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Use of Chimeric Muscarinic Receptors to Investigate Epitopes Involved in Allosteric Interactions

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SUMMARY

All five (m1-m5) muscarinic receptors are sensitive to allosteric regulation, but gallamine is considerably more potent in slowing the dissociation of *N*-[^{3}H]methylscopolamine (NMS) from the m2 subtype than from the m3 or m5 subtypes. To study the structural basis for the preference of gallamine for the m2 subtype, we evaluated [^{3}H]NMS-gallamine interactions with chimeric receptors in which segments of the m5 receptor were systematically replaced with the corresponding m2 sequence. Substitutions that included the sixth transmembrane domain and third extracellular loop resulted in marked increases in the potency of gallamine, but substitutions that did not include these regions were without effect. A similar substitution was investigated using m2/m3 chimeric receptors, in which a segment extending from the middle of the sixth transmembrane domain to the carboxyl terminus was exchanged. As with the m2/m5 constructs, substitution of the m2 carboxyl-terminal segment into the m3 subtype significantly increased the potency of gallamine. Furthermore, the converse substitution reduced the potency of gallamine dramatically, to approximately that seen for the m3 subtype itself. It appears that this portion of the receptor is a critical determinant for the binding of gallamine and/or the allosteric interactions between gallamine and [^{3}H]NMS.

The five subtypes of muscarinic acetylcholine receptors (m1-m5) are all members of the seven-transmembrane domain receptor family and initiate responses via activation of G proteins (1). All of the muscarinic receptor subtypes are activated by the endogenous agonist acetylcholine, and this activation is antagonized with high affinities by classical competitive antagonists such as atropine, NMS, and quinuclidinylbenzilate. The affinities of these antagonists do not differ across subtypes. Other antagonists have been found to differentiate very well between certain subtypes, for example, pirenzepine (m1/m2) and AQ-RA 741 (m2/m5). However, in spite of a great deal of effort over the last decade, there are as yet no pharmacological agents capable of unambiguously identifying a single muscarinic subtype from a mixture of unknown composition (1).

There is also another class of ligands for the muscarinic receptor subtypes. These compounds act at allosteric sites on the receptors, inhibiting function and ligand binding of the receptors in ways that do not follow the mass-action predictions of competitive interactions (2–4). Gallamine has been the most intensively studied of these muscarinic allosteric ligands. We have shown that all five muscarinic receptor subtypes are sensitive to allosteric regulation by gallamine, with its greatest potency being at the m2 subtype, intermediate potency at the m1 and m4 subtypes, and lowest potency at the m3 and m5 subtypes (5).

Acetylcholine and muscarinic receptors are widely distributed in the central nervous system and are believed to be involved in a number of central nervous system disorders, most notably the memory disturbances of Alzheimer's disease. It is believed that subtype-selective drugs may be the key to safe and effective treatment of these conditions (6, 7). If the allosteric sites are located distantly from the acetylcholine binding site, it is possible that there may be greater potential for selectivity via allosteric modulation, because of the need for all of the subtypes to maintain a similar structure around acetylcholine. Additionally, allosteric agents offer intrinsic therapeutic advantages (6, 8).

The construction of chimeric receptors is an approach that is particularly well suited to the investigation of differences between closely related receptors (9). We have used this approach to investigate the structural features that confer higher affinity toward gallamine on the m2 muscarinic receptor, relative to the m3 and m5 subtypes. The results suggest that an important element of this selectivity lies within a fairly short segment of the receptor, made up of most of the sixth transmembrane region and the third extracellular loop.

Experimental Procedures

Materials. Atropine sulfate and gallamine triethiodide were obtained from Sigma Chemical Co. (St. Louis, MO), methoctramine

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ABBREVIATIONS: NMS, *N*-methylscopolamine; PB, phosphate buffer; CHO, Chinese hamster ovary.

tetrahydrochloride from Research Biochemicals Inc. (Wayland, MA), and obidoxime chloride from Schweizerhall (South Plainfield, NJ). [^{3}H]NMS chloride (81.5 Ci/mmol) was from NEN DuPont (Boston, MA).

Chimeric receptors. The construction and verification of the m2/m5 and m2/m3 chimeric muscarinic receptor genes used in this study have been described previously (10, 11). Plasmids containing the various gene constructs were transfected into COS-7 cells by calcium phosphate precipitation (11). Cells were harvested 72 hr after transfection by scraping into 5 mM PB, pH 7.4, homogenization, and collection of 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. Binding assays were conducted in 5 mM PB at 25°. Membranes (approximately 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 gallamine, 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 and Schuell, Keene, NH), 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 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 gallamine and divided by the true rate constant (k_{off}), determined in the presence of 3 μM atropine only. The resulting number was designated the fold shift in the off-rate, such that a value of 2 would indicate a dissociation of [^{3}H]NMS that was twice as fast as the control rate and a value of 0.5 would be slower than control. The concentrations of gallamine 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 gallamine on the dissociation of [^{3}H]NMS are proportional to the occupancy of the allosteric site, as previous studies have confirmed (12). Therefore, data from the fold shift experiments were fitted to the following equation:

$$FS = 1 - \frac{mL}{L + K}$$

where *FS* is the fold shift (described above), *L* is the concentration of gallamine, *m* is the maximal reduction in the rate constant that can be produced by gallamine, and *K* is the apparent equilibrium dissociation constant (for the interaction between gallamine and the NMS-bound form of the receptor). Curve-fitting was carried out with the MINSQ program (MicroMath, Salt Lake City).

Results

Muscarinic allosteric ligands exhibit the greatest selectivities between the m2 receptor subtype and the m5 or m3 subtypes. Previous studies have found that methoctramine possesses very high affinity for the m2 receptor (13), whereas only gallamine and obidoxime have been definitely shown to interact at a well defined allosteric site on a muscarinic receptor subtype (m2) (12). Under our assay conditions, gallamine was found to have about 10 times better m2/m5 and m2/m3 selectivities than methoctramine or obidoxime (Fig. 1). Therefore, gallamine was selected as the ligand of choice with which to detect the contributions of receptor epitopes to the affinity of allosteric interaction.

m2/m5 chimeric constructs. The structures of m2/m5 (and m2/m3) chimeras that were used in this study are shown in Fig. 2 in schematic form; the exact sequences are given in the legend to Fig. 2. As we reported previously (5), [^{3}H]NMS dissociated much more rapidly from the m2 subtype than from the m5 subtype (Fig. 3). Two of the m2/m5 chimeric receptors

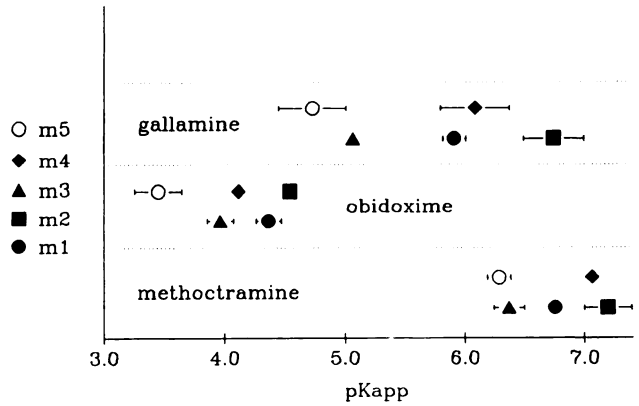


Fig. 1. Apparent affinities of gallamine, methoctramine, and obidoxime at muscarinic receptor subtypes. CHO-K1 cells that had been transfected with individual muscarinic receptor subtypes were harvested and membranes were prepared according to the procedures given for COS-7 cells. The affinities with which gallamine, methoctramine, and obidoxime modulated the rate of dissociation of [^{3}H]NMS from the various subtypes were determined at least three times and are expressed as apparent p*K* values (mean ± standard error). Where error bars are not seen, they are smaller than the *symbols*. See Experimental Procedures for details of the methods.

had rates even slower than that of the m5 subtype, but only CR6 differed from m5 by much more than a factor of 2. This construct (CR6), which consisted of a central core of m5 subtype sandwiched between amino- and carboxyl-terminal m2 domains, exhibited a dissociation rate slightly faster than that of the m2 subtype itself. The slowest dissociation was observed when a smaller m2 core was flanked by m5 domains at both terminals (CR2). When the m2 insert was enlarged, compared with CR2, to extend back to the amino terminus (CR5), the dissociation rate was just slightly faster than that of the m5 subtype. A summary of the rates of dissociation of [^{3}H]NMS (in the absence of gallamine) from the chimeric and wild-type receptors is given in Table 1.

The presence of gallamine slowed the dissociation of [^{3}H]NMS from the m2 and m5 receptors with about the same potencies that were observed in CHO cells (compare Fig. 1 with Figs. 4 and 6). Four of the chimeric constructs exhibited apparent affinities for gallamine that were nearly identical to that of the m5 subtype. The pK_{app} values for the remaining two, CR4 and CR6, were similar to each other and near the mean of the pK_{app} values for the m2 and m5 subtypes (see Figs. 4 and 6). Notably, CR4 contains just 31 amino acids of m2 sequence (of 532 total), and that piece of m2 sequence is completely included in the larger m2 sequence of CR6.

m2/m3 chimeric constructs. [^{3}H]NMS dissociated from the (rat) m3 receptor transiently expressed in COS-7 cell membranes (Fig. 5; Table 1) somewhat more slowly than from human m3 receptors stably expressed in CHO cells (5). However, the apparent affinity with which gallamine altered the rate of dissociation of [^{3}H]NMS from the rat m3 receptor (Figs. 5 and 6) was essentially identical to that found for the human m3 receptor (Fig. 1). Exchanging the amino-terminal regions of the receptors significantly affected the dissociation of [^{3}H]NMS, such that the construct that was mostly m2 receptor with the amino terminus of the m3 receptor performed like the m3 receptor, and vice versa (Fig. 5; Table 1). In terms of apparent affinity for gallamine, the m3N2 construct was consistent with the CR4 and CR6 m2/m5 constructs. That is, a

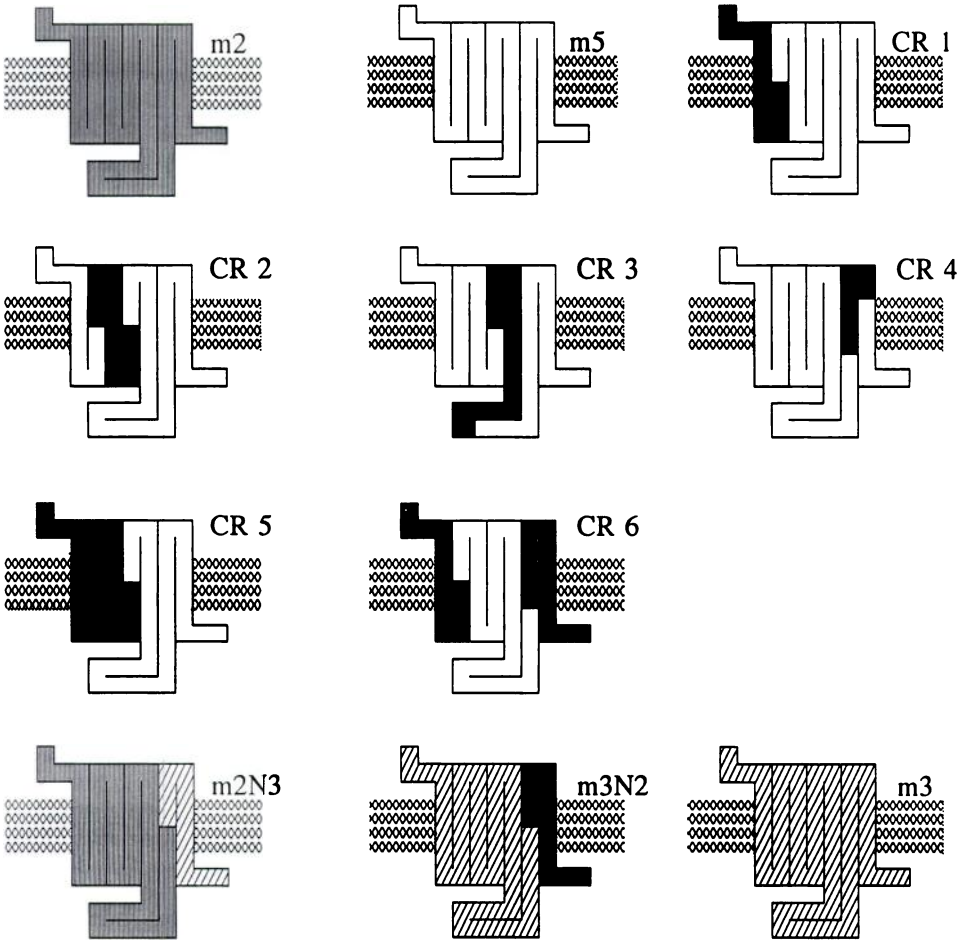


Fig. 2. Schematic representations of chimeric receptors used in this study, drawn so that the amino-terminal domain is extracellular (*top left*) and the carboxyl-terminal domain is cytoplasmic (*bottom right*). Chimeric receptors studied were made up of amino acid sequences from the human m2, human m5, and rat m3 subtypes, as indicated. The exact sequences are as follows: 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; m2N3, m2 1–400, m3 504–589; m3N2, m3 1–503, m2 401–466.

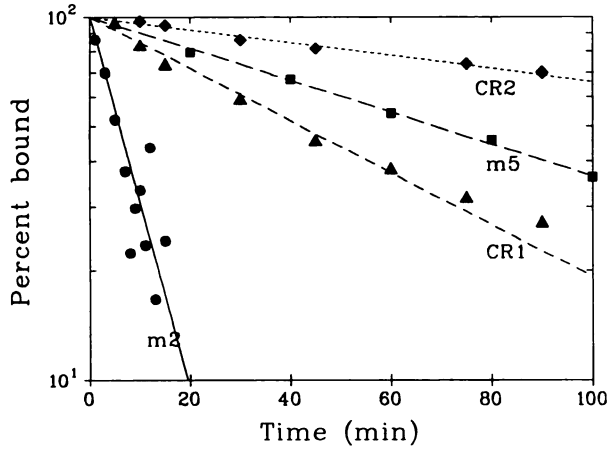
relatively small segment of m2 sequence was able to increase the affinity of gallamine significantly, so that the pK_{app} value was about the average of the values for m2 and m3. On the other hand, the analogous segment from the m3 subtype was even more effective in disrupting the higher affinity of the m2 subtype. Thus, the pK_{app} value for the m2N3 construct was nearly identical to that of the m3 subtype. A summary of the spread of the pK_{app} values from all experiments is given in Fig. 6.

Discussion

Gallamine is undoubtedly the most well studied muscarinic allosteric ligand and is well known to slow the off-rate of [^{3}H] NMS from cardiac muscarinic receptors. Gallamine is also among the most selective of muscarinic ligands, preferring the m2 subtype over m3 and m5 by about 2 orders of magnitude. Furthermore, it is known to interact with a specific allosteric site, a characteristic that has not been established for most muscarinic allosteric ligands (14). The major finding of this study is that a large portion of the m2/m5 selectivity of gallamine can be attributed to a small segment of the receptor, consisting of the third outer loop and most of the sixth transmembrane region.

The use of chimeric receptors offers advantages and disadvantages for the investigation of receptor structure/function relations. The chimeric approach cannot be used at all to investigate elements that are common to both receptor subtypes involved. That is, because all of the muscarinic subtypes are sensitive to allosteric regulation by gallamine, it is possible that they may share a common active site for gallamine. In that case, that site would be present in all of the chimeric constructs and the stretch identified in CR4 would represent an auxiliary binding site that confers the m2 selectivity of gallamine. Investigation of a site common to all muscarinic receptor subtypes would require a different approach. Also, even when analogous domains are substituted between similar receptors, incompatible regions may be encountered that alter function through relatively vague mechanisms, such as by prohibiting the necessary folding structure of the protein.

On the other hand, the chimeric approach can allow the investigation of a library of broad domains and can therefore be a good first step in the analysis of structure-function relations. The interpretation of a chimeric study is facilitated when a minority of the domains studied are found to alter function or when reciprocal chimeric substitutions result in reciprocal changes in function. In the present study, the lack of effect of most of the substitutions (in the m2/m5 chimeras) is as striking as the gain in affinity produced by the two substitutions that included the segment of CR4; the reciprocity of the similar substitutions in the m2/m3 chimeras further supports the involvement of this region of the receptor in conferring the m2 selectivity of gallamine. In neither case, however, does the epitope from the m2 subtype confer m2 affinity completely. There may be multiple features of the m2 subtype, spread out



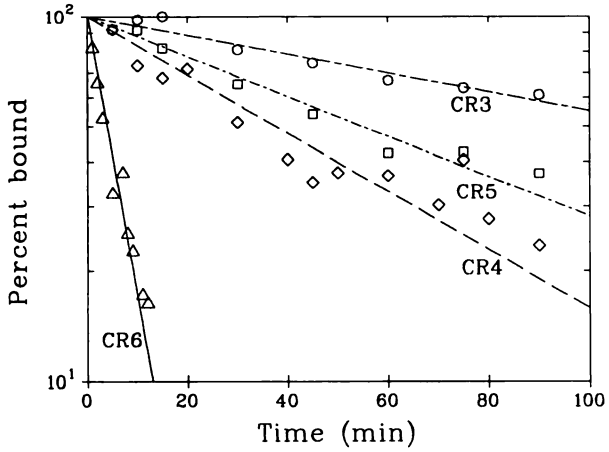
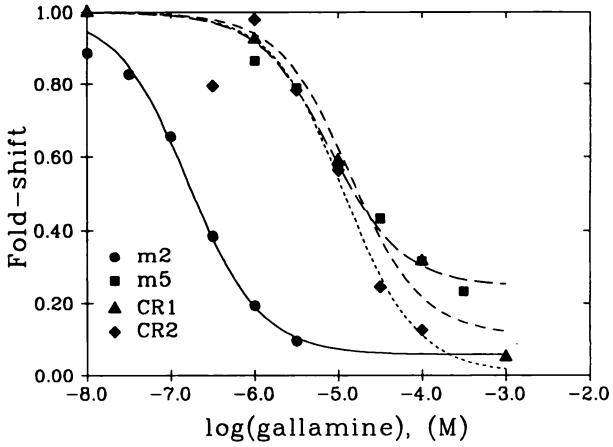


Fig. 3. Rates of dissociation of [^{3}H]NMS from m2/m5 chimeric muscarinic receptors. Assays were conducted as described in Experimental Procedures. After prelabeling of COS-7 membranes containing the indicated chimeric receptor, [^{3}H]NMS was allowed to dissociate for the times shown. The binding remaining at that time is expressed as a percentage of the binding at time 0. Data from one experiment (representative of three experiments) are shown. Mean ± standard error values for the half-time of dissociation of [^{3}H]NMS are given in Table 1.



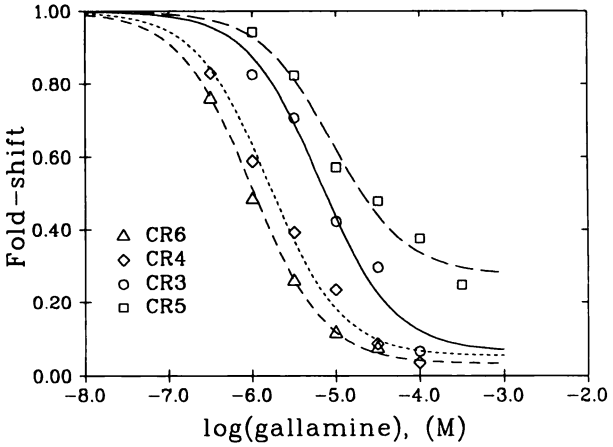


Fig. 4. Concentration-dependent modulation by gallamine of the dissociation of [^{3}H]NMS from m2/m5 chimeric muscarinic receptors. Experiments were conducted and analyzed as described in Experimental Procedures. Fold shift values of <1 indicate slowing of the dissociation of [^{3}H]NMS. Data shown are from representative experiments; summary data for the apparent affinities are given in Fig. 6.

TABLE 1

Half-times of dissociation of [^{3}H]NMS from chimeric and wild-type receptors

Values are the means ± standard errors, in the absence of gallamine, derived from three to five independent experiments.

Receptor construct	Dissociation $t_{1/2}$, ([^{3}H]NMS)
	min
m5	84 ± 8
CR1	45 ± 2
CR2	179 ± 24
CR3	131 ± 5
CR4	46 ± 3
CR5	63 ± 4
CR6	3.9 ± 0.02
m2	6.4 ± 0.04
m3N2	11 ± 1
m2N3	44 ± 2
m3	78 ± 8

around the receptor, that are too subtle to be detected individually by our chimeric approach; for example, they may serve, in aggregate, to align the crucial epitope precisely. If that were so, it would be expected that removal of that epitope would produce the most dramatic effect, and results obtained with the m2N3 chimer are consistent with that expectation.

There are several possible approaches to the analysis of allosteric interactions. Resultant analysis, dose ratio additivity, and Schild analysis essentially measure deviations from competitive behavior without quantitating the allosteric interaction *per se*. Ehlert (15) has described quantitative methods of analysis for a straightforward allosteric model that can be used to yield the affinity (K_a) with which an allosteric ligand interacts with a receptor and the degree of cooperativity (α) between that ligand and another ligand that binds to another site on the receptor. All of these methods require data obtained under equilibrium conditions, a condition that can be difficult to achieve for the m3 and m5 subtypes, from which [^{3}H]NMS dissociates with a half-time of 80 min (which is greatly slowed further in the presence of gallamine). In the present study, we have taken the ability of gallamine to slow the off-rate of [^{3}H]NMS as a measure of its allosteric effect. The change in off-

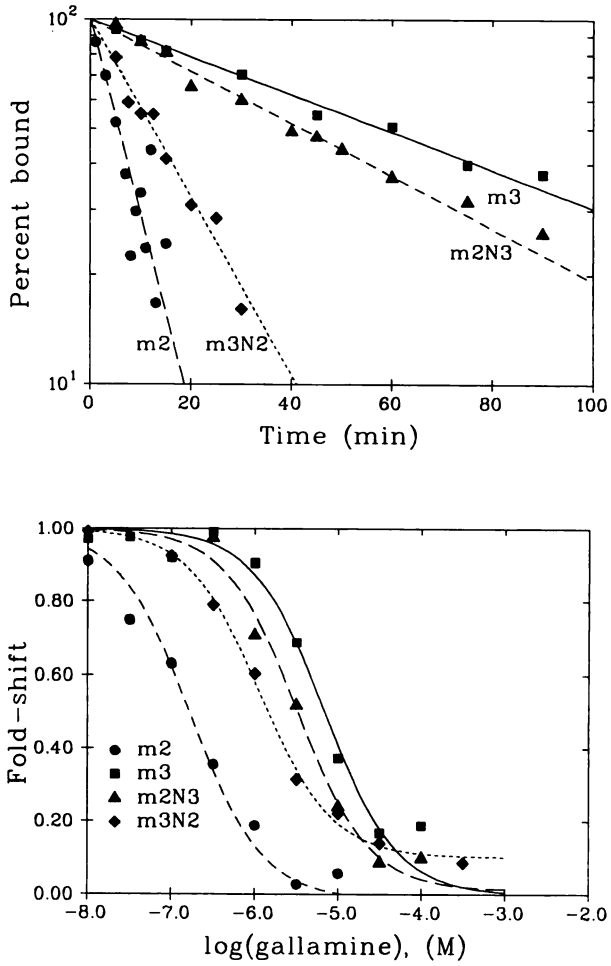


Fig. 5. Dissociation of [^{3}H]NMS from m2/m3 chimeric muscarinic receptors (*top*) and modulation of the dissociation rate by gallamine (*bottom*). Experiments were conducted and analyzed as in Figs. 3 and 4, except that each *curve* is the average of three experiments. The values for the half-times of dissociation of [^{3}H]NMS (control) and the apparent affinities of gallamine are given in Table 1 and Fig. 6, respectively.

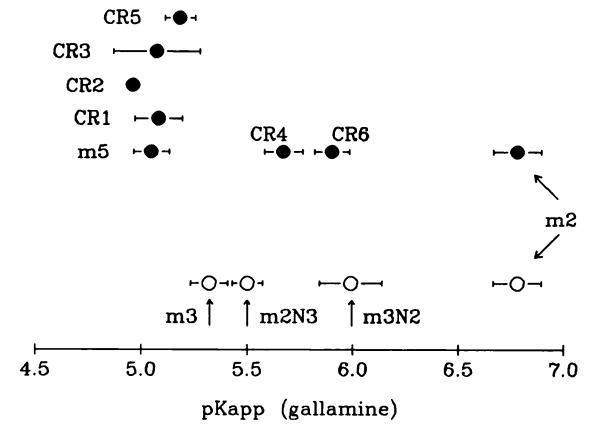


Fig. 6. Summary data on the apparent affinities with which gallamine modulates the rate of dissociation of [^{3}H]NMS from m2/m5 and m2/m3 chimeric muscarinic receptors. pK_{app} values were obtained from the data in Figs. 4 and 5 by the curve-fitting methods described in Experimental Procedures. Each *symbol* represents the mean ± standard error of three or four experiments.

rate is easily and reproducibly measured and is independent of the affinity or off-rate of [^{3}H]NMS. This approach ensures that possible concomitant competitive interactions (3, 4) between gallamine and NMS do not obscure the analysis. In a sense, we have considered the ability of gallamine to alter the off-rate of [^{3}H]NMS as a response, although we remain cognizant of the potentially applicable model. This is a useful approach because it allows us to analyze a definitively allosteric effect whether the model is correct or not. That is, in the worst case, our value for K_{app} empirically represents the concentration of gallamine that produces a half-maximal slowing of the rate of dissociation of [^{3}H]NMS. On the other hand, if gallamine does bind exclusively to a single site that interacts allosterically with the site to which NMS binds, then K_{app} is equal to αK_g, where K_g is the affinity of gallamine for the free receptor and α is the degree of cooperativity between gallamine and NMS (according to Ehlert's model; see above). Preliminary studies in our laboratory suggest that, for the interaction between NMS and gallamine at the m2 subtype, K_{app} is indistinguishable from αK_g, when α and K_g are determined by Ehlert's method.[1] However, this remains an open question for other subtypes, other ligands, and other assay conditions. For example, tetrahydroaminoacridine appears to interact with more than one allosteric site at m2 and other subtypes of muscarinic receptors (12, 16), and alcuronium must interact with at least two sites on cardiac muscarinic receptors (17). Additionally, in a report that included estimation of α and K_g and dissociation studies (of gallamine), it appeared certain that K_{app} did not equal αK_g at the m3 subtype when [^{3}H]NMS was the labeled ligand (13). In such cases, the physical meaning of the best-fit values of α and K_a would not be clear.

The rate of dissociation of [^{3}H]NMS from the receptors was found to be sensitive to epitopes that were similar, but not identical, to those that influenced allosteric selectivity. In the m2/m3 chimeras, dissociation rate correlated very well with the potency of gallamine, but the m2/m5 chimeras revealed a clear difference. Thus, whereas CR4 and CR6 were similar in terms of potency for gallamine, CR1, CR4, and CR5 were very similar in terms of rate of dissociation of [^{3}H]NMS. Much of the dissociation data can be explained if there is an epitope between the CR4 region and the carboxyl terminus that strongly influences the rate of dissociation of [^{3}H]NMS. However, rate constants are less likely to have discrete structural counterparts than are state functions (e.g., affinity). For example, the very slow kinetics associated with CR2 and CR3 may be reflections of incompatibilities between domains from different subtypes (discussed above). The foreign domains may put a crimp in the pocket of the receptor that would be dependent on the nature of adjacent or associated domains. This kind of change would slow both the entrance and egress of ligands but would not affect equililbrium binding parameters. Whether or not the substitutions of CR2 and CR3 do in fact rearrange the receptor so that there is a more tortuous path for [^{3}H]NMS to its binding site, observations indicate that the binding site itself, the site for gallamine, and the interaction between the two sites all appear to be unaffected, relative to m5.

Previous investigations of these same m2/m5 chimeras (assaying inhibition of [^{3}H]NMS binding under equilibrium conditions) suggested that multiple receptor domains were involved

[1] M. Stadtmauer and J. Ellis, unpublished observations.

in the preferences of himbacine and AQ-RA 741 for the m2 subtype (11). Likewise, the m5-preferring antagonist silahexocyclium was moderately sensitive to many receptor domains. Interestingly, UH-AH 37, which has about 10-fold higher affinity for the m5 subtype than for the m2 subtype, was selectively sensitive to the same chimeric substitutions that affected gallamine, namely, CR4 and CR6. Previous studies of UH-AH 37 did not find any indications for an allosteric mode of action (18) and, even though strongly (negatively) cooperative interactions can be indistinguishable from competitive interactions (15), there is no compelling reason to expect UH-AH 37 to be allosteric. Indeed, what is clear is that gallamine and UH-AH 37 must bind differently to the portion of the receptor exchanged in CR4, because they have preferential affinities for different subtype-specific structural elements in that region. Conversely, himbacine is known to exert allosteric effects at muscarinic receptors (13) but was strikingly insensitive to the CR4 chimera (11). That study may or may not have been measuring allosteric effects (see above), and it remains to be seen whether allosteric ligands other than gallamine are sensitive to the same restricted region (CR4). Of the 31 amino acids in the region exchanged in CR4, nine differ between m2 and m5. Three of these differences lie within the sixth transmembrane region, whereas the other six lie in the third outer loop. It is generally accepted that ligands for G protein-coupled receptors are most likely to bind in a pocket formed by the transmembrane regions (19, 20), but it certainly remains possible that some ligands, especially allosteric ones, might bind to adjacent extracellular epitopes. We are currently using site-directed mutagenesis to attempt to identify the distinct amino acids that are responsible for the subtype-selective binding of gallamine. Such findings would have implications for the orientation of gallamine on the receptor and for the mechanism by which it alters receptor conformation and function; this knowledge is essential to the design of ligands that would alter function in specific ways.

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