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Role of Acidic Amino Acids in the Allosteric Modulation by Gallamine of Antagonist Binding at the m2 Muscarinic Acetylcholine Receptor

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SUMMARY

The purpose of this study was to explore the role of acidic amino acids in the allosteric behavior of gallamine at the m2 receptor. This was achieved by first mutating the acidic residues to neutral residues by site-directed mutagenesis. Both the parent and mutated receptors were expressed in mouse fibroblast A9L cells and characterized pharmacologically. The two main methods used were (i) Schild analysis of equilibrium binding data and (ii) study of the effect of gallamine on the dissociation kinetics of *N*-methylscopolamine. The Schild analysis gave an estimate of the affinity of gallamine for the allosteric site (K_{dA}) and also a measure of the level of cooperativity (α) between the allosteric and primary binding sites. For the receptors studied, a good agreement was found between the αK_{dA} values calculated from the Schild analysis and the IC_{50} values for the effect of gallamine on the *N*-methylscopolamine off-rate. One mutated receptor, in which the acidic EDGE (Glu-Asp-Gly-Glu) sequence of the putative third outer domain was changed to the neutral LAGQ (Leu-Ala-Gly-Gln) sequence, displayed an 8-fold reduction in affinity for gallamine at the allosteric site, in comparison with the parent receptor. The level of cooperativity between the allosteric and primary binding sites in this mutant was 46% of that of the parent receptor. A second mutated receptor, in which Asp-97 (near the top of putative transmembrane domain 3) was changed to asparagine, was found to have a level of cooperativity between sites 58% of that of the parent but was found not to be affected with respect to the affinity of gallamine for the allosteric site. When all of the acidic groups on the outer side were changed to neutral residues, there was still only an 8.6-fold reduction in gallamine affinity for the allosteric site, but the level of cooperativity was reduced to 19% of that found in the parent receptor. The results suggest that the allosteric site for gallamine binding in the m2 receptor resides at or near the putative third outer domain and that both the EDGE motif and Asp-97 play an essential role in the interaction between the two sites. However, none of the acidic amino acids mutated were found to be critical for binding at the allosteric site.

The muscarinic acetylcholine receptors are part of a large family of membrane-bound receptors that are postulated to contain seven membrane-spanning domains and that interact with a variety of G proteins (1). The first evidence that the muscarinic receptors were not a homogeneous class came from the observation that gallamine blocked the action of acetylcholine on the heart but did not appreciably affect the action of acetylcholine in other tissues (2). Since then, other selective ligands have been discovered, and this has enabled the muscarinic receptors to be divided into three pharmacologically distinct classes, M1-M3 (3, 4). The use of gene cloning, however, has now revealed the existence of at least five distinct subtypes of muscarinic receptors, m1-m5 (5, 6). Expression and characterization of these receptors showed that two of the subtypes, m2 and m4, were coupled to the inhibition of adenylyl cyclase, whereas the other three subtypes, m1, m3, and m5, were coupled to inositol phospholipid metabolism (3, 5). The genetically defined m1-m3 subtypes were found to correspond to the pharmacologically defined M1-M3 (3).

Examination of the effect of gallamine on the negative inotropic responses to acetylcholine and carbachol in the heart led Clark and Mitchelson (7) to conclude that gallamine was acting as an allosteric antagonist. This work was extended by Stockton *et al.* (8), who found that gallamine binds to a site distinct from the principal ligand binding site and that the bound gallamine modulates ligand binding at the latter site. Since then, many compounds other than gallamine have been shown to allosterically affect binding at the primary site. These include not

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ABBREVIATIONS: CHO, Chinese hamster ovary; Hm1 and Hm2 receptors, human muscarinic acetylcholine receptors 1 and 2; o1, o2, and o3 domains, outer (extracellular) domains 1, 2, and 3; i2 and i3 domains, inner (cytoplasmic) domains 2 and 3; AF-DX 116, [11-[[2-(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMS, *N*-methylscopolamine.

only small molecules (9) but also polysaccharides, peptides, and proteins (10, 11, 12).

Early studies with various tissues demonstrated that the effect of gallamine was most evident with the heart M2 receptor (2, 8) but gallamine was a poor antagonist at muscarinic receptors in other tissues, such as bladder or salivary glands (2, 13, 14). Stockton *et al.* (8), however, showed that gallamine behaved as an allosteric antagonist in membranes from a number of rat tissues, but the strongest effect was still found with rat heart membranes. The cloned m1-m5 receptors, each expressed in stably transfected CHO cell lines, have now been tested (15). For the regulation by gallamine of the dissociation of [^{3}H]NMS from these receptors, the strongest effect was again found with the m2 receptor. However, all five subtypes showed allosteric behavior, with the strength of the behavior being m2 > m4 > m1 > m3 > m5 (15).

Because allosteric compounds such as gallamine are positively charged, or would be positively charged at physiological pH values, a reasonable working hypothesis was that the allosteric site contains an acidic amino acid that is critical for binding. To test this hypothesis, a mutagenesis program was undertaken, in which all of the acidic amino acids of the Hm2 receptor were to be replaced with neutral residues. The only acidic amino acids not to be mutated were Asp-103, postulated to be the central counter-ion in the primary binding site (16, 17), and the acidic residues of the i3 domain, with the exception of Glu-382. While this work was in progress, results from the study of allosteric behavior in three aspartate/asparagine mutant receptors derived from the rat m1 receptor were reported (18).

Experimental Procedures

Materials. Tissue culture reagents were from GIBCO BRL. The A9L cell line was from the American Type Culture Collection. [^{3}H]NMS (75–85 Ci/mmol) was from New England Nuclear/DuPont. Restriction endonucleases were from New England Biolabs. Hm2pCD was from the National Technical Information Service (Springfield, VA). pBluescriptII KS was from Stratagene, and the pSelect mutagenesis system was from Promega. All other reagents were from Sigma.

Construction of the expression vectors. To convert Hm2pCD (19) to a high-copy number vector, the Hm2 gene was first removed by *Xho*I digestion, to give pD6. The mutation in pBluescriptII KS thought to be responsible for high copy number (20) was isolated as a 1.0-kilobase *Pvu*I-*Hae*II fragment, and this was ligated to a 1.9-kilobase *Pvu*I-*Hae*II fragment from pD6, to give pD7. The *Xho*I fragment containing the Hm2 gene was ligated back into pD7, to give pD12. This change resulted in an approximately 8-fold increase in DNA yield from large-scale plasmid preparations. For future flexibility, the Hm2 gene in pD12 was deleted as a *Pst*I-*Bam*HI fragment and replaced by a polylinker, forming the general purpose, high-copy number vector pD30, a derivative of the pcD-X vector (21). The polylinker sequence inserted was *Pst*I-*Eco*RI-*Sac*I-*Apa*I-*Sma*I-*Xba*I-*Kpn*I-*Bcl*I-*Xho*I-*Bgl*II-*Not*I-(*Bam*HI destroyed).

Site-directed mutagenesis. For mutagenesis of the front end of the Hm2 receptor (Glu-22/Gln, Asp-69/Asn, and Asp-97/Asn), a 0.35-kilobase *Pst*I-*Nhe*I fragment from pD12 was first subcloned into the pSelect plasmid, using an *Nhe*I-*Eco*RI linker. Similarly, for mutagenesis of the middle region, either a 0.30-kilobase *Nhe*I-*Bcl*I [Asp-120/Asn and Glu-Asp-Gly-Glu-172–175/Leu-Ala-Gly-Gln (EDGE172–175/LAGQ)] or a 0.62-kilobase *Sac*I (Glu-382/Gln) fragment was subcloned into pSelect, using linkers where necessary. Then, to convert aspartate to asparagine, the guanine of GAC was converted to an adenine, at bases 205, 289, and 358, by oligonucleotide-directed mutagenesis, as recommended by the manufacturer (Promega). To convert glutamate to glutamine, the GAA was converted either to CAG (bases 64–66, Glu-22/Gln) or to CAA (bases 1144–1146, Glu-382/Gln), as recommended by Lathe (22), using the mutagenesis method described above. For mutation of the EDGE sequence, the amino acid sequence was changed to the equivalent Hm1 sequence LAGQ, by conversion of the base sequence GAGGATGGGGAG (bases 514–525) to CTGGCTGGGCAG by mutagenesis. The mutagenized fragments were subcloned back into the pD12 vector and the sequences were confirmed by double-stranded dideoxy sequencing.

Transient transfections. A9L cells were seeded into 10-cm plates at a density of 6×10^5 cells/plate, in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The plates were incubated at 37° in a 5% CO_2 incubator. Twenty-four hours later, cells were transfected with 15 μg of plasmid DNA/plate, by using the calcium phosphate precipitation method (23). Twenty-four hours later, the medium was replaced with fresh medium containing 4 mM butyrate (24). The cells were harvested 48 hr later.

Membrane preparation. The cells were washed twice with Dulbecco's phosphate-buffered saline (GIBCO), harvested in cold HME buffer (20 mM Na-HEPES, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), homogenized with a Brinkmann Polytron (setting 6, 20 sec), and then centrifuged at $25{,}000 \times g$ for 40 min. The pellet was resuspended in 50 mM sodium/potassium phosphate buffer, pH 7.4, by passage through a 23-gauge needle and then a 27-gauge needle and was then either used immediately or stored at −80°. Protein concentrations were determined by the method of Bradford (25), using bovine γ-globulin as the standard.

Ligand binding assays. For saturation experiments, membranes (0.05–0.1 mg of protein) were incubated with increasing concentrations (0.08–8 nM) of [^{3}H]NMS in duplicate, in a final volume of 1 ml of 50 mM sodium/potassium phosphate buffer, pH 7.4, for 60 min at 22°. Nonspecific binding was determined in the presence of 2 μM atropine. The binding reaction was terminated by filtration under vacuum through GF/B glass fiber filters (Whatman), using a Brandel cell harvester. The radioactivity on the filters was quantitated by liquid scintillation counting. For each Schild analysis, eight saturation experiments were carried out, in the absence or presence of gallamine (0.1–100 μM), for 120 min at 22°; membranes were then filtered as described above.

For dissociation kinetic studies, membranes were preincubated in duplicate with 1 nM [^{3}H]NMS, in a final volume of 0.95 ml of 50 mM sodium/potassium phosphate buffer, for 60 min at 22°. Dissociation of the [^{3}H]NMS-receptor complex was initiated by the addition of 40 μM atropine (50 μl), in the same buffer. The dissociation reaction was terminated by filtration at the times indicated in the figure legends. For studies on the effect of gallamine on dissociation, increasing concentrations of gallamine were added at the same time as atropine and then the reaction was terminated when approximately 70% of the initially bound [^{3}H]NMS would have dissociated in the absence of gallamine, as determined previously.

Data analysis. Data were analyzed with GraFit curve-fitting software (26). Saturation experiment results were fitted to the ligand-binding one-site equation of that software. For Schild analysis, the dissociation constant values for [^{3}H]NMS binding obtained from the saturation experiments performed in the presence of gallamine were fitted to the equation $DR - 1 = (\alpha - 1) \cdot [A]/(\alpha K_{dA} + [A])$, where DR (dose response, or K_d shift) $= K_d'/K_d$, K_d is the dissociation constant for [^{3}H]NMS binding in the absence of gallamine, K_d' is the dissociation constant for [^{3}H]NMS binding in the presence of gallamine, K_{dA} is the dissociation constant for binding of the allosteric ligand A to the allosteric site, and α is the cooperativity factor. The equation was derived from the work of Ehlert (27).

Results from dissociation kinetic experiments with NMS were fitted to the single-exponential decay equation of the GraFit software. The concentration of gallamine required to slow the dissociation of [^{3}H]NMS by 50% (IC_{50}) was calculated with the IC_{50} four-parameter logistic

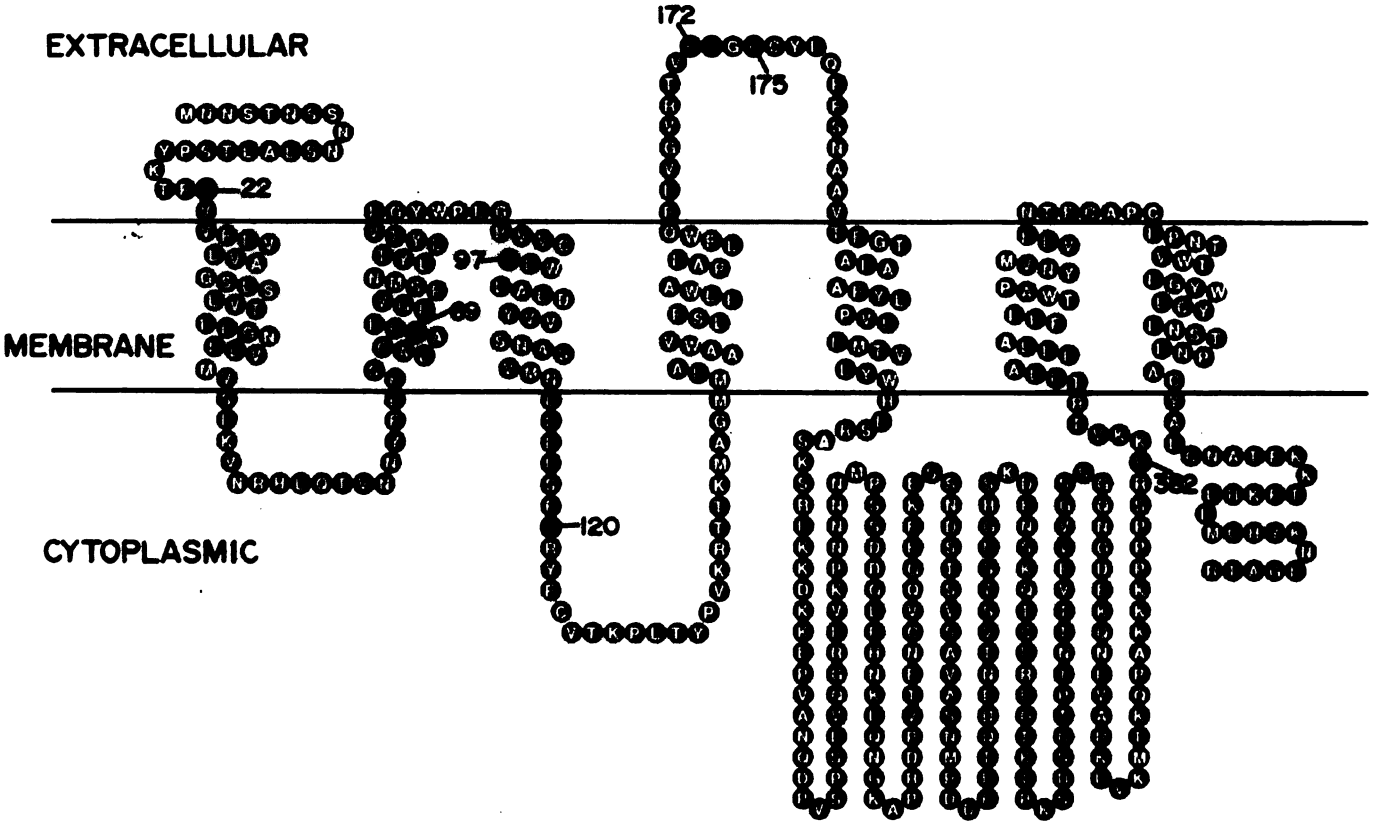

Fig. 1. Schematic representation of the Hm2 receptor. The amino acid sequence of the receptor is from Ref. 5 and is indicated by the single-letter code. The postulated seven transmembrane domains are from Refs. 29 and 37. The eight acidic amino acids mutated are shown in *black*.

equation of the GraFit software. For statistical analysis of the data, Student's unpaired *t* test was used.

Results

To explore the contribution of specific acidic amino acids to allosterism at the Hm2 receptor, the amino acids were converted to neutral residues by site-directed mutagenesis (Fig. 1). For the subsequent pharmacological characterization of the mutant receptors, the use of membrane preparations from transiently transfected cells was planned. Because this requires relatively large quantities of DNA, the vector encoding the gene for the parent Hm2 receptor, Hm2pCD, was modified before mutagenesis, to produce the high-copy number derivative pD12. This change resulted in an approximately 8-fold increase in DNA yield from large-scale plasmid preparations.

Equilibrium binding studies. The muscarinic receptor antagonist [^{3}H]NMS bound specifically to membranes prepared from A9L cells that had been transiently transfected with the various wild-type or mutant muscarinic receptor genes (Table 1). In all cases, the binding was saturatable, and the Scatchard plots were linear. The K_d values found for the mutated receptors were close to the parent value, indicating that none of the mutations had a major effect on NMS binding at the primary binding site. The large errors quoted for many of the B_{max} values are a reflection of the variability inherent in the efficiency of the transient transfection methodology. However, two of the mutated receptors (Asp-69/Asn and Asp-120/Asn) gave consistently low B_{max} values, indicating that the mutations appear to have an adverse effect on the levels of expression.

TABLE 1

K_d and B_{max} values for [^{3}H]NMS binding to Hm1 and Hm2 wild-type and Hm2 mutant receptors

Values are means ± standard errors from binding studies performed with *n* independent membrane preparations. The membranes were from transiently transfected A9L cells.

Mutation	Location of mutation[a]	*n*	K_d	B_{max}
			pM	*fmol/mg of protein*
Hm1 wild-type		3	52 ± 5	1710 ± 690
Hm2 wild-type (pD12)		5	132 ± 12	1590 ± 450
Glu-22/Gln	o1/TM1	3	114 ± 9	750 ± 160
Asp-69/Asn	TM2	4	58 ± 4	210 ± 50
Asp-97/Asn	o2/TM3	7	197 ± 14	1660 ± 740
Asp-120/Asn	i2	5	92 ± 3	160 ± 20
EDGE172-175/LAGQ	o3	6	107 ± 5	940 ± 200
Glu-382/Gln	i3 end	4	137 ± 23	1000 ± 450
Glu-22/Gln + Asp-97/Asn + EDGE172-175/LAGQ	All outer acidic amino acids	1	130 ± 4	540 ± 10

[a] TM, transmembrane domain.

To characterize the allosteric binding site and the interaction of that site with the primary binding site, sets of equilibrium saturation experiments with [^{3}H]NMS were carried out, with each set being in the absence or presence of a given level of the allosteric ligand gallamine. The dissociation constants obtained were subjected to a Schild-type analysis (27), and the results obtained are summarized in Table 2. One of the mutant receptors, that containing the mutated EDGE sequence, was found to display an 8-fold reduction in the affinity of gallamine for the allosteric binding site and a halving of the cooperativity

TABLE 2

Schild analysis of [^{3}H]NMS equilibrium binding in the presence of gallamine

Values are means ± standard errors of two or three independent experiments, with each experiment being performed with a different membrane preparation. The fold change in the values is relative to the Hm2 receptor.

Mutation	K_{d_A}		Cooperativity	
	Value	Fold change	α	Fold change
	nM			
Hm2 wild-type (pD12)	41.7 ± 2.8	1.0	74 ± 4	1.0
Glu-22/Gln	53.1 ± 8.0	1.3	80 ± 20	1.1
Asp-69/Asn	66.4 ± 3.3[a]	1.6	144 ± 5[b]	1.9
Asp-97/Asn	46.0 ± 2.4	1.1	43 ± 2[a]	0.58
Asp-120/Asn	56.3 ± 0.3[a]	1.4	81 ± 9	1.1
EDGE172-175/LAGQ	339 ± 28[b]	8.1	34 ± 5[a]	0.46
Glu-382/Gln	42.6 ± 0.5	1.0	63 ± 1	0.85
Glu-22/Gln + Asp-97/Asn + EDGE172-175/LAGQ	359 ± 66[a]	8.6	14 ± 3[b]	0.19
Hm1 wild-type	648 ± 64[b]	15.5	130 ± 16	1.8

[a] $p < 0.05$ versus the value obtained for the Hm2 wild-type receptor.
[b] $p < 0.01$ versus the value obtained for the Hm2 wild-type receptor.

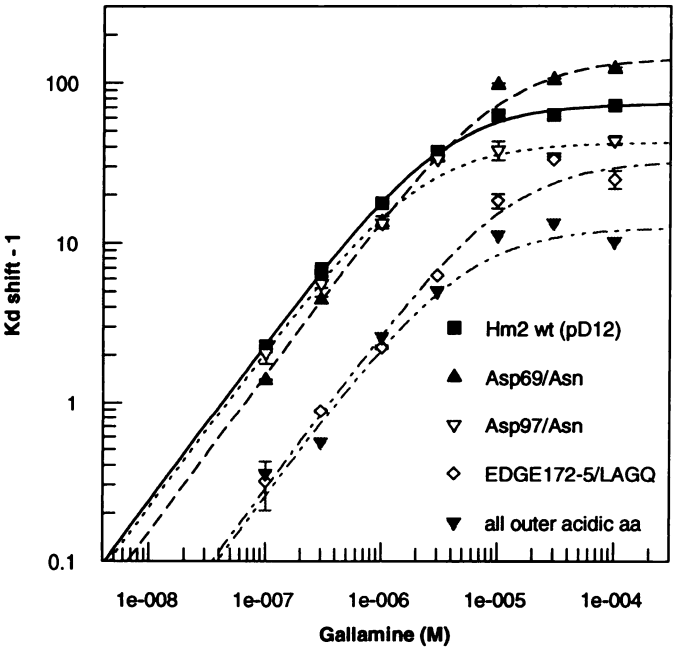

Fig. 2. Schild plots for the effects of gallamine on [^{3}H]NMS binding at the wild-type (*wt*) and Asp-69/Asn, Asp-97/Asn, and EDGE mutant Hm2 receptors. Plotted data points (mean ± standard error) are from the individual experiments described in Table 2. The *points* were fitted as described in Experimental Procedures. An absence of error bars indicates that the symbol is larger than the associated error. *aa*, amino acids.

between that site and the primary binding site (Fig. 2; Table 2). Two other mutated receptors were found to be only slightly affected with respect to their affinity at the allosteric site but to have significantly different levels of cooperativity between the two sites. For one receptor, that containing the Asp-97/Asn mutation, the cooperativity was reduced by 42%, whereas for the other receptor, that containing the Asp-69/Asn mutation, the cooperativity was almost doubled. When all of the mutations on the extracellular side were combined in one receptor, the affinity of gallamine for the allosteric site was close to that found for the EDGE mutant receptor but the level of cooperativity was reduced to only 19% of that found for the parent Hm2 receptor (Fig. 2; Table 2).

Effect of gallamine on [^{3}H]NMS dissociation. [^{3}H]NMS dissociation data for both wild-type and mutant receptors could be fitted by a single-exponential equation, which gave a good fit for the loss of at least 90% of the bound [^{3}H]NMS (Fig. 3). Some deviation from linearity could be seen, however, in the loss of the final 10% of the bound [^{3}H]NMS (Fig. 3). This deviation from linearity was not affected by the inclusion of GTPγS (0.1 mM) in the incubation of the parent Hm2 receptor (data not shown). The explanation for the deviation from linearity was not pursued further in the current work. The dissociation rate constants derived are given in Table 3. The rates found for the two glutamate mutants were close to the rate for the parent Hm2 receptor (Table 3); of the aspartate mutants, one (Asp-97/Asn) had a slightly faster NMS off-rate (1.4-fold), whereas the other two had approximately 2-fold slower off-rates, as did the EDGE172–175/LAGQ mutant.

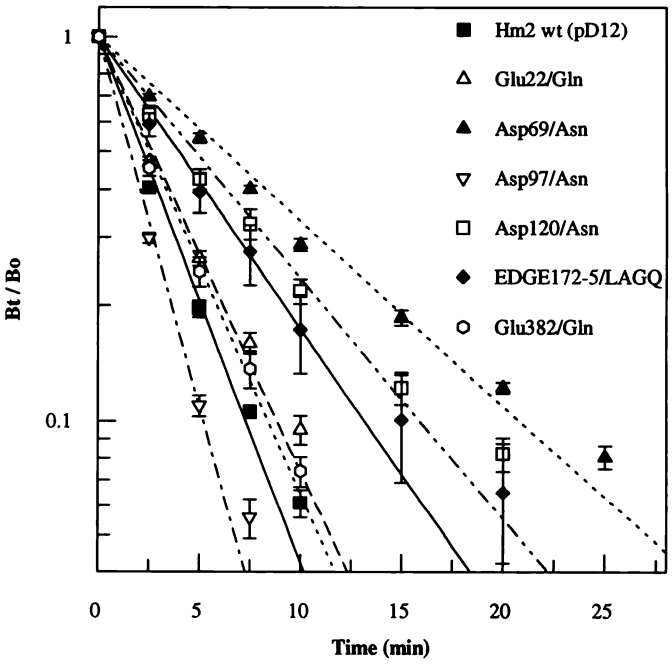

Fig. 3. Dissociation of [^{3}H]NMS from wild-type and mutant receptors. [^{3}H]NMS (1 nM) was equilibrated for 1 hr with membranes containing the individual receptors, and then atropine (2 μM) was added at time 0. The reactions were terminated by filtration at the times stated. The [^{3}H]NMS bound at time *t* is expressed as a ratio to that bound at time 0.

TABLE 3

[^{3}H]NMS off-rate results from membranes containing wild-type or mutant muscarinic receptors

Values are means ± standard errors of three independent experiments, with each experiment being performed with a different membrane preparation. The rate constant values were obtained by fitting the data with a single-exponential equation.

Mutation	Rate
	min^{-1}
Hm2 wild-type (pD12)	0.316 ± 0.017
Glu-22/Gln	0.261 ± 0.014
Asp-69/Asn	0.111 ± 0.004[a]
Asp-97/Asn	0.443 ± 0.032
Asp-120/Asn	0.145 ± 0.007[b]
EDGE 172-175/LAGQ	0.175 ± 0.011[b]
Glu-382/Gln	0.274 ± 0.012

[a] P < .01 versus the rate value obtained for the Hm2 wild-type receptor.
[b] P < .05 versus the rate value obtained for the Hm2 wild-type receptor.

To examine the effect of gallamine on the dissociation of bound [^{3}H]NMS, membrane preparations were incubated with [^{3}H]NMS for 1 hr and then dissociation was initiated by the addition of atropine plus various levels of gallamine. This was allowed to proceed for the time period previously determined to be needed for approximately 70% of the bound [^{3}H]NMS to dissociate in the absence of gallamine. The dissociation was then terminated by rapid filtration. The results obtained from the analysis of the data are shown in Table 4. Two of the mutated receptors (Asp-69/Asn and EDGE172–175/LAGQ) were found to differ significantly from the parent receptor, and the effect of gallamine on their NMS off-rates is shown in Fig. 4.

Discussion

In the present study, the role of the acidic amino acids of the Hm2 receptor in allosteric behavior was explored. This was achieved by first changing the acidic amino acids by site-directed mutagenesis and then characterizing the mutant receptors pharmacologically. Of the eight acidic amino acids that were mutated, five were changed individually, the glutamates to glutamines and the aspartates to asparagines. However, in the putative o3 domain there is a block of four amino acids, Glu-Asp-Gly-Glu (EDGE), of which three are acidic (5, 6) (Fig. 1). This acidic sequence is found only in the m2 receptor subtype, whether from human, rat, or pig (5, 6, 28). It was decided to change these as a group, from the acidic m2 sequence to the neutral sequence found in the o3 domain of the m1 receptor, Leu-Ala-Gly-Gln (LAGQ). These eight amino acids cover all of the acidic amino acids of the Hm2 receptor, with the exception of Asp-103 and those of the putative i3 domain. Mutagenic studies on Asp-105, the rat m1 equivalent of the Asp-103 of the Hm2 receptor (16), and labeling studies on muscarinic receptors in rat forebrain and striatum with [^{3}H] propylbenzilylcholine mustard (17) indicate that the aspartic acid in this position is essential for ligand binding at the primary binding site, where the positively charged nitrogen atom of the ligand probably links to the carboxylate anion of the aspartate. If correct, then this aspartate would not form part of a separate allosteric binding site. In the putative i3 domain of the Hm2 receptor, on the other hand, there are 24 acidic residues. However, only one of these residues is within 12 amino acids of either end of this domain; that residue is Glu-382, which is found six residues from the postulated start of transmembrane domain 6 (6, 29) (Fig. 1). This residue is conserved across all five muscarinic receptor subtypes (5, 6), so it was included in the mutagenesis program.

The mutated receptors were transiently expressed in mouse fibroblast A9L cells and initially characterized by [^{3}H]NMS binding in membrane preparations (Table 1). The K_d values found for all of the mutated receptors were in the range of 58–197 pM, close to the value found for the parent Hm2 receptor (132 pM) (Table 1). This suggests that none of the amino acids mutated plays a major role in NMS binding at the primary binding site. The strongest binding was found for the receptor containing the Asp-69/Asn mutation (K_d, 58 pM), 2.3-fold stronger than that for the parent. The equivalent amino acid in the rat m1 receptor has also been mutated (Asp-71/Asn), and this was also found to only slightly affect antagonist binding (16, 18). However, with the agonist carbachol there was a 5.5-fold increase in affinity but a marked attenuation of its effect on the stimulation of phosphoinositide hydrolysis (16). It was thus suggested that this residue may mediate receptor-G protein interactions (16). The weakest binding was found for the receptor containing the Asp-97/Asn mutation (K_d, 197 pM), 1.5-fold weaker than that for the parent receptor. The equivalent mutation in the rat m1 receptor, Asp-99/Asn, also produced a receptor with a decreased affinity for ligands, ranging from 1.5-fold and 1.7-fold for AF-DX 116 and NMS, respectively, to 4.1-fold for quinuclidinylbenzilate and 5.5-fold for carbachol (16).

To examine allosteric behavior, and the effect of mutations on that behavior, perhaps the two most effective approaches are (i) the use of a Schild-type analysis of equilibrium binding data and (ii) the study of the effect of a postulated allosteric ligand on the off-rate of a radiolabeled ligand from the primary binding site (9). For the Schild-type analysis, the (K_d shift − 1) values obtained from sets of [^{3}H]NMS saturation experiments, with each set being performed in the presence of a given concentration of the secondary ligand gallamine, are plotted against the gallamine concentration (Fig. 2). If both ligands compete for the same site on the receptor, then a straight line is obtained in a log/log plot (27). However, if there is an allosteric interaction between two sites and the cooperativity factor is not too large, then a curvilinear line is obtained, as was found here (Fig. 2) and by others (8, 18, 30, 31) for these

TABLE 4

Effect of gallamine on [^{3}H]NMS dissociation

Values are means ± standard errors of two to four independent experiments, with each experiment being performed with a different membrane preparation. The NMS bound at the end of the time stated was expressed as a percentage of total bound, and the IC_{50} values were calculated. The k_{off} values were also derived from the dissociation experiments, and the IC_{50} values were calculated (Fig. 4).

Mutation	Time	Percentage bound curve		k_{off} curve	
		IC_{50}	Hill slope	IC_{50}	Hill slope
	min	*μM*		*μM*	
Hm1 wild-type	30	78.4 ± 6.2[a]	1.52 ± 0.05[a]	54.2 ± 3.8[a]	1.48 ± 0.06[a]
Hm2 wild-type	4	12.1 ± 0.3	1.00 ± 0.03	6.8 ± 0.2	0.97 ± 0.03
Glu-22/Gln	5	10.7 ± 1.1	1.18 ± 0.05	6.7 ± 1.0	1.15 ± 0.04
Asp-69/Asn	11	30.4 ± 3.4[b]	1.64 ± 0.06[b]	21.2 ± 1.3[a]	1.60 ± 0.06[b]
Asp-97/Asn	4	11.3 ± 1.1	1.05 ± 0.01	4.7 ± 0.2[b]	0.99 ± 0.01
Asp-120/Asn	9	9.7 ± 0.6	1.08 ± 0.01	6.2 ± 0.6	1.05 ± 0.01
EDGE172-175/LAGQ	8	40.6 ± 3.7[b]	1.74 ± 0.23[b]	28.2 ± 0.5[a]	1.70 ± 0.25[b]
Glu-382/Gln	5	11.9 ± 1.5	0.95 ± 0.24	6.2 ± 0.1	0.92 ± 0.23

[a] $p < 0.01$ versus the value obtained for the Hm2 wild-type receptor.
[b] $p < 0.05$ versus the value obtained for the Hm2 wild-type receptor.

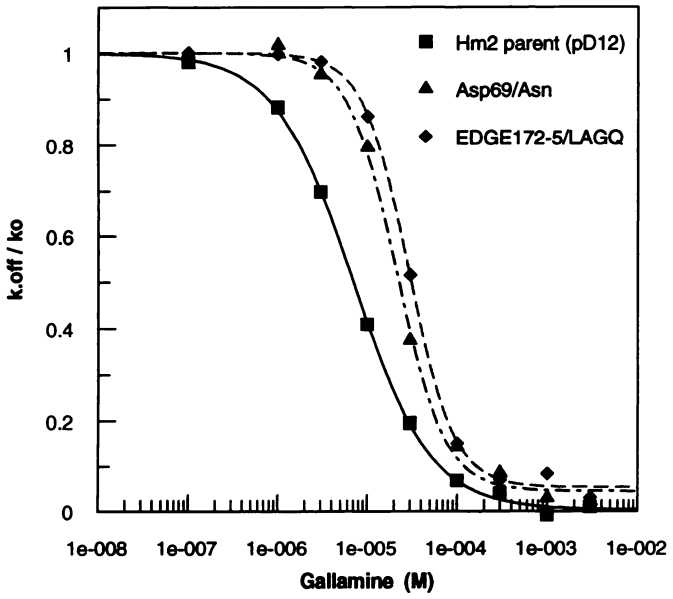

Fig. 4. Inhibition by gallamine of [^{3}H]NMS dissociation from wild-type and Asp-69/Asn and EDGE mutant Hm2 receptors. [^{3}H]NMS (1 nM) was equilibrated for 1 hr with membranes containing the individual receptors, and then atropine (2 μM) and gallamine were added at time 0. The k_{off} values calculated for the off-rates in the presence of gallamine are expressed as a ratio of k_{off} to k_o (the k_{off} value in the absence of gallamine).

ligands. From such an analysis, two estimates are obtained, (i) the K_{dA} value for the binding of the allosteric ligand at the allosteric site in the absence of any ligand binding at the primary binding site and (ii) the cooperativity factor α (27). In the current work, the affinity of gallamine for the allosteric site in the Hm2 receptor was found to be 41.7 ± 2.8 nM, and the cooperativity between the two sites was found to be 74 ± 4 (Table 2). By comparison, with the Hm1 receptor the affinity for gallamine was 15.5-fold weaker (K_{dA} = 648 ± 64 nM), but the cooperativity was 1.75-fold stronger (α = 130 ± 16) (Table 2). The values found for the Hm1 receptor are a little stronger than those reported by Lee *et al.* (18) for the rat m1 receptor expressed in CHO cells (K_{dA} = 1140 nM, α = 74.5). One disadvantage of the Schild analysis, however, is that if the cooperativity factor is too high then it may not be possible to achieve a high enough concentration of the competing ligand to see the curve begin to plateau. This was found in the case of the rat m3 receptor (30).

In the second approach, the effect of various concentrations of a competing ligand on the off-rate of a tracer ligand from the primary binding site is examined. A change in the off-rate is probably the most straightforward way of demonstrating an allosteric interaction (9). However, if both a mutated receptor and its unmutated parent display the same off-rate change in the presence of the allosteric ligand, this does not necessarily mean that the amino acid mutated is not involved in the allosteric interaction, as shown below. The binding results obtained are normally reported as a percentage (or as a ratio) of the tracer ligand bound at time 0, but a more meaningful representation (in terms of the underlying mechanism) involves determination of the k_{off} values (Fig. 4). The IC_{50} results calculated from the k_{off} values were found to be lower than those calculated using the percentage bound values, but the Hill slopes were found to be essentially unchanged (Table 4).

With the off-rate studies, because one would be observing only the effect of the binding of gallamine to those receptors already occupied by NMS, then the dissociation constant for gallamine at the allosteric site would be equal to αK_{dA}. When the αK_{dA} values calculated from the Schild analysis data (Table 2) were compared with the IC_{50} results obtained from the k_{off} values (Table 4), only an approximately 2-fold difference was found (Table 5). The correlation was even better when the fold changes, relative to the parent Hm2 receptor, were compared for the two methods (Table 5). Consequently, if a mutant receptor is affected in its allosteric behavior, as revealed in a Schild analysis, but the αK_{dA} calculated for the mutant is close to that calculated for the unmutated parent receptor, then the IC_{50} for the gallamine effect on the off-rate should be approximately the same for both receptors. An example of this situation was found with the Asp-97/Asn mutant (see below); other examples have been reported by Lee *et al.* (18). The actual IC_{50} values calculated here from the percentage bound data for the Hm1 receptor (78.4 μM) and the Hm2 receptor (12.1 μM) are comparable to those reported by Lee and El-Fakahany (30) for the rat m1 receptor expressed in CHO cells (157 μM) and for the rat heart m2 receptor (14 μM).

Of the first mutant receptors constructed, one was found to have an 8-fold reduction in the affinity for gallamine at the allosteric site (Fig. 2; Table 2). In that mutant, the acidic EDGE sequence in the putative o3 domain of the Hm2 receptor had been changed to the equivalent neutral LAGQ sequence of the Hm1 receptor (5, 6). The K_{dA} value calculated from the Schild analysis for the mutant receptor was 339 nM, 8.1-fold larger than that for the parent Hm2 receptor and 1.9-fold smaller than that for the Hm1 receptor. Thus, the EDGE sequence can in large part explain the difference between the affinities of gallamine for the allosteric sites of the Hm1 and Hm2 receptors. The cooperativity between the allosteric and primary binding sites in this mutant receptor was less than half that found for the parent receptor (Table 2). This is in the opposite sense to that found for the Hm1 receptor, because the level of cooperativity found for the Hm1 receptor was 1.75-fold higher than that for the Hm2 receptor. Thus, the EDGE sequence may play a role in the interaction between the sites in the Hm2 receptor, but different amino acids would be involved in intersite cooperativity in the Hm1 receptor. When the effect of gallamine on the NMS off-rate was tested with the EDGE mutant receptor, a change was still observed (Fig. 4) but the IC_{50} was 28 μM, 4-fold larger than that for the Hm2 parent (Tables 4 and 5). The Hill slope for the gallamine

TABLE 5

Comparison of the affinities derived from the Schild analyses with the IC_{50} values obtained from the dissociation experiments

Values are the means ± standard errors of two to four independent experiments, with each experiment being performed with a different membrane preparation. The IC_{50} values are from the k_{off} data (Table 4). The fold change in the values is relative to the Hm2 parent receptor.

Mutation	αK_{dA}		IC_{50}	
	Value	Fold change	Value	Fold change
	μM		μM	
Hm2 wild-type (pD12)	3.1 ± 0.1	1.0	6.8 ± 0.2	1.0
Glu-22/Gln	4.1 ± 0.4	1.3	6.7 ± 1.0	1.0
Asp-69/Asn	9.5 ± 0.8	3.1	21.2 ± 1.3	3.1
Asp-97/Asn	2.0 ± 0.1	0.6	4.7 ± 0.2	0.7
Asp-120/Asn	4.6 ± 0.5	1.5	6.2 ± 0.6	0.9
EDGE172-175/LAGQ	12.2 ± 3.2	4.0	28.2 ± 0.5	4.1
Glu-382/Gln	2.7 ± 0.0	0.9	6.2 ± 0.1	0.9

concentration-effect curve was also changed (Fig. 4; Table 4), from 1.00 for the parent receptor to 1.74 for the mutant receptor. The reason for this is unknown, but it is of interest to note that the only two mutant receptors with a significant increase in the Hill slope are those two displaying a significant increase in the IC_{50} for gallamine (Table 4). More detailed studies on the off-rates would be needed to understand this Hill slope change. Also needed is an examination of individual amino acid mutations in the EDGE sequence, to determine which of the acidic amino acids are responsible for the changes discussed above.

Two additional mutant receptors were found to be affected with respect to the interaction between the primary and allosteric sites (Table 2). However, both were largely unaffected with respect to the affinity of gallamine for the latter site. For one, Asp-97/Asn, the cooperativity was about 40% lower than that for the parent Hm2 receptor (Table 2). The IC_{50} for the effect of gallamine on the NMS off-rate, on the other hand, was close to that found for the Hm2 receptor (Table 4), which at first was surprising, considering that the cooperativity was weakened. However, the αK_{dA} values calculated for the mutant and parent receptors are close (Table 5), so the IC_{50} values are explicable. The Hill slope for the gallamine concentration-effect curve is close to unity (Table 4), so this mutant differs from the other two mutant receptors with a significantly altered level of cooperativity. The equivalent mutation in the rat m1 receptor, Asp-99/Asn, has also been studied (18). The results reported were similar to those found here, except that the affinity of gallamine for the allosteric site was increased approximately 2-fold.

The second mutant receptor to be affected with respect to the interaction between the two sites was Asp-69/Asn. For this receptor the level of cooperativity was almost doubled, and there was a slight but significant increase in the K_{dA} value (Table 2). The effect of gallamine on the NMS off-rate was also weakened (Fig. 4; Table 4), in line with the αK_{dA} value (Table 5). The Hill slope for the gallamine concentration-effect curve was also increased significantly, as discussed above. These results are in contrast to those found for the equivalent rat m1 mutant, Asp-71/Asn (18). With that mutated receptor, the cooperativity was one third that of the m1 parent and the K_{dA} value was 3.1-fold higher, but the effect of gallamine on the NMS off-rate, with regard to either the IC_{50} or the Hill slope, was essentially the same as for the parent m1 receptor. Additional work will be needed to determine the reason for these differences.

Because of the aforementioned results, all of the extracellular mutations (Glu-22/Gln, Asp-97/Asn, and EDGE172–175/LAGQ) were combined in one receptor and characterized by Schild analysis (Fig. 2; Table 2). The affinity of gallamine for the allosteric site was close to that found for the EDGE mutant alone, thus supporting the individual mutant results showing that neither Glu-22 nor Asp-97 is involved in gallamine binding at the allosteric site. However, the combined mutant had only 19% of the level of cooperativity of the Hm2 parent, implying that both the EDGE sequence and Asp-97 are involved in intersite interactions.

In the primary binding site, experimental evidence suggests that the Asp-103 residue in the middle of the third transmembrane domain, or its equivalent in other muscarinic or adrenergic receptors, is the central point of attachment for ligands (16, 17, 32) and that its replacement with asparagine by mutagenesis results in loss of detectable binding (16, 32). No such dramatic loss of gallamine binding was found for any of the mutant receptors discussed in this paper, indicating that none of the acidic amino acids mutated is critical for binding at the allosteric site. This leaves three main alternatives. Firstly, more than one acidic amino acid may be involved in the binding, especially with the triply charged gallamine molecule. Indeed, the only mutant receptor found to have a reduced affinity for gallamine was the EDGE mutant, with three acidic residues. However, the results discussed in the previous paragraph would argue that, for more acidic amino acids to be involved, gallamine would have to bind to both the outer EDGE sequence and internal acidic residues, thus spanning the primary binding site. The second alternative is that amino acids other than acidic ones are central to gallamine binding. The construction of chimeric receptors would help to clarify this possibility. The third possibility is that one or more of the 23 acidic amino acids in the i3 domain not examined here is involved in allosteric binding. This is considered to be unlikely, mainly because the results with the EDGE mutation suggest that the allosteric site is on the outer side of the receptor, at or near the o3 region.

Thus, the results suggest that there may be at least three sites in the muscarinic receptor for allosteric modulation of ligand binding. The first site would be for the binding of compounds such as gallamine. The EDGE mutation results indicate that, in the case of the m2 receptor at least, this site is on the outer surface of the receptor, possibly at or near the o3 region. In addition, the Asp-97/Asn mutant results suggest that the interaction of this allosteric site with the primary site involves the Asp-97 residue, located near the top of transmembrane domain 3. The second allosteric site would be for the binding of compounds such as heparin (10) and protamine (11). The evidence suggests that this site is on the inner surface of the muscarinic receptor (10, 11) and, in the case of the rat m1 receptor (11), may involve (with protamine) the Asp-122 residue, which is located in the i2 domain. The region containing this aspartate may also be one of the areas involved in the G protein-binding domain of muscarinic and adrenergic receptors (33), and it has been proposed that heparin acts by interfering with receptor-G protein coupling (10). The equivalent residue in the Hm2 receptor, Asp-120, was found here to have little, if any, role in the allosteric interaction of the receptor with gallamine. The third site would be for the interaction of the receptor with anions such as Na^+. Such anions modulate the binding of ligands to muscarinic receptors (10, 34, 35). It has been shown by Horstman *et al.* (36) in the α_2-adrenergic receptor that the site for allosteric regulation by Na^+ is the Asp-79 residue. The equivalent residue in the Hm2 receptor is Asp-69; thus, this could be the site for Na^+ regulation in the Hm2 receptor. The Asp-69/Asn mutation in the Hm2 receptor was found here to have only a slight effect on gallamine affinity, indicating that this residue is not part of the gallamine allosteric binding site.

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