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Probing of the Location of the Allosteric Site on m1 Muscarinic Receptors by Site-Directed Mutagenesis

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SUMMARY

In an attempt to locate the allosteric site on muscarinic receptors to which gallamine binds, 21 residues in the putative external loops and loop/transmembrane helix interfaces have been mutated to alanine. These residues are conserved in mammalian m1–m5 receptors. All mutant receptors can be expressed in COS-7 cells at high levels and appear to be functional, in that acetylcholine binding is sensitive to GTP. The gallamine binding site does not appear to involve the first, second, and most of the third extracellular loops. Tryptophan-400 and -101 inhibit gallamine binding when mutated to alanine or to phenylalanine and may form part of the allosteric site. Several mutations also affect antagonist binding. Surprisingly, tryptophan-91, a residue conserved in monoamine and peptide receptors, is important for antagonist binding. This residue, present in the middle of the first extracellular loop, may have a structural role in many G protein-coupled receptors. Antagonist binding is also affected by mutations of tryptophan-101 and tyrosine-404 to alanine or phenylalanine. In a helical wheel model, tryptophan-101 and tyrosine-404, in conjunction with serine-78, aspartate-105, and tyrosine-408, form a cluster of residues that have been reported to affect antagonist binding when mutated, and they may therefore be part of the antagonist binding site. It is suggested that the allosteric site may be located close to and just extracellular to the antagonist binding site. The binding of methoctramine, an antagonist with allosteric properties, is not substantially affected by mutations at tryptophan-91, -101, and -400 and tyrosine-404, and thus these amino acids are not important for its binding. The binding of himbacine, another antagonist with allosteric properties, is affected by these mutations but in a manner different from that of gallamine or competitive antagonists. It has not been possible to determine whether methoctramine and himbacine bind exclusively to the allosteric site or to both the competitive site and the allosteric site.

The binding site for ACh and competitive antagonists on muscarinic receptors appears to be located within the seven α-helical transmembrane segments characteristic of all G protein-coupled receptors (1, 2). In particular, protein labeling and sequencing studies using the alkylating antagonist [^{3}H]propylbenzilylcholine mustard and the ACh analogue [^{3}H]ACh mustard have demonstrated that aspartate-105 of the m1 muscarinic receptor is covalently modified (3–5). These findings provide strong evidence that aspartate-105 is the primary site of interaction with the positively charged nitrogen of ACh and competitive muscarinic antagonists. Indeed, this residue is conserved not only in muscarinic receptors but also in all G protein-coupled receptors that bind small, positively charged ligands such as adrenaline, serotonin, and histamine. Furthermore, mutation of this residue to asparagine in the m1 receptor (6) and the β-adrenoceptor (7) produces a large decrease in the binding of agonists and antagonists.

In the case of muscarinic receptors, there is a second binding site (1, 8). Ligands such as gallamine can bind to this site and allosterically change the binding affinity of agonists and antagonists for the competitive site. Very little is known about the location of the allosteric site or the amino acids that are important for the binding of gallamine. However, from the fact that impermeant, highly charged ligands such as gallamine can rapidly produce their allosteric effects in whole cells or tissues, it may be deduced that the allosteric site is located on the extracellular face of the receptor structure. In addition, the binding of many allosteric ligands profoundly slows the association and dissociation rates for the competitive antagonist NMS, in a concentration-dependent manner (8), to the extent that in some instances a compulsory order of ligand binding may exist. In other words, when, for example, gallamine is bound to the receptor, association and dissociation of NMS from the receptor is prevented.

Our hypothesis is thus that the allosteric site is located extracellularly to the competitive site. Because the allosteric site is found in all muscarinic receptors, it was decided to mutate residues conserved in all five subtypes, concentrating on the conserved aromatic and polar residues in the postulated extracellular loops. The Hm1 receptor was chosen because of

ABBREVIATIONS: ACh, acetylcholine; NMS, *N*-methylscopolamine; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; QNB, 3-quinuclidinylbenzilate; WT, wild-type; PCR, polymerase chain reaction; Hm1, human m1.

the possible therapeutic applications of agents acting at this receptor in the treatment of Alzheimer's disease. The effects of these mutations on the binding of ACh, antagonists, and gallamine were examined with the aim of locating residues that are important for the binding of gallamine and its allosteric interaction with competitive muscarinic agents.

Our results highlight the importance of tryptophan-400 and tryptophan-101 for the binding of gallamine. The evidence suggests that the allosteric and competitive sites may be close to each other. Most surprisingly, mutation of tryptophan-91, in the middle of the first extracellular loop, produces substantial decreases in the binding of ACh and competitive antagonists.

Materials and Methods

Chemicals. [^{3}H]NMS was obtained from Amersham International. GTP, gallamine triethiodide, and ACh bromide were obtained from Sigma Chemical Co. Methoctramine was obtained from Research Biochemicals (Natick, MA). QNB was synthesized by B. Peck (Laboratory of Molecular Structure, National Institute for Medical Research). 4-DAMP, Pirenzepine, and himbacine were kind gifts from Dr. R. B. Barlow (Newcastle University, England, UK), Dr. R. Hammer (Boehringer-Ingelheim, Germany), and Dr. W. Taylor (University of Sydney, Sydney, Australia), respectively.

Introduction of mutations. The expression vector pCDm1, containing the Hm1 muscarinic receptor cDNA (9), was kindly provided by Dr. N. J. Buckley. The mutagenesis was performed using a PCR-mediated method (10). For example, in the case of the W400A mutant the following oligonucleotides were used as primers: A, d(TGATCGAGCTGGCAAGGGCCAGAA) (positions 1002–1025); B, d(GTAGCCCAGCTCGGCCAGGGTCTCGGG) (positions 1212–1186); C, d(CCCGAGACCCTGGCCGAGCTGGGCTAC) (positions 1186–1212); D, d(AAAGCTAGTAGAGTCTCTCTGGGC) (positions 1545–1522). Oligonucleotides B and C contained the desired mutation (underlined) and are complementary. Oligonucleotides A and D are complementary to nucleotides 1002–1025 and 1545–1522 of the cDNA sequence, respectively, flank *Pvu*II (position 1045) and *Bst*EII (position 1506) cleavage sites, and flank the mutation site at positions 1198–1200 (TGG changed to GCC). Two independent PCRs were carried out using, firstly, primers A and B and, secondly, primers C and D. The two purified PCR products were mixed and one cycle of the PCR was carried out. Oligonucleotides A and D were then added to this product and another PCR was performed (25 cycles). The final PCR product was digested with *Pvu*II and *Bst*EII and inserted into digested pCDm1 to replace the WT fragment with the mutant fragment. The introduction of the desired mutation and the absence of the mismatches caused by lack of fidelity of *Thermus aquaticus* polymerase were confirmed by DNA sequencing, and an appropriate clone was prepared on a large scale. The restriction sites used to construct mutations in the first and second extracellular loops were at *Nhe*I (position 109)/*Nhe*I (position 520) and *Kpn*I (position 228)/*Pvu*II (position 819) sites, respectively, of the cDNA sequence.

Strategy. The strategy was to mutate to alanine, separately, all of the conserved aromatic, charged, proline, glycine, and potential hydrogen-bonding residues in the putative extracellular domains of the Hm1 receptor (see Fig. 7). In addition, two residues, tryptophan-101 and tyrosine-404, thought to be close to the extracellular domain/transmembrane α-helix interface were mutated.

These constructs were expressed transiently in COS-7 cells and the binding of ACh, the antagonist [^{3}H]NMS, and gallamine to the membranes was examined. If these binding properties of specific mutant receptors were substantially changed from those of the WT receptor, the structure-binding relationships of these receptors were characterized in further detail using additional antagonists. For certain tyrosine and tryptophan residues, more conservative mutations of these residues (tyrosine to phenylalanine and tryptophan to phenylalanine) were made to maintain the aromatic nature of the side chain, which was missing in the alanine mutants, and to possibly lessen the structural perturbations produced by the mutation.

Transfection of mutant genes into COS-7 cells. The purified mutant genes were introduced into COS-7 cells by electroporation. Twenty micrograms of plasmid DNA were used for 8×10^6 COS-7 cells. Three days after transfection, the cells were collected in 20 mM HEPES buffer, pH 7.4, containing 10 mM EDTA, sonicated, washed twice with 20 mM HEPES buffer, pH 7.4, containing 0.1 mM EDTA, suspended in the same buffer, and used for assays.

Binding assays. All assays were performed for 2 hr at 30° in 20 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 10 mM $MgCl_2$, in a volume of 1 ml. Bound radiolabel was collected by filtration on Whatman GF/B filters (presoaked in 0.1% polyethyleneimine), using a Brandel cell harvester. The filters were rapidly washed three times with ice-cold water and the radioactivity was counted. The dissociation constants of NMS for each mutant were calculated from the results of [^{3}H]NMS saturation experiments. The dissociation constants of ACh, gallamine, himbacine, methoctramine, pirenzepine, and 4-DAMP were calculated using the data from [^{3}H]NMS inhibition curves, corrected appropriately for [^{3}H]NMS occupancy. Nonspecific binding was determined using 10^{-6} M QNB.

Dissociation rate assays. A dissociation rate assay was conducted to estimate the affinity of ligands for the [^{3}H]NMS-occupied receptor. A membrane preparation for each mutant was labeled with [^{3}H]NMS by incubation at 30° for 30 min in the same buffer as used for the binding assay. The labeled membranes were then mixed with different concentrations of ligand in the presence of 10^{-6} M QNB (a potent antagonist) at 30°. The time for dissociation (t) was chosen to be approximately 2–3 half-lives of [^{3}H]NMS dissociation, based on the rate found in the absence of allosteric ligand. Because the off-rates for [^{3}H]NMS in the presence or absence of allosteric ligands are monoexponential under these and similar conditions, at least for the ligands we have examined, the binding at time 0 and at time t can be used to calculate a rate constant. The change in rate constant with ligand concentration can be shown to be a simple function of the occupancy of the [^{3}H]NMS-occupied receptor by the allosteric ligand.[1]

Data analysis. All data were fitted by nonlinear least-squares methods, using the appropriate equations with the programs Enzfitter (Sigma) and SigmaPlot (Jandel).

Results

Initial screening of the alanine mutants. All mutant receptors were expressed in COS-7 cells at high levels (1–6 pmol/mg of protein), which were comparable to those found with the WT receptor (data not shown). The logarithm of the affinity constant of [^{3}H]NMS for the WT receptor was 9.92 ± 0.02 (two experiments). This affinity constant was changed by less than a factor of 2 in most of the mutants (Table 1). The exceptions were the Y404A mutant, for which the [^{3}H]NMS affinity was 50-fold lower, W101A (9-fold lower), and W91A (8-fold lower) (Fig. 1).

ACh binding was somewhat more sensitive to the effects of the mutations (Table 1). In the absence of GTP, ACh binding was decreased to the greatest extent with Y404A (50-fold), W91A (40-fold), and W101A (20-fold) (Fig. 2), with smaller effects being observed for the W400A and W164A mutants. In the case of some mutants, e.g., Q181A and C394A, a small increase in ACh affinity (~3-fold) was observed.

The binding of ACh to the WT receptor was decreased in the presence of GTP (Table 1). This was due to the formation

[1] S. Lazareno, N. J. M. Birdsall, The detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G-protein-coupled receptors, manuscript in preparation.

TABLE 1
Binding constants of m1 receptor mutants for [^{3}H]NMS and ACh

Gene	pK_d [^{3}H]NMS	pK_i ACh, −GTP	pK_i ACh, +GTP
WT	9.92 ± 0.02	5.25 ± 0.07	5.02 ± 0.01
T83A	9.94	5.29	5.02
Y85A	9.67 ± 0.19	4.92 ± 0.04	4.56 ± 0.06
W91A	9.02 ± 0.14	3.68 ± 0.12	3.36 ± 0.05
W91F	9.13 ± 0.16	4.59 ± 0.14	4.29 ± 0.16
G94A	9.77 ± 0.02	5.39 ± 0.07	4.60 ± 0.06
W101A	8.97 ± 0.02	3.91 ± 0.01	3.64 ± 0.05
W101F	9.83 ± 0.04	5.80 ± 0.03	5.34 ± 0.04
W164A	9.75 ± 0.11	4.43 ± 0.10	4.28 ± 0.10
Q165A	9.84	5.33	4.87
G169A	9.54 ± 0.01	4.61 ± 0.06	4.22 ± 0.08
R171A	9.40 ± 0.07	4.68 ± 0.10	4.40 ± 0.03
T172A	9.85 ± 0.01	5.13 ± 0.14	4.82 ± 0.13
Q181A	9.88	5.68	5.33
F182A	9.44 ± 0.03	5.01 ± 0.02	4.78 ± 0.09
S184A	10.00 ± 0.01	4.87 ± 0.03	4.62 ± 0.11
T389A	9.86 ± 0.04	5.03 ± 0.02	4.90 ± 0.13
F390A	9.77 ± 0.05	5.13 ± 0.04	4.96 ± 0.14
C391A	9.94	5.53	5.33
C394A	10.04	5.81	5.35
P396A	10.05	5.13	4.96
T398A	9.94 ± 0.02	5.10 ± 0.02	4.87 ± 0.11
W400A	9.75 ± 0.03	4.29 ± 0.04	4.12 ± 0.05
W400F	9.84 ± 0.12	4.34 ± 0.02	4.22 ± 0.13
Y404A	8.19 ± 0.03	3.60 ± 0.16	3.25 ± 0.03
Y404F	9.06 ± 0.02	3.89 ± 0.06	3.61 ± 0.04

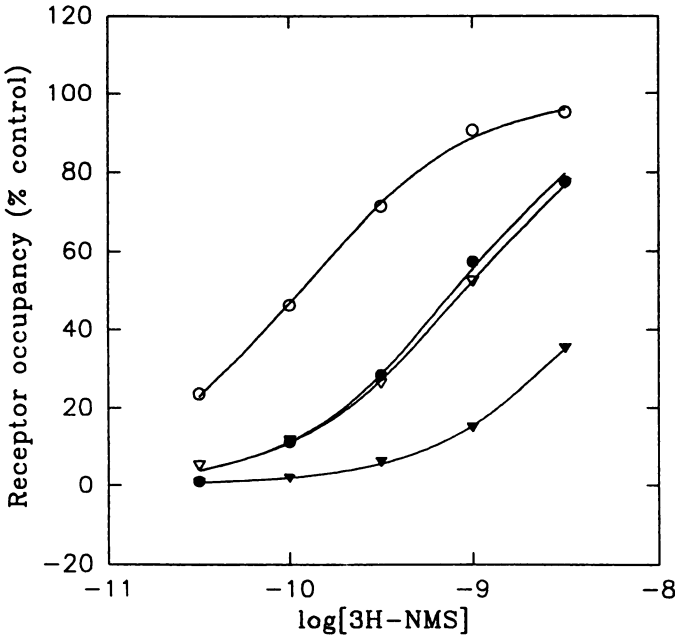

Fig. 1. [^{3}H]NMS saturation binding curves for WT, W91A, W101A, and W404A Hm1 receptors. Assays were carried out using concentrations of [^{3}H]NMS as described in Materials and Methods. The *curves* represent the nonlinear least-squares fits to the data, with the estimated binding capacity being normalized to 100%. The individual fits are as follows: WT (○), pK_d = 9.94, B_{max} = 4.9 pmol/mg of protein; W91A (●), pK_d = 9.10, B_{max} = 1.7 pmol/mg of protein; W101A (▽), pK_d = 9.05, B_{max} = 5.7 pmol/mg of protein; Y404A (▼), pK_d = 8.22, B_{max} = 7.2 pmol/mg of protein. The data points represent the means of two replicates from one experiment, which was repeated at least twice.

of high affinity ACh-receptor-G protein complexes, which are disrupted by GTP to form lower affinity ACh-receptor complexes, a phenomenon found for all G protein-coupled receptors. The decrease in ACh potency produced by GTP was small but of a magnitude expected for Hm1 receptors. However, the

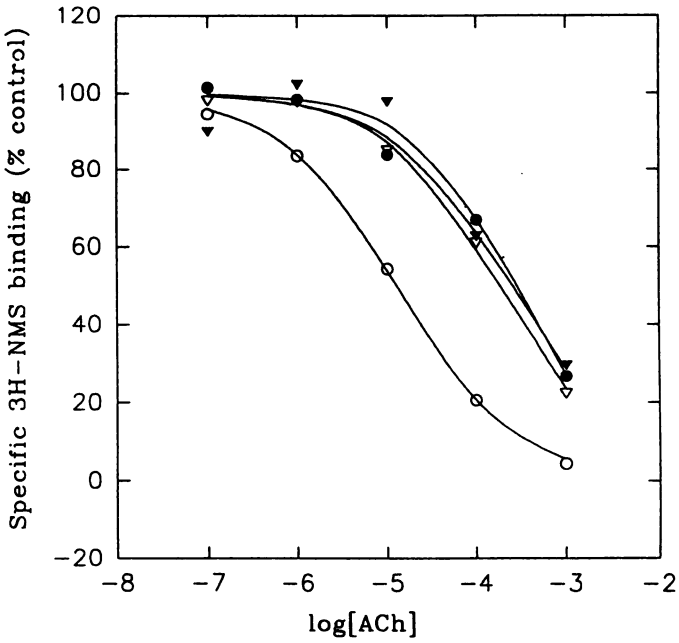

Fig. 2. ACh/[^{3}H]NMS inhibition curves for WT, W91A, W101A, and Y404A Hm1 receptors. The [^{3}H]NMS concentration was ~0.8 K_d for all receptor species. The *curves* are the nonlinear least-squares fits of the untransformed data to a logistic function. The individual fits are as follows: WT (○), pIC_{50} = 4.90, slope factor = 0.65; W91A (●), pIC_{50} = 3.62, slope factor = 0.64; W101A (▽), pIC_{50} = 3.76, slope factor = 0.67; Y404A (▼), pIC_{50} = 3.58, slope factor = 0.74. The data points represent the means of two replicates from one experiment, which was repeated at least twice.

phenomenon was very reproducible and GTP decreased ACh potency to comparable extents in every experiment with the WT and mutant receptors (Table 1). This indicates that all of the mutant receptors are capable of coupling to and uncoupling from G proteins in an agonist- and GTP-dependent manner and may therefore, by this criterion, be considered functional receptors.

Binding of gallamine to the mutated receptors. Two affinity constants are required to describe the binding of an allosteric ligand, D, to a receptor, R. One affinity constant, K_1, is that for the binding of the allosteric ligand to the unliganded receptor. The second affinity constant, K_2, is that for the binding of the allosteric ligand to the receptor when the competitive site is occupied by a competitive agonist or antagonist, L. The ratio K_2/K_1 gives the cooperativity between L and D. Both K_2 and K_2/K_1 are dependent on the nature of L as well as D (8).

$$\begin{array}{ccc} & K_3 & \\ R + L & \rightleftharpoons & R \cdot L \\ + & & + \\ D & & D \\ K_1 \Updownarrow & & \Updownarrow K_2 \\ & K_4 & \\ D \cdot R + L & \rightleftharpoons & D.R.L \end{array}$$

In this section, the cooperative interaction between [^{3}H]-NMS and gallamine was investigated. Because gallamine and

[^{3}H]NMS have relatively high negative cooperativity ($K_2/K_1 \ll 1$), a gallamine-[^{3}H]NMS inhibition curve can provide a good estimate of K_1 (11). As shown in Fig. 3 and Table 2, the affinity of gallamine for the unliganded receptor was decreased substantially only for the W101A (30-fold) and W400A (20-fold) mutants. The affinity of gallamine for the unliganded Y404A receptor was unchanged, in contrast to the large effects seen on ACh and NMS binding.

The affinity of gallamine for the [^{3}H]NMS-occupied receptor, K_2, was estimated from the dose-response relationship for the ability of gallamine to slow the rate of [^{3}H]NMS dissociation from the receptor (12–14).[2] This value was decreased to a similar extent as was K_1 for the W101A (10-fold) and W400A (10-fold) mutants (Fig. 4). Hence, the negative cooperativity of [^{3}H]NMS and gallamine was relatively unchanged by these mutations. It was technically very difficult to demonstrate an effect of gallamine on the rate of [^{3}H]NMS dissociation from the Y404A receptor. This was because of the extremely fast rate of [^{3}H]NMS dissociation from the receptor ($t_{1/2} \leq 5$ sec).

[^{3}H]NMS kinetics of the mutated receptors. Associated with the methodology for the determination of log K_2 values was the estimate of [^{3}H]NMS off-rates for the different receptor species. The rate constant for [^{3}H]NMS dissociation from the WT Hm1 receptors in these experiments was 0.073 min^{-1} ($t_{1/2}$ ~ 9 min). This was decreased to ~0.03 min^{-1} ($t_{1/2}$ ~ 20 min) for the W91A, W101A, and W400A receptors, all of which exhibited unchanged or decreased NMS affinity. This implies that larger decreases in the NMS association rate constants (up to 20-fold) were produced by these mutations. Small decreases in dissociation rate were found for R171A and F182A, with these changes also being accompanied by small decreases in NMS affinity, thus implying that these mutations, in contrast to those described above, had little effect on the [^{3}H]NMS association rate constant. The largest increase in [^{3}H]NMS dissociation rate (≥100-fold) was found for Y404A but, because the NMS affinity for this receptor was 50-fold lower than that for the WT receptor, this means that the NMS association rate constant was not decreased and may even have been increased.

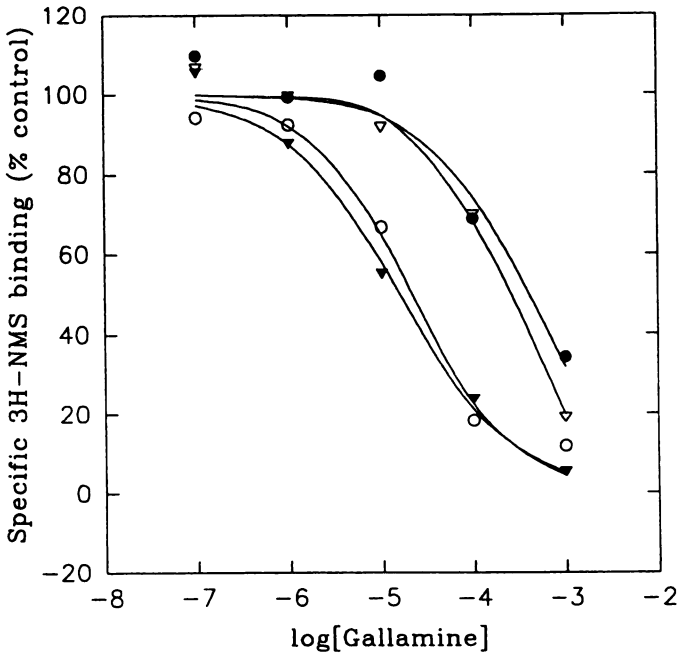

Fig. 3. Gallamine/[^{3}H]NMS inhibition curves for WT, W101A, W400A, and Y404A Hm1 receptors. The [^{3}H]NMS concentration was ~0.8 K_d for all receptor species. The *curves* are the nonlinear least squares fits of the untransformed data (excluding the estimates of nonspecific binding) to a mass action inhibition curve. The individual fits are as follows: WT (○), pIC_{50} = 4.67; W101A (●), pIC_{50} = 3.42; W400A (▽), pIC_{50} = 3.65; Y404A (▼), pIC_{50} = 4.81. The data points represent the means of two replicates from one experiment, which was repeated two to six times. Note that for some receptor species all of the specific [^{3}H]NMS binding is not inhibited by high concentrations of gallamine, as would be expected from the negatively cooperative interactions.

Screening of W91F, Y101F, W400F, and Y404F mutants. From the experiments described in the previous sections, it is clear that the largest effects on binding were produced by mutation to alanine of the aromatic residues tryptophan-91, tryptophan-101, tryptophan-400, and tyrosine-404. These residues were also mutated to phenylalanine (more conservative mutations). The binding constants of NMS, ACh, and gallamine for these receptors are included in Tables 1 and 2.

The W400F mutant behaved similarly to W400A in its NMS, ACh, and gallamine binding affinities, as well as in its NMS kinetics. An analogous pattern was exhibited by the Y404F mutant. In contrast, the binding properties of the W101F mutant were more similar to those of the WT receptor than W101A.

Antagonist binding properties of selected mutants. The mutants in which the NMS or gallamine binding properties were altered substantially, namely W91A/F, W101A/F, W400A/F, and Y404A/F, were examined further. The ligands chosen were the selective antagonists pirenzepine and 4-DAMP, which behave like NMS as competitive antagonists, and two M_2-selective antagonists of unusual structure, methoctramine and himbacine, which are usually considered to be competitive antagonists. At high concentrations, however, methoctramine and himbacine inhibit the dissociation of [^{3}H]-NMS from muscarinic receptors in the same way as does gallamine. These two effects are usually thought to be mediated by different mechanisms (competition at low concentrations in the equilibrium assay and allosterism at the higher concentrations used in the off-rate assay). It is equally possible, however, that they reflect a common allosteric interaction, because a high degree of negative cooperativity is indistinguishable from a competitive interaction. The binding data for the four antagonists are given in Table 3.

The simplest predictions are that 1) the binding of pirenzepine and 4-DAMP, as competitive antagonists, would be affected by the mutations in a similar way as that of NMS and, 2) if himbacine and methoctramine bind to the allosteric site using the same residues as gallamine, then their binding properties would be affected by mutations in a manner similar to that of gallamine binding.

Pirenzepine and 4-DAMP binding were generally affected in the same way as NMS binding. A decrease in affinity for the W91A mutant was also found for the W91F mutant, whereas the substantial decrease in affinity seen for the W101A mutant was not observed for the W101F mutant. The W400A/F mutants showed only small changes in antagonist binding properties, relative to those of the WT receptor. The Y404A mutant exhibited a 10-fold decrease in pirenzepine and 4-DAMP affinity (compared with 50-fold for [^{3}H]NMS). A difference between the three ligands was observed for the Y404F mutant; the

[2] S. Lazareno, N. J. M. Birdsall, unpublished observations.

TABLE 2

Effects of mutations on the [^{3}H]NMS off-rate and on the binding constants of gallamine for the unoccupied and [^{3}H]NMS-occupied receptors (K_1 and K_2, respectively)

Gene	[^{3}H]NMS off-rate ($t_{1/2}$)	Affinity constant		Cooperativity, K_2/K_1
		Unoccupied receptor, log K_1	[^{3}H]NMS-occupied receptor, log K_2	
	min			
WT	9.4 ± 0.4	5.21 ± 0.04	3.67 ± 0.03	0.029
T83A	3.8	5.09	3.74	0.045
Y85A	7.5 ± 0.9	4.77 ± 0.04	3.74 ± 0.23	0.093
W91A	16.6 ± 1.6	4.65± 0.11	3.93 ± 0.05	0.191
W91F	7.5 ± 0.9	5.19 ± 0.07	3.99 ± 0.02	0.063
G94A	8.4	4.72	3.31	0.039
W101A	20.6 ± 1.6	3.79 ± 0.05	2.60 ± 0.04	0.065
W101F	14.1 ± 2.1	4.46 ± 0.03	3.53 ± 0.05	0.117
W164A	6.4 ± 0.4	5.16	3.85 ± 0.13	0.049
Q165A	6.3	4.98	3.55	0.037
G169A	6.8	4.61	3.55	0.087
R171A	4.2 ± 1.6	5.12	4.15	0.107
T172A	9.4	5.16	3.66	0.032
Q181A	6.4	5.08	3.65	0.037
F182A	4.8 ± 0.8	5.19	3.73 ± 0.14	0.035
S184A	12.8	5.23	3.68	0.028
T389A	8.9	4.98	3.51	0.033
F390A	9.6 ± 2.4	5.04	3.76 ± 0.22	0.052
C391A	6.8	4.70	3.63	0.085
C394A	8.1	5.03	3.51	0.030
P396A	7.4	5.01	3.75	0.055
T398A	9.9	5.16	3.60	0.028
W400A	21.2 ± 1.3	3.95 ± 0.03	2.68 ± 0.02	0.054
W400F	28.5 ± 2.4	4.21 ± 0.08	2.96 ± 0.01	0.056
Y404A	<0.1	5.33 ± 0.12	[a]	[a]
Y404F	0.8 ± 0.1	5.15 ± 0.04	3.00 ± 0.03	0.007

[a] Cannot be estimated, because of the fast [^{3}H]NMS off-rate.

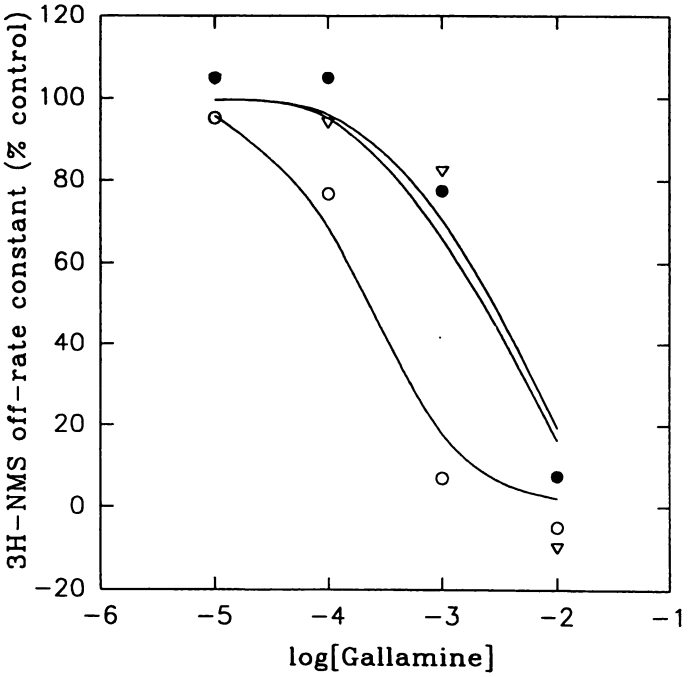

Fig. 4. Gallamine inhibition of the [^{3}H]NMS dissociation rate for WT, W101A, and W400A Hm1 receptors. The *curves* are nonlinear least-squares fits of the estimated rate constants to simple mass action inhibition curves, with the following log K_2 values: WT (○), 3.66; W101A (●), 2.62; W400A (▽), 2.71. The data are the results of one experiment, which was repeated two to six times.

pirenzepine affinity for the Y404F mutant was unchanged, relative to the WT receptor, the 4-DAMP affinity was similar to that for Y404A, and the NMS affinity was between that of the WT receptor and Y404A. As would be expected for competitive antagonists, pirenzepine and 4-DAMP, at the concentrations examined (up to 10^{-3} to 10^{-2} M), did not significantly affect the off-rate of [^{3}H]NMS with any of the mutants examined.

In contrast, methoctramine, himbacine, and gallamine binding were affected by the mutations in a manner different from that observed for the competitive antagonists. Methoctramine binding was relatively little affected by all the mutations, with the biggest change being a 5-fold decrease in affinity for the unoccupied W101A receptor. The negative cooperativity (K_2/K_1) between NMS and methoctramine varied only between 0.02 and 0.003, with the extreme values being found for W101A and W101F. Methoctramine did not slow the [^{3}H]NMS off-rate to an immeasurably low value, as found for gallamine and most other allosteric agents. It appeared to maximally decrease the [^{3}H]NMS off-rate only 2–10-fold, depending on the mutant (Fig. 5A).

Himbacine binding was more sensitive to the mutations than methoctramine. Its binding to the unoccupied receptor was decreased for W91A/F, W400A/F, and Y404A (but not substantially for Y404F), whereas it appeared to be slightly increased for W101A/F. The negative cooperativity with [^{3}H]-NMS varied from ~0.006 (W400A/F) to 0.0001 (Y404F and W101A). The most dramatic change was found at the NMS-occupied receptor of the tryptophan-101 mutants; the affinity for W101A was decreased to >10-fold, whereas that for W101F

TABLE 3

Muscarinic antagonist affinity constants (log K_a values) measured in [^{3}H]NMS competition assays and potencies (pIC_{50} values) in inhibiting the [^{3}H]NMS dissociation rate at selected mutant receptors

Gene	Himbacine		Methoctramine		Pirenzepine		4-DAMP	
	log K_a	pIC_{50}	log K_a	pIC_{50}	log K_a	pIC_{50}	log K_a	pIC_{50}
WT	7.06 ± 0.09	3.94 ± 0.04	6.83 ± 0.02	4.45 ± 0.08	8.09 ± 0.06	<2.5	8.89 ± 0.03	<3
W91A	5.92 ± 0.03	3.23 ± 0.15	6.35 ± 0.07	4.48 ± 0.05	7.20 ± 0.04	ND[a]	7.33 ± 0.09	<3
W91F	6.47 ± 0.04	3.91 ± 0.05	6.52 ± 0.07	4.69 ± 0.21	7.19 ± 0.12	ND	8.08 ± 0.12	<3
W101A	7.35 ± 0.10	<3	6.09 ± 0.08	4.33 ± 0.08	5.90 ± 0.01	<2	7.00 ± 0.11	<3
W101F	7.40 ± 0.01	5.10 ± 0.02	6.58 ± 0.01	4.00 ± 0.09	7.70 ± 0.10	ND	8.76 ± 0.19	ND
W400A	5.83 ± 0.06	3.74 ± 0.07	6.20 ± 0.04	4.43 ± 0.06	7.54 ± 0.06	<2.5	9.00 ± 0.04	<3
W400F	6.07 ± 0.01	3.90 ± 0.13	6.32 ± 0.02	4.59 ± 0.04	7.52 ± 0.06	<2.5	8.93 ± 0.02	<3
Y404A	5.70 ± 0.01	[b]	6.70 ± 0.09	[b]	7.19 ± 0.01	[b]	7.90 ± 0.05	[b]
Y404F	6.71 ± 0.01	3.22 ± 0.04	6.86 ± 0.02	4.27 ± 0.07	8.14 ± 0.03	ND	8.10 ± 0.12	ND

[a] ND, not determined.
[b] Cannot be estimated, because of fast [^{3}H]NMS off-rate.

was increased >10-fold (Fig. 6). As was observed for methoctramine, himbacine produced only a <20-fold maximal decrease in the [^{3}H]NMS off-rate. This partial effect was particularly noticeable for the W91A mutant (Fig. 5B).

Discussion

On the basis of the known profound inhibitory actions of gallamine and other, highly positively charged, allosteric ligands on the on- and off-rate constants for the binding of competitive muscarinic antagonists, we thought that allosteric ligands might bind to residues on the putative extracellular loops of muscarinic receptors. This could provide a 'cap' on the receptor that physically prevents the access of antagonist to or egress of antagonist from its binding site in the extracellular one third of the putative transmembrane domains (1, 15).

Accordingly, we mutated to alanine all of the aromatic, polar, proline, and glycine residues in the putative extracellular loops, which are conserved in the Hm1–m5 muscarinic receptor sequences (Fig. 7). The only conserved residues not mutated were the hydrophobic residues leucine-93, valine-168, valine-173, alanine-175, and isoleucine-180 and the two cysteine residues at positions 98 and 178, which form a disulfide bond (4) and which, when mutated to serine, do not result in a functional receptor (16).

In general, these mutations produced only small changes in the binding properties of NMS, ACh, and gallamine (Fig. 8). The major changes clearly occurred at tryptophan-91, tryptophan-101, tryptophan-400, and tyrosine-404, with minor perturbations of binding (2–3-fold) being produced by mutations at tyrosine-85, tryptophan-164, glycine-169, arginine-171, phenylalanine-182, cysteine-391, and cysteine-394.

In all cases, the ACh binding curves had Hill slopes of <1 (~0.8) and the binding curves were shifted to lower potency by GTP, suggesting that, in the presence of ACh, the receptors were capable of coupling to G proteins and thus were functional. The lack of major effects on ACh and NMS binding of the C391A and C394A mutants agrees with the lack of effect of C391S and C394S mutations on the antagonist and agonist binding properties of rat m1 receptors (16).

However, mutation of tryptophan-91, in the middle of the first extracellular loop, to alanine most unexpectedly produced a receptor that had a substantially lower affinity for antagonists and for ACh. The decrease in binding was most pronounced for 4-DAMP and ACh (~40-fold) and least for methoctramine (3-fold) (Fig. 9; Table 1). The more conservative mutation W91F produced equally large decreases in pirenzepine and NMS binding, compared with those observed for W91A, but intermediate decreases of 4-DAMP, himbacine, and NMS binding. In contrast, gallamine binding was relatively unaffected with the W91A mutant and was unchanged with the W91F mutant, relative to the WT receptor.

The only mutation in the extracellular loops (at least as portrayed in Fig. 7) that affected gallamine binding involved tryptophan-400. Binding at unoccupied and NMS-occupied receptors was ~10-fold lower at the W400A and W400F receptors. The binding of himbacine and other allosteric agents to these mutants was also strongly inhibited.[3] In contrast, competitive antagonist binding was unaffected (NMS and 4-DAMP) or only weakly inhibited (pirenzepine). This evidence highlights the importance of a tryptophan residue at position 400 for high affinity binding of allosteric agents and is compatible with, but does not prove, the idea that tryptophan-400 is a component of the allosteric site.

The evidence so far discussed suggests that the allosteric site for gallamine binding is not located in the middle of the extracellular loops, because none of the mutations in these regions affects gallamine binding. It remains possible that residues (typified by tryptophan-400) at the loop/transmembrane helix interface (according to Fig. 7) may form part of this site. This raises the possibility that the allosteric site may be close in space to the agonist/antagonist site, which is thought to be in the outer one third of the transmembrane domains (1, 15). The tryptophan-101 mutants as well as the tryptophan-400 mutants show decreased gallamine binding. However, the former mutants also show strongly decreased binding of competitive antagonists. This latter effect might be expected, because tryptophan-101 is approximately one α-helical turn extracellular to aspartate-105, which is considered to be part of the competitive binding site (see Fig. 7).

Comparison of the binding properties of the W101A and W101F receptors with those of the WT receptor suggests that an aromatic residue at position 101 is compatible with high affinity antagonist (and ACh) binding, whereas a tryptophan residue at position 101 is important for high affinity gallamine binding. The importance of this residue for binding to the allosteric site is illustrated by the fact that himbacine binding to the [^{3}H]NMS-occupied receptor is decreased, relative to the WT receptor, for W101A but increased for W101F.

[3] H. Matsui, S. Lazareno, and N. J. M. Birdsall, unpublished observations.

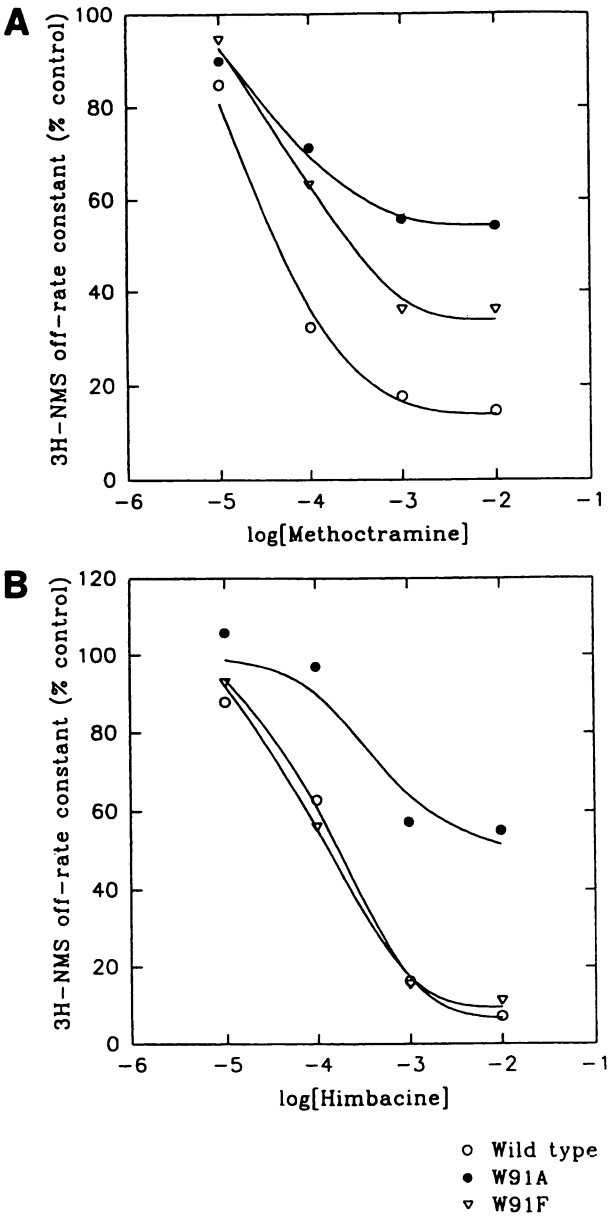

Fig. 5. Incomplete inhibition of [^{3}H]NMS dissociation rate by high concentrations of methoctramine (A) and himbacine (B) at WT and mutant Hm1 receptors. The *curves* are simple mass action inhibition curves. For the WT receptor the log K_2 values are 4.44 (A) and 3.90 (B), with minimum values of 15% and 7%, respectively. The data are the results of one experiment, which was repeated three times.

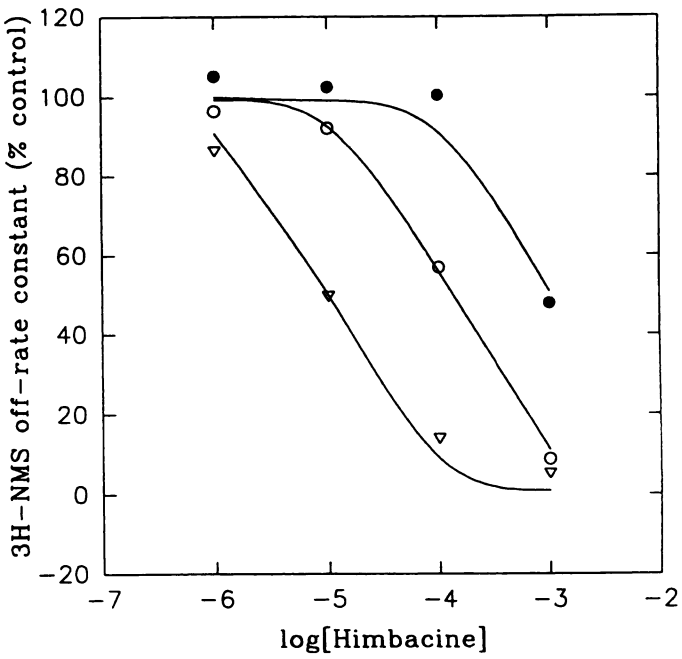

Fig. 6. Inhibition by himbacine of [^{3}H]NMS dissociation rate at WT, W101A, and W101F receptors. The *curves* are nonlinear least-squares fits of the estimated rate constants to simple mass action inhibition curves, with the following log K_2 values: WT (○), 3.95; W101A (●), 2.96; W101F (▽), 5.08.

An analogous situation is found for the Y404A mutant, for which antagonist binding but not gallamine binding to the unoccupied receptor is inhibited. This selective inhibitory effect on competitive antagonist binding is attenuated with the Y404F mutant, as has been found for the equivalent m3 mutant receptor, Y529F (17). More specifically, the presence of a tyrosine at position 404 is important for 4-DAMP, NMS, and ACh binding, whereas an aromatic residue (tyrosine or phenylalanine) at that position suffices for high affinity pirenzepine and himbacine binding. The nature of residue 404 does not appear, at least from the mutants examined, to be important for gallamine or methoctramine binding. This is another example of two residues one helical turn apart, residues 400 and 404, being important for gallamine and competitive antagonist binding, respectively. These data are compatible with, but again do not prove, the concept that the gallamine binding site is just extracellular to the competitive site.

Experiments using receptors with asparagine mutations of the conserved aspartate residues 71, 99, and 122 have indicated that gallamine affinity for the [^{3}H]NMS-occupied receptor is unaltered, relative to the WT receptor (18). The affinity for the unoccupied receptor is slightly attenuated (3-fold) in the D71N mutant and enhanced (2-fold) in the D99N mutant. These small effects are not compatible with the positively charged gallamine forming a strong ionic interaction with these aspartate residues. The D105N mutant, for which the binding of NMS and ACh is strongly inhibited, also has only a slightly lowered affinity for gallamine.[4]

Epitopes that may be important for the expression of the m2 selectivity of gallamine have been explored recently by the generation and investigation of m2/m5 and m2/m3 chimeric receptors (19). The authors concluded that inclusion of small segments of transmembrane domains 6/7 and the third extracellular loop into m3 and m5 receptors conferred increased binding affinity of gallamine for the NMS-occupied receptors. Gallamine affinity for the unoccupied receptors was not investigated. These constructs were used to investigate sequences/conformations that were different between m2 and m3/m5 receptors and not the features that were common to the allosteric site, as in our study. It is, however, of interest that the region of the m2 receptor that increased gallamine affinity at m5 and m3 receptors did include tryptophan-400.

The possible location of tryptophan-101 and -400, relative to those transmembrane domain residues that, when mutated, affect the binding of one or more antagonists, is illustrated in

[4] J. Proška and N. J. M. Birdsall, unpublished observations.

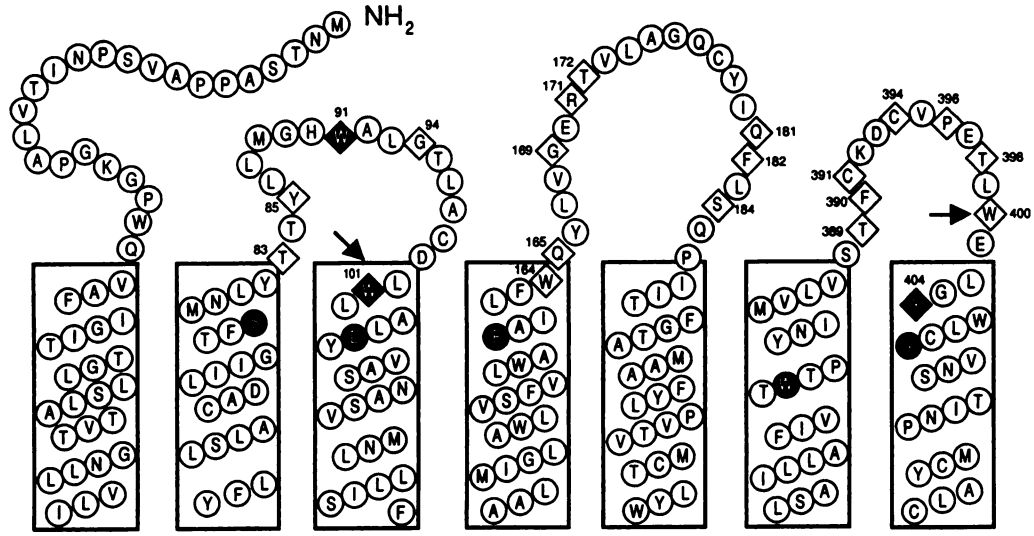

Fig. 7. Model of the external loops and transmembrane α-helices (*boxed*) of the Hm1 receptor. *Diamonds*, residues that have been mutated in this study. *Arrows*, residues to which the binding of gallamine is inhibited. *Reverse mode*, residues that in this study and other published studies on m1 and m3 receptors (15–17, 21) show altered antagonist binding when mutated.

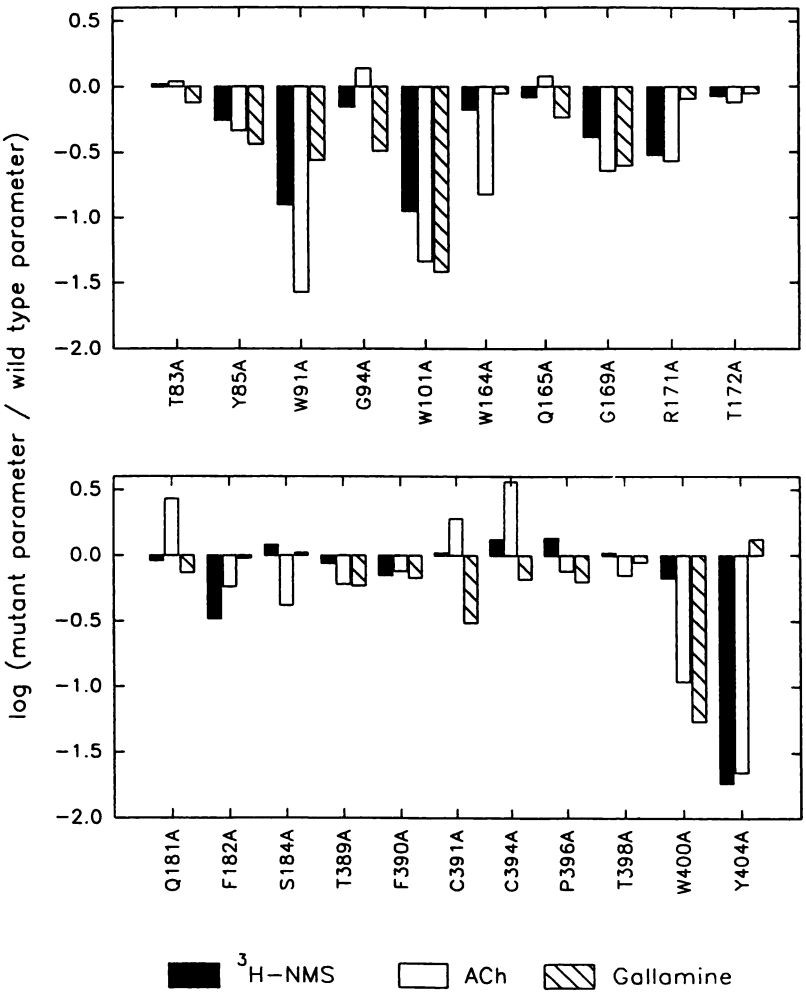

Fig. 8. Changes in mutant m1 receptor affinity for NMS, ACh, and gallamine (unoccupied receptor) as a function of the position of mutation. The changes are expressed as the logarithm of the fold change in affinity, compared with the WT receptor.

a helical wheel model of muscarinic receptors (Fig. 10). With the exception of the proline in transmembrane domain 4 (20), the mutation of which could be expected to produce major changes in helix 'bend' and interhelical packing, and tryptophan-378, which is highly conserved in G protein-coupled receptors and might have a structural role, the residues that regulate antagonist binding (serine-78, aspartate-105, tyrosine-404, and tyrosine-408) are associated with the same region as tryptophan-101 and -400 (Fig. 10) and are immediately intracellular to these two residues (Fig. 8). This picture is in accord with the ability of gallamine to decrease the [^{3}H]NMS off-rate and to provide a cap to block entrance to and egress from the

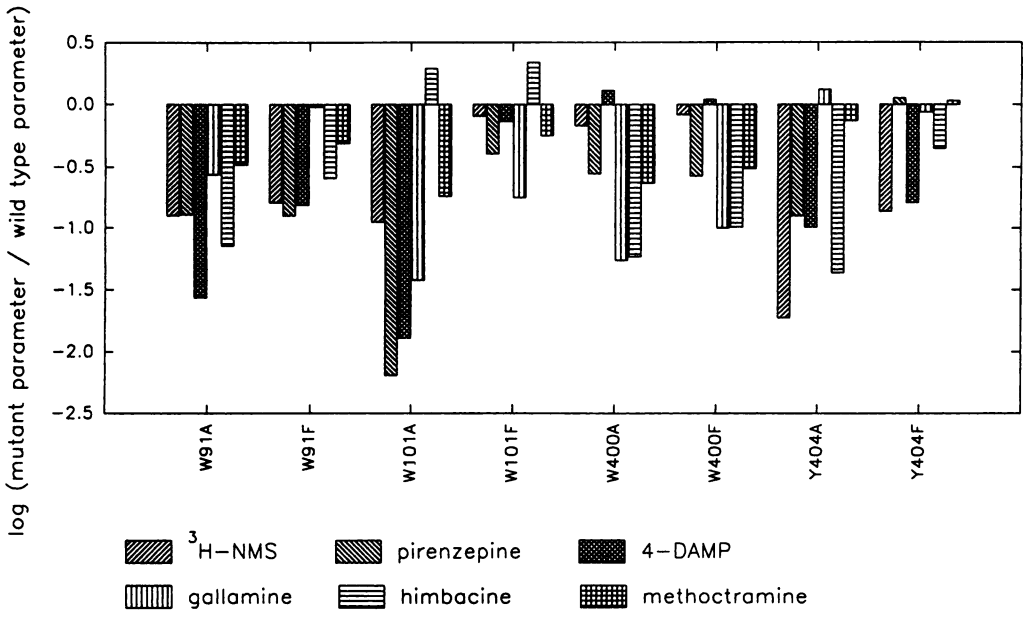

Fig. 9. Changes in mutant m1 receptor affinity for [^{3}H]NMS, pirenzepine, 4-DAMP, gallamine (unoccupied receptor), himbacine, and methoctramine, compared with the WT receptor.

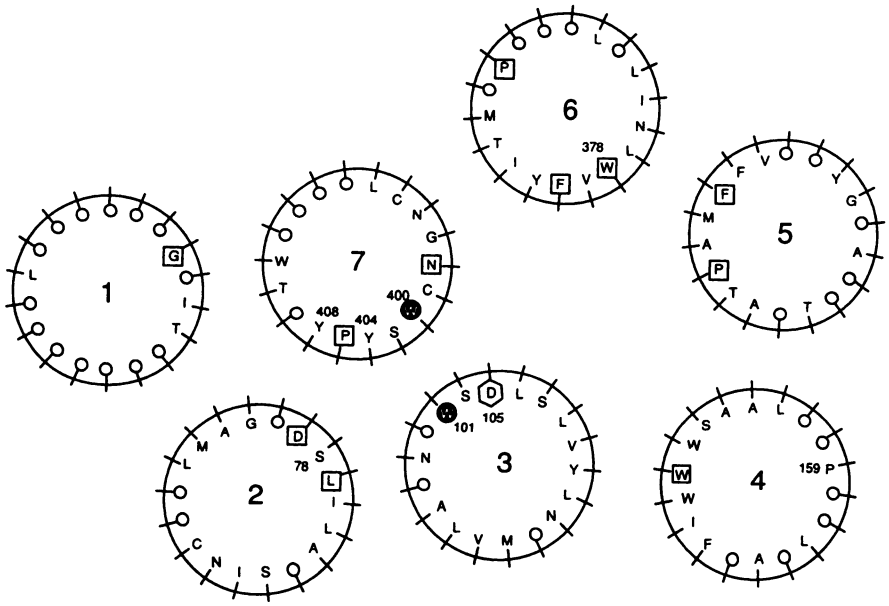

Fig. 10. Projection view of a helical wheel model of the seven transmembrane α-helices of the putative transmembrane domains of muscarinic receptors. Sequences from mammalian m1–m5 receptors are considered. Each helix is depicted as a helical wheel (100° angle). *Short lines*, orientation of the side chains. Only the extracellular 18 residues of the α-helices shown in Fig. 7 are portrayed. [For the seventh transmembrane helix, tryptophan-400 and glutamate-401 (Hm1 sequence) are included in this helical wheel.] The helices are viewed from the extracellular domain and are positioned approximately in the relative positions suggested by the projection map of rhodopsin (27). *Residues in single-letter code*, residues identical in all mammalian sequences. *Squares*, residues found in most G protein-coupled receptors. *Hexagon*, the aspartate residue in transmembrane region 3 that is conserved in all monoaminergic receptors. *Open circles*, residues that are not conserved between subtypes and species; these are generally hydrophobic and located on the exterior of the model. *Numbered residues*, residues (in the Hm1 sequence) that, in this and other studies (16, 17 20), show differing antagonist binding properties when mutated. *Reverse mode circles*, tryptophan-101 and -400.

competitive site. However, gallamine has been reported to enhance the dissociation of [^{3}H]QNB (but not [^{3}H]NMS) from muscarinic receptors (13, 14), and this finding is not compatible with the model described above. This unusual gallamine/QNB interaction is found only at low ionic strength, with gallamine slowing the off-rate of [^{3}H]QNB at higher ionic strengths. Because gallamine binding is very sensitive to ionic strength (8, 12, 13, 21), it is possible that the mode of binding of gallamine at low ionic strength may be somewhat different. Equally, QNB may bind to muscarinic receptors in a manner somewhat different from that of NMS. Indeed, there is evidence of a mutation of a transmembrane domain residue (tyrosine-381 to alanine) that strongly inhibits [^{3}H]NMS binding but has little effect on [^{3}H]QNB binding.[5] The studies in this paper concentrate on ligand-receptor interactions at higher ionic strength.

The [^{3}H]NMS kinetics were sensitive to the site of mutation. For certain mutants, e.g., Y404A and Y404F, the affinity of NMS appeared to be regulated by the dissociation rate constant. In the case of the Y404A mutant, the off-rate was so fast that it precluded accurate estimations of the affinity of gallamine for the [^{3}H]NMS-occupied receptor by measurement of the dose-response relationships for slowing [^{3}H]NMS dissociation. In contrast, the [^{3}H]NMS kinetics were slowed by mutations at tryptophan-91, -101, and -400. One may speculate that substitution of smaller amino acids at these three residues results in a constriction in the access of NMS to its binding site, which is more deeply buried in the membrane. This is counterintuitive if the tryptophan side chains provide just bulk and not structure or interaction with NMS during the binding process. These tryptophan residues may form the lining of a

[5] H. Matsui, S. Lazareno, and N. J. M. Birdsall, unpublished observations.

cleft leading to the ACh binding site, by analogy with the aromatic gorge in acetylcholinesterase, which is thought to be responsible for the facilitated access of ACh to the buried active site (22, 23). If there is a structure important for maintaining ready access of ligands to the binding site, then the loss of a transient NMS-π electron interaction or partial collapse of the structure by substitution of a smaller amino acid could explain the slowing of the kinetics.

An attempt has also been made to address the question of whether archetypal competitive antagonists such as pirenzepine and 4-DAMP are affected by the mutations in a manner similar to that of NMS and thus may bind in a similar pocket. In general, these antagonists do behave similarly, with binding being inhibited by mutations at positions 91, 101, and 404 and less so by mutations at position 400.

A complementary question is whether antagonists such as himbacine and methoctramine, which can allosterically inhibit the dissociation of [^{3}H]NMS, are affected by the same mutations that modulate gallamine binding. The prediction would be that the binding to W101A, W400A, and W400F would be strongly inhibited, whereas binding to W91A and Y404A/F should be less affected.

Himbacine, methoctramine, and gallamine binding are clearly affected by the mutations in different ways, compared with the competitive antagonists examined. The amino acid determinants for methoctramine binding to the occupied or unoccupied receptor have not been elucidated but do not seem to be tryptophan-91, tryptophan-101, tryptophan-400, or tyrosine-404. Himbacine binding to the unoccupied receptor is inhibited by mutations at tryptophan-91, tryptophan-400, and tyrosine-404, whereas gallamine binding is primarily affected by mutations at tryptophan-400 and -101 and competitive antagonists are affected by mutations at tryptophan-91, tyrosine-404, and tryptophan-101. Interestingly, himbacine binding to the unoccupied receptor is slightly and equally increased with W101F and W101A. A more dramatic effect is observed at the NMS-occupied receptor, where himbacine binding is decreased >10-fold with W101A and increased 15-fold with W101F (Fig. 6). It is not possible to say whether himbacine binds preferentially to the allosteric or competitive site, although it probably binds in the same region of the receptor (see below).

Another difference between himbacine, methoctramine, and gallamine binding is that, in certain mutants, himbacine and methoctramine are capable of only partially inhibiting [^{3}H]-NMS dissociation (Fig. 5), as has been reported for obidoxime (24), whereas other allosteric agents completely inhibit this process.

There is always a problem of interpretation of the effects of site-directed mutagenesis, in terms of changes in direct interactions or generation of conformationally induced effects, especially in the absence of high-resolution, three-dimensional, structural information. Interpretation in this study is further complicated both by 1) the very considerable variations in the structures of the ligands examined and 2) the strong negative cooperativity between the allosteric ligands and NMS. The former means that individual amino acids in a given binding site have varying contributions to the binding of different ligands. The latter phenomenon indicates that a considerable change is induced in the allosteric site by the binding of NMS (and, conversely, gallamine induces a conformational change in the competitive site). Therefore, the structure of one binding site is affected by occupancy of the other site.

This study does, however, suggest that the gallamine allosteric site is not associated with the first, second, and most of the third extracellular loops. Residues 101 and 400 are important for gallamine binding and may form part of the binding site, which, in our model, appears to be just extracellular to the antagonist binding pocket, which could contain the clustered residues aspartate-105, tyrosine-404, tyrosine-408, tryptophan-101, and serine-78. This picture of the antagonist binding site is based on both the results of this study and those of other mutagenesis studies (15–17, 20) and is somewhat similar to that suggested by modeling studies (25, 26).

The role of tryptophan-91 in binding is unexpected and indicates either that the current models of the receptor structure are incorrect, if tryptophan-91 is part of the competitive binding site, or, more likely, that this residue, which is conserved in monoamine and peptide receptors, has a structural role that is disrupted by mutation.

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