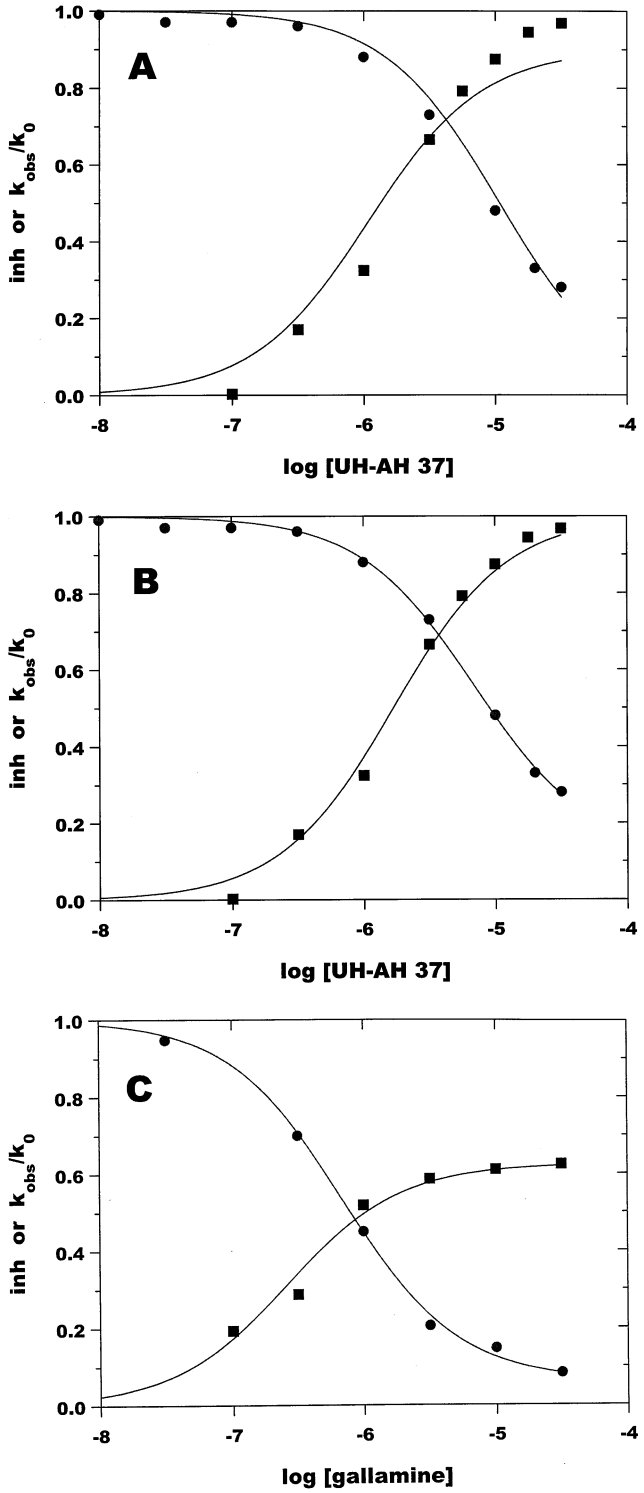

FIG. 3. Effects of UH-AH 37 and gallamine on the equilibrium binding and on the rate of dissociation of [^{3}H]NMS at m2 muscarinic receptors. In equilibrium experiments (■), CHO membranes containing m2 receptors were incubated for 3 hr with a high concentration (8 nM) of [^{3}H]NMS, with or without the indicated concentrations of inhibitors, and then filtered as described in Materials and Methods. These data are presented as the fractional inhibition of the binding of [^{3}H]NMS. Dissociation experiments (●) were conducted as in Fig. 2. (A) Data for UH-AH 37 (for one experiment, representative of 2) were fitted to a single model with shared parameters. The apparent affinity of UH-AH 37 observed in the dissociation assay (K_{app}) was constrained to be equal to the product of its affinity for the free receptor (K_A) and its cooperativity factor toward NMS (α). The curves represent the best fit to the shared model (see Materials and Methods for details of models used and text for parameters obtained). (B) The same data as in A, fitted to an unconstrained model, in which K_{app} is independent of K_A and α. This fit is significantly better than the constrained model in A [F (1,13) = 58, P<0.0001]. (C) Data were obtained and analyzed for gallamine as described in A, above. The best-fit shown is for the constrained model; in this case, the unconstrained model did not provide a significantly better fit [F (1,8) = 0.928, P > 0.25].

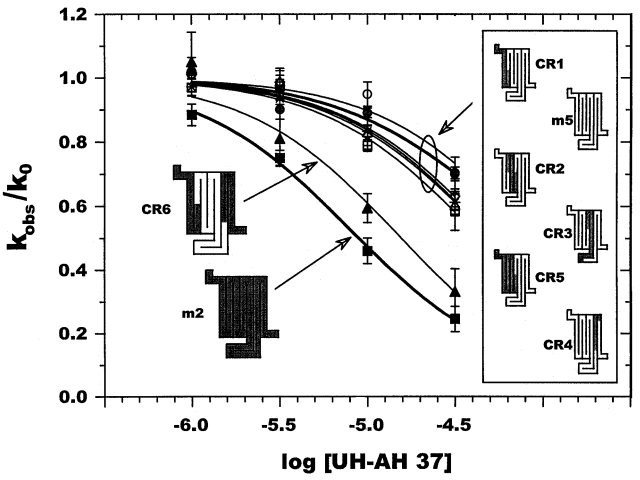

FIG. 4. Effects of UH-AH 37 on the dissociation of [^{3}H]NMS from m2/m5 chimeric muscarinic receptors. Experiments were performed as described in Fig. 2, using COS-7 cell membranes that contained m2 receptors, m5 receptors, or chimeric receptors. In the schematic representations of the receptors, the shaded regions are m2-sequence and the unshaded regions are m5-sequence (see exact sequences below). In the box on the right, the schematic representations are shown in the same order as their respective curves in the oval; that is, CR1 is the top curve and CR4 is the bottom curve. The curves for the wild-type receptors are drawn with thicker lines than are those for the chimeric receptors. The points shown are the means ± SEM of three experiments. The sequences of the chimeric receptors follow. CR1: m2 1–69, m5 77–532; CR2: m5 1–76, m2 70–155, m5 163–532; CR3: m5 1–162, m2 156–300, m5 336–532; CR4: m5 1–445, m2 391–421, m5 477–532; CR5: m2 1–155, m5 163–532; CR6: m2 1–69, m5 77–445, m2 391–466.

DISCUSSION

One of the rationales for the investigation of muscarinic allosteric ligands is that allosteric ligands may exhibit greater selectivity for the various muscarinic receptor subtypes. This possibility is based on the reasoning that ligands that are competitive with acetylcholine are likely to bind to epitopes close to the acetylcholine-binding core of the receptor; allosteric ligands must bind elsewhere and more distal epitopes seem less likely to be highly conserved among the subtypes. However, while some allosteric ligands have been identified that are in fact quite selective, so far this selectivity has nearly always been for the m2 subtype [2]. The only dramatic exceptions have been snake venom

toxins [10–13]; other than these peptides, the universal pattern has been that selective allosteric ligands have highest affinity for the m2 subtype and lowest affinity for the m5 subtype. Thus, it was very interesting that the subtype-selectivity of UH-AH 37 (m5 > m2) was sensitive to an epitope in the same very small region of the receptor that affected the allosteric activity of gallamine [5, 6]. Although previous studies have indicated that UH-AH 37 interacts competitively with acetylcholine and classical muscarinic antagonists [7], it can be very difficult to differentiate between allosteric and competitive interactions when the degree of negative cooperativity is very great [9]. We found that, in fact, UH-AH 37 did inhibit the rate of dissociation from all five muscarinic subtypes (Fig. 2). However, the order of potency across the subtypes was very different for this definitively allosteric effect (m2 ≈ m4 > m1 > m3 > m5, Fig. 2) than has been observed under equilibrium conditions, wherein the m2 subtype has the lowest affinity. The striking discrepancy in the orders of potencies between the two assays suggests that different mechanisms might be at work, but it is not conclusive; the difference could be due to different cooperativity factors, which play a major role in the apparent affinities determined in dissociation assays [2] but have only marginal effects in equilibrium assays under typical conditions. Nonetheless, such dramatically different potency orders (i.e. the reversal of m2 from least potent to most potent) are very unusual. For example, gallamine and alcuronium both have much higher affinity at the m2 subtype than at m5, in both dissociation and equilibrium assays (Ellis, unpublished data; [14]). Lee and El–Fakahany found the same orders of potency in the two types of assays for gallamine and a number of other allosteric ligands at the m1, m2, and m3 subtypes [15]; even among low-selectivity agents, in no case did one subtype display highest affinity in one assay and lowest in the other.

To determine a cooperativity factor for the interaction between UH-AH 37 and NMS at the m2 subtype, we carried out equilibrium binding assays at very high concentrations (approximately 100 × K_D) of [^{3}H]NMS. Under these conditions, an allosteric ligand with a cooperativity factor (α) of 100 versus NMS will only be able to inhibit the binding of the labeled ligand by about 50% at saturating concentrations (see gallamine, Fig. 3C). On the other hand, an allosteric ligand with much greater negative cooperativity will still be able to inhibit virtually all of the binding of the labeled ligand, precisely as would a competitive inhibitor. To further differentiate these possibilities, and as a test of the simple allosteric model, we also compared equilibrium assays at high [^{3}H]NMS concentrations with dissociation assays (which are independent of the concentration of [^{3}H]NMS). Given the reasonable assumption that the allosteric ligand is in rapid equilibrium in the dissociation assay, the apparent affinity of the allosteric ligand in that assay will equal αK_A [2]. It is apparent in Fig. 3A that there is a systematic discrepancy between the data for UH-AH 37 and the simple allosteric model. The problem is that in order for the midpoints of the curves to be reasonably well-fit, α must be about 800, which leads to a plateau in the inhibition curve at about 90%. For the inhibition curve to climb to 100%, as it appears to, α must approach infinity. One way to rationalize these data are that there are two separate processes at work, one that is very highly negatively cooperative (i.e. competitive) that predominates in the equilibrium assay and another that is allosteric and modulates the dissociation of [^{3}H]NMS. The analysis in Fig. 3B is based on this possibility and gives a fit that is better to a highly significant extent ($P < 0.0001$; see analyses in Results and figure legends).

The analysis of combined equilibrium and dissociation assays for gallamine indicated that a single allosteric mechanism was consistent with the data. This is in agreement with a previous study from our laboratory that employed a similar approach, but relied on a more complex data set [2]. We prefer the method used in the present study because the goodness of fit can be readily appreciated from the graphical display as well as from a statistical analysis. It also requires fewer data points.

Previous studies [6] have found that the equilibrium binding of UH-AH 37 is uniquely sensitive to an epitope present in the region of the receptor that is highlighted in CR4 (Fig. 4). This region is also present in the m2 sequence of CR6. We found that the allosteric potency of UH-AH 37 was sensitive to an epitope present in CR6, but not in any of the other chimeric receptors. This epitope cannot be within the N-terminal region of the receptor, because CR1 contains that sequence. Likewise, it is not within the TM6-o3 region, because CR4 contains that sequence. So, it would be either in TM7 or the carboxyl tail of the receptor. In keeping with data that suggest that allosteric ligands bind to the outermost regions of muscarinic receptors [2, 4], we expect it to be near the extracellular end of TM7. It remains possible that there may be interactions between residues within the receptor that modulate a more general structural change, which in turn affects ligand affinity, as has been found, for example, between TM2 and TM7 of the gonadotropin-releasing hormone receptor [16]. However, the most important molecular aspect of this study is the finding that the epitopes implicated in equilibrium assays and dissociation assays are different. At the present time, we believe that the simplest scenario that explains all of our data is that UH-AH 37 interacts with one epitope within the CR4 region that contributes to a high-affinity competitive interaction versus NMS, and with a different epitope, probably near the top of TM7, that contributes to its lower-affinity allosteric interaction with NMS. Subsequent studies of receptor constructs with point mutations in these regions are expected to shed further light on the mechanisms of the interactions.

It is somewhat ironic that gallamine was suspected of acting both competitively and allosterically a decade ago. More recent studies have made gallamine one of the few ligands that has considerable evidence for a purely allosteric mode of action, at least at the m2 subtype (this study; [2,

17]). However, studies of the other muscarinic ligands in Fig. 1 have been more equivocal. Pirenzepine is commonly considered to be the prototype m1-selective competitive antagonist, although its selectivity is not as great as could be desired [1]. Furthermore, Kenakin and Boselli [18] have used resultant analysis to demonstrate that pirenzepine acts allosterically. This analysis does not rule out the possibility that pirenzepine might interact with two sites on the receptor, as its analog, UH-AH 37, appears to in the present study. AF-DX 116 is well-known to allosterically modulate the dissociation of NMS from muscarinic receptors [15], but in equilibrium studies has been found to bind preferentially to the classical muscarinic binding site [17]; this author suggested that AF-DX 116 had much weaker affinity for the accessory (allosteric) site, just as we suggest for UH-AH 37.

It has been suggested recently that AF-DX 384 binds to both the classical muscarinic site and to the allosteric site simultaneously, based on extensive studies of the interactions among labeled and unlabeled AF-DX 384, the allosteric ligand W84, and classical muscarinic ligands [19]. This suggestion nicely explains the dramatically greater negative cooperativity found between W84 and [^{3}H]AF-DX 384, compared with W84 and [^{3}H]NMS. These authors also speculate that many m2 selective ligands may derive their preferential affinities in this manner and, indeed, many m2 selective ligands do display allosteric activity. However, it is difficult to distinguish between the case wherein a ligand binds at two overlapping sites from that in which it binds at two distinct sites. We prefer the model of two distinct sites for UH-AH 37 for now, because of its reverse selectivities in equilibrium and dissociation assays. It is hard to argue that the allosteric attachment site confers higher affinity toward m5 when the dissociation assays indicate highest apparent affinity for m2. Also, the chimeric studies indicate that different epitopes can modulate these affinities independently. However, we recognize that both of our arguments are complicated by our inability to determine the necessary cooperativity factors (because we have demonstrated that the simple allosteric model does not apply). Future studies with more discrete mutations may be useful in deciding between these alternatives, for both ligands.

In summary, we have compared the binding properties of UH-AH 37 in equilibrium and non-equilibrium assays from three points of view: subtype selectivity, simultaneous modeling, and epitope sensitivity. All three of these viewpoints suggest that UH-AH 37 binds to the classical muscarinic binding site with high affinity and to a second (allosteric) site with lower affinity. These findings may be relevant to understanding the complex interactions of structurally related ligands with muscarinic receptors.

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