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Site-Directed Mutagenesis Reveals Two Epitopes Involved in the Subtype Selectivity of the Allosteric Interactions of Gallamine at Muscarinic Acetylcholine Receptors

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ABSTRACT

Gallamine allosterically modulates the binding of classical muscarinic ligands with a potency order of $M_2 > M_1,M_4 > M_3,M_5$. We have suggested previously that the M_2/M_5 and M_2/M_3 selectivities are attributable to an epitope in the sixth transmembrane region or third outer loop (o3) region of the receptor. In this study, analysis of numerous point mutations in this region of the M_5 receptor found that a mutation of V → N resulted in an increased affinity toward gallamine, suggesting that the asparagine residue at M_2^{419} is responsible for gallamine's M_2/M_5 selectivity. Mutations in the other subtypes indicated that the acidic residues found at this position in M_1 and M_4 are associated with slightly higher affinity toward gallamine, whereas the valine and lysine residues of M_5 and M_3, respectively, are associated with significantly lower affinity. In the o2 region, replacement of an acidic sequence of M_2 (EDGE) by the corresponding neutral sequence of M_1 (LAGQ) reduced the affinity toward gallamine, as reported previously by others; the converse substitution of the acidic sequence into M_1 significantly increased affinity for gallamine. Substitution of the M_1 sequence into this region of M_5 markedly reduced affinity toward gallamine, whereas substitution into M_4 had no effect. All of the above mutations are consistent with gallamine binding with a similar orientation at each subtype, such that it interacts with acidic residues in the o2 region of M_3 and M_5 and with acidic residues in the o3 region of M_1 and M_4; gallamine appears to interact with both regions of the M_2 subtype.

Muscarinic acetylcholine (ACh) receptors belong to the superfamily of receptors that share structural homology with rhodopsin and couple to G proteins. All five subtypes of muscarinic receptors bind the endogenous agonist ACh and have similar affinities for classical competitive antagonists such as atropine and scopolamine. In addition, muscarinic receptors are capable of binding a second ligand at a well defined allosteric site (Ellis and Seidenberg, 1992; Waelbroeck, 1994). Allosteric interactions between ligands are quite common at receptors that possess intrinsic ion channels; a notable example is the γ-aminobutyric $acid_A$ receptor, with its associated sites for benzodiazepines and other drugs. From another viewpoint, the hallmark of seven-transmembrane-domain (7TM) receptors is the allosteric interaction between agonist and receptor/G protein coupling. However, ligand–ligand allosteric interactions at the external surface of 7TM receptors have been well documented only at muscarinic receptors, α_2-adrenergic receptors, and A_1-adenosine receptors (Birdsall et al., 1995; Leppik et al., 1998). Thus, at present, ACh appears to be the only transmitter for which a complete family of 7TM receptors is sensitive to ligand–ligand allosteric interactions.

The very large number of receptors in the 7TM superfamily and the high degree of conservation of some TM residues throughout the family have allowed for a significant amount of sharing of structural and functional information across receptors (Baldwin et al., 1997; Schwartz et al., 1997). Within subgroups of the superfamily, even greater structure/function homology may exist. For example, within the subset of biogenic amine receptors, which includes the muscarinic receptors, many studies have pointed to the crucial importance of an aspartate residue in TM3 (Strader et al., 1987, 1994). This conclusion has been supported by mutagenic studies of muscarinic receptors (Fraser et al., 1989; Page et al., 1995) and by peptide analysis of receptors labeled by irreversible muscarinic agonists and antagonists (Curtis et al., 1989; Kurtenbach et al., 1990; Spalding et al., 1994). On the other hand, the rarity of ligand–ligand interactions at 7TM receptors and the lack of irreversible allosteric ligands have hampered progress in delineating the structural features of the

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ABBREVIATIONS: ACh, acetylcholine; 7TM, seven-transmembrane-domain; TM, transmembrane region of the receptor; o2, o3, the second and third outer (extracellular) loops of the receptor; NMS, *N*-methylscopolamine; CR, chimeric receptor; PB, sodium-potassium-phosphate buffer, pH 7.4.

binding site(s) for muscarinic allosteric agents. Nonetheless, a number of observations have suggested that muscarinic allosteric ligands bind near to the extracellular entrance to the ACh-binding pocket of the receptor. This location is in agreement with the nearly universal property of these ligands to dramatically slow the kinetics of binding of classical muscarinic antagonists (Stockton et al., 1983; Proska and Tucek, 1994); the binding of allosteric ligands also appears to protect the classical binding site from protein-modifying reagents (Jakubik and Tucek, 1994).

Gallamine was the first muscarinic allosteric ligand to be identified (Clark and Mitchelson, 1976; Stockton et al., 1983) and has been the ligand studied most intensively. All of the mutagenic studies of the muscarinic allosteric site that have been published to date have used gallamine as the allosteric ligand (Lee et al., 1992; Ellis et al., 1993; Leppik et al., 1994; Matsui et al., 1995). Of these studies, two have taken advantage of the subtype selectivity of gallamine. We examined chimeric receptors composed of pieces of high-affinity subtype sequence (M_2) embedded in a background of low-affinity sequence (M_5). From these studies, we identified a small portion of the receptor sequence, which included the third outer loop (o3; between TM6 and TM7), that seemed to be uniquely important in conferring M_2/M_5 selectivity toward gallamine (Ellis et al., 1993). Leppik et al. (1994) found that they could markedly reduce affinity toward gallamine by replacing a four-residue segment of o2 in the M_2 subtype with the corresponding sequence of the M_1 receptor. From one point of view, this finding seemed to conflict with our chimeric studies, because different epitopes were implicated in the binding of gallamine to the M_2 receptor. However, based on comparative sequences, we have noted that the combined results might be explained by the presence of a common gallamine-binding epitope within o2 if it were present in both M_2 and M_5, but absent in M_1 (Ellis, 1997).

This study extends our chimeric study by identifying a specific residue in o3 that influences gallamine binding. It also confirms and extends the importance of the sequence identified by Leppik et al. (1994) in o2. Finally, it appears likely that each of the muscarinic receptor subtypes derives affinity toward gallamine from one or the other of these epitopes in the outer loops, except for M_2, in which both epitopes appear to be important. Some of these results have been reported recently in preliminary form (Ellis et al., 1999).

Experimental Procedures

Materials. Atropine sulfate and gallamine triethiodide were obtained from Sigma Chemical Co. (St. Louis, MO). Labeled *N*-methylscopolamine chloride ([^{3}H]NMS; 82–85 Ci/mmol) was obtained from NEN-DuPont (Boston, MA).

Mutagenesis and Expression. Site-directed mutagenesis was performed with muscarinic receptor DNA in pcD plasmids, either with the Altered Sites II kit (Promega, Madison, WI) or the QuikChange kit (Stratagene, Inc., La Jolla, CA). Briefly, oligonucleotides containing the desired base changes were synthesized and allowed to anneal with a vector containing the appropriate muscarinic receptor DNA sequence. A high-fidelity polymerase then extended these synthetic oligonucleotides. With the Altered Sites II kit, an appropriate region of the receptor gene was first cloned into the pAlter-Ex1 vector, and single-stranded DNA was produced with a helper phage. After mutagenesis, mutated double-stranded DNA was cloned back into the pcD vector. With the QuikChange kit, mutagenesis was conducted directly in the pcD vector, and parental DNA was digested by a methylation-specific endonuclease. In either case, mutations were confirmed by sequencing.

Plasmids containing wild-type or mutated receptor genes were purified from bacterial cultures and transfected into COS-7 cells by calcium phosphate precipitation. Cells were harvested 72 h after transfection by scraping into 5 mM sodium-potassium-phosphate buffer (PB), pH 7.4. After homogenization and centrifugation at 50,000*g* for 20 min, membranes were resuspended in 5 mM PB and stored as aliquots at −70°C.

Dissociation Binding Assays. Binding assays were conducted in 5 mM PB at 25°C. Membranes (~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 the indicated concentration of gallamine, and the incubation was allowed to continue for the appropriate time. The incubation was terminated by filtration through S & S no. 32 glass fiber filters (Schleicher & Schuell, Keene, NH), followed by two rinses with 40 mM PB (0°C). Nonspecific binding was determined by the inclusion of 3 μM atropine during the prelabeling period.

Dissociation assays were used throughout, because they guarantee that allosteric effects are being measured; the data from the dissociation assays 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 (k_{obs}) and divided by the true rate constant (k_0), determined in the presence of 3 μM atropine only. Thus, the resulting number indicates a dissociation of [^{3}H]NMS slower than the control rate when it is <1. 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 an allosteric ligand on the dissociation of [^{3}H]NMS should be proportional to the occupancy of the allosteric site, as previous studies have confirmed (Ellis et al., 1992, 1997). Therefore, data from these experiments were fitted to the following equation:

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

where A is the concentration of the allosteric modulator (gallamine), m is the maximal reduction in the rate constant that can be exerted by the modulator, and K_{app} is the apparent equilibrium dissociation constant (for the interaction between gallamine and the NMS-bound form of the receptor). The rate constants for the dissociation of [^{3}H]NMS from the receptors in the absence of allosteric ligands (k_0) correspond to half-times that range from <5 min (M_2) to >1 h (M_5). For these studies, values for m were routinely constrained to lie between 0 and 1, and K_{app} was required to be a positive number. Curve-fitting was performed with the Scientist program (MicroMath, Salt Lake City, UT).

Results

The sequences of the M_2 and M_5 subtypes of muscarinic receptors show a large degree of identity in the TM6 region, but less in the o3 region. Of the 31 amino acids in the segment of the receptors that was swapped in chimeric receptor (CR) 4 in a previous study (Ellis et al., 1993), all but 9 are identical (Fig. 1). A series of mutant M_5 receptors was created, such that one of these nine residues was mutated in isolation in each receptor, except that the Val ⇒ Ile mutation at the position corresponding to M_2^{417} was not tested. Additionally, in the two cases in which there are adjacent differences between M_2 and M_5 (i.e., the positions corresponding to $M_2^{409\text{-}410}$ and $M_2^{414\text{-}415}$), double mutations were also created. Thus, a total of 10 mutated M_5 receptors were investigated. These discrete mutations were evaluated for their effects on the ability of gallamine to allosterically regulate the receptors, relative to gallamine's effects on the wild-type M_2 and M_5 receptors and the chimeric M_2/M_5 receptor CR4 (Fig. 2). Most of these mutations resulted in receptors with characteristics that did not differ significantly from the wild-type M_5 receptor. Three mutations resulted in significantly different affinities. The replacement of the M_5 Ser with Asn (at position M_2^{410}) gave a very low affinity for gallamine (Fig. 3). This anomalous result may be related to the importance of this position in constitutive activation of the receptor (Spalding et al., 1997), but clearly cannot be the explanation for gallamine's preference for the M_2 subtype over the M_5 subtype (see *Discussion*). The replacement of the M_5 Lys with Pro (at position M_2^{415}) resulted in a receptor with an affinity equal to that of CR4. This raised the possibility that the unique structural effects of proline might be an organizing factor in the preference of gallamine for the M_2 subtype (see *Discussion*). However, in this case, the double (adjacent) mutation provides additional insight. Where $M_2^{414-415}$ is Ala-Pro, the homologous M_5 sequence is Asp-Lys. Mutating the M_5 aspartate to alanine had no effect on gallamine's affinity by itself, but that mutation prevented the effect of the Lys ⇒ Pro mutation (Fig. 3). Thus, it appears that the insertion of the proline into M_5 allows an anomalous interaction with the aspartate that does not occur in the wild-type M_5. Furthermore, this interaction cannot be responsible for the greater affinity toward the M_2 subtype, because M_2 lacks that aspartate (see *Discussion*). This leaves us with the asparagine at M_2^{419}. When the valine residue at this position in the M_5 receptor is replaced by asparagine, the resulting receptor exhibits an affinity approximately equal to that of CR4. The sequences of the o3 regions of the five muscarinic receptor subtypes are shown in Fig. 4. The M_1 and M_4 receptors contain acidic amino acids at the positions corresponding to the M_2^{419} Asn, whereas the corresponding M_3 residue is lysine. Previous studies with chimeric M_2/M_3 receptors have demonstrated that swapping regions containing the o3 loops between these two subtypes yields results that are in agreement with the M_2/M_5 chimeric receptors (Ellis et al., 1993). Substitutions at the M_2^{419} position produced results that are consistent with these previous studies. Replacement of the asparagine in M_2 by lysine markedly reduced the affinity toward gallamine, whereas replacement of the lysine in M_3 by asparagine markedly increased the affinity toward gallamine (compare Fig. 5, A and B). On the other hand, the acidic amino acid aspartate is associated with somewhat higher affinity toward gallamine than is asparagine, in both M_2 and M_4 (compare Fig. 5, A and C); the affinity of M_2 toward gallamine also is slightly enhanced when the asparagine is replaced by the glutamate found in M_1 (Fig. 5A).

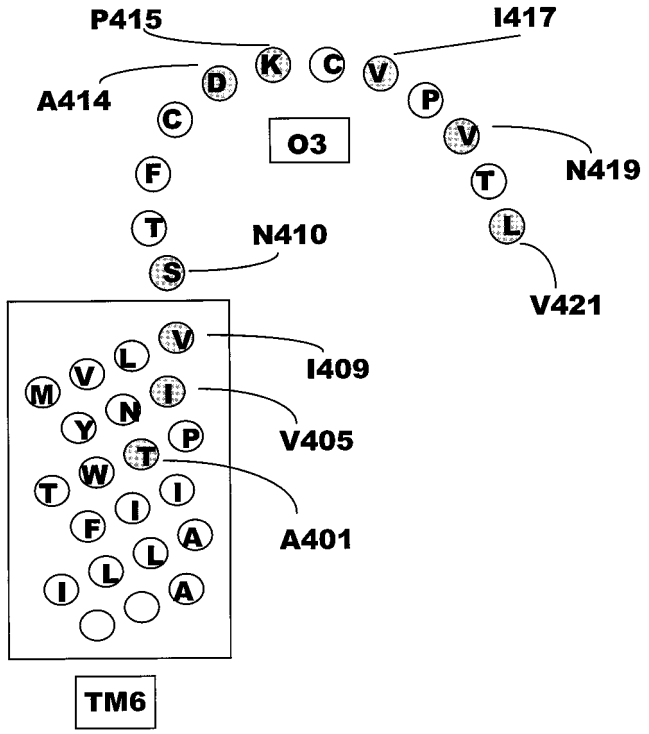

Fig. 1. Amino acid residues that differ between the M_2 and M_5 subtypes, in TM6 and o3. The circled letters represent the M_5 sequence. The numbered letters denote the sequence (and residue number) of the M_2 receptor where it differs from the M_5 receptor.

Although our chimeric studies did not indicate an involvement of o2 in the subtype specificity of gallamine for M_2 over M_5, other studies have suggested that a cluster of acidic amino acids in this region of the M_2 receptor is related to gallamine's affinity. Leppik et al. (1994) found that the replacement of the M_2 sequence Glu-Asp-Gly-Glu (Fig. 6) by the corresponding M_1 sequence (Leu-Ala-Gly-Gln) resulted in a significant reduction in affinity toward gallamine. We have confirmed their result (Fig. 7B) and extended it to the reverse substitution in M_1. That is, insertion of the acidic sequence into the M_1 receptor results in a significant increase in affinity toward gallamine (Fig. 7A). These regions of the M_1 and M_4 receptors appear to be equivalent with regard to gallamine's allosteric actions, because replacement of the M_4 sequence by the M_1 sequence produced no effect (Fig. 7C). We have suggested that the reason that this region was not implicated in our M_2/M_5 chimeras may be that the M_5 subtype contains a similar gallamine-binding epitope in this region (Ellis, 1997). In agreement with this suggestion, the affinity toward gallamine was reduced markedly when the acidic amino acids in this region of the M_5 subtype (Asp-Glu) were replaced by the corresponding M_1 sequence (Gly-Gln) (Fig. 7D).

The effects of mutation on the dissociation rate of [^{3}H]NMS (k_0) and on the parameters m and K_{app} are summarized in Table 1. The affinity of [^{3}H]NMS did not appear to be affected very much by these mutations but was not determined at every construct; this affinity does not enter into the analysis of the dissociation data generated in this study in any way. A

few mutations did produce small but noticeable changes in k_0 and m, but we do not think that they are relevant to this study (see *Discussion*).

Discussion

Two different approaches have been adopted in previous mutagenic studies of the binding of gallamine to muscarinic allosteric sites. One approach has assumed that there is a core binding component common to all five subtypes, analogous to the aspartate residue in TM3 of biogenic amine receptors; investigators using this approach have targeted likely conserved residues (Lee et al., 1992; Matsui et al., 1995). Another approach, which we have used, assumes that the ligand adopts a common orientation at each subtype, regardless of whether there is a common binding component. This strategy uses chimeric receptors to look for epitopes that may represent structural bases for subtype-specific differences in affinity. Although this chimeric approach cannot locate a common core binding component directly, it does offer the advantage of providing a natural structure to mutagenic studies. In our case, we expected to be able to increase the affinity of low-affinity M_5 and M_3 receptors by providing a structural feature from the high-affinity M_2 receptor, and we found that to be the case. We also demonstrated the reciprocal effect of making the converse chimera (Ellis et al., 1993). On the other hand, mutations of conserved residues are very much more likely to reduce affinity than to increase affinity, and there is no "converse" mutation to be considered. Because of this, it is more difficult to discriminate specific disruption of a binding epitope from a nonspecific deleterious effect on the receptor.

Leppik et al. (1994) adopted an intermediate approach, mutating both conserved and subtype-specific residues. The most striking effect of their study was found by an essentially chimeric approach, wherein they converted a four-residue sequence of the M_2 receptor to its M_1 counterpart and significantly reduced affinity toward gallamine. For the reasons outlined above, we were anxious to examine the converse chimera. In this study, we have observed the same affinity-

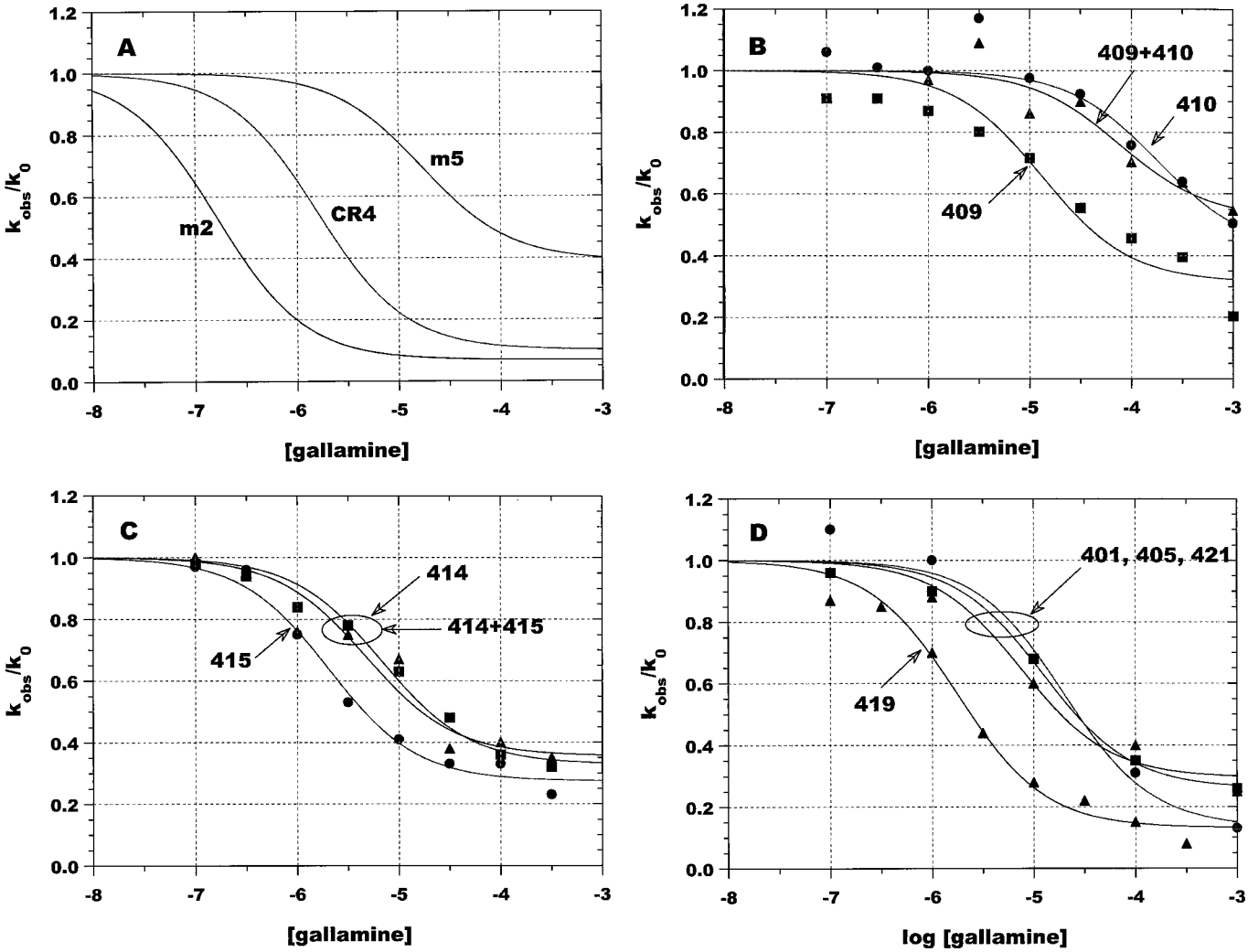

Fig. 2. Gallamine's allosteric effects on mutated M_5 receptors. The abilities of gallamine to slow the rate of dissociation of [^{3}H]NMS from the various receptors were determined as described in *Experimental Procedures*. A, gallamine's potencies at the wild-type M_2 and M_5 receptors and at CR4, described previously (Ellis et al., 1993). B–D, representative experiments performed with mutated M_5 receptors in which one or two residues have been replaced with the homologous residue from the M_2 receptor. Summary data are presented in Fig. 3. The numbers refer to the M_2 receptor to be compatible with the diagram in Fig. 1. For example, 401 indicates a receptor that has M_5 sequence everywhere except for the threonine in the middle of TM6, which has been replaced by alanine.

reducing effect of eliminating the acidic (EDGE) sequence from the M_2 receptor that had been reported. Moreover, we found a similarly significant increase in affinity toward gallamine upon inserting that acidic sequence into the homologous position of the M_1 receptor. These results are entirely consistent with the suggestion that gallamine adopts essentially the same orientation at the M_1 and M_2 subtypes.

Another advantage of the chimeric approach is that after an important region of the receptor structure has been identified, the essential component may be found by making smaller chimeric inserts or point mutations; the nature of the mutations is dictated by the comparative sequences of the receptors or subtypes being studied. Thus, in this work, we built on our M_2/M_5 chimeric study by converting individual residues in the M_5 receptor to the corresponding M_2 residue (Figs. 4 and 5). Only three of these point mutations affected the affinity toward gallamine to a significant extent. The first of these, the substitution of asparagine for serine near the top of TM6, reduced affinity toward gallamine. It is immediately apparent that this result cannot be taken as support for the involvement of this residue in the higher affinity of M_2.

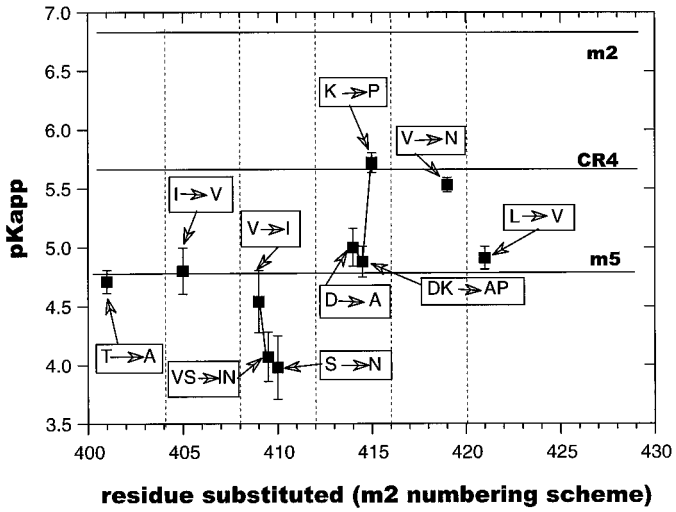

Fig. 3. Apparent affinities for gallamine at mutated M_5 receptors. Experiments were performed as in Fig. 2 and analyzed as described in *Experimental Procedures*. The apparent affinities obtained from those analyses were expressed as the negative logarithm (pK_{app}) and are shown as the mean ± S.E. of three to five experiments. The *x*-axis represents the position of the mutation according to the M_2 numbering scheme, and the substitutions made at each symbol are indicated in boxes (see also Fig. 1) In two cases, double mutants were studied. They were assigned average positions and are connected to the related single mutants by solid lines. For example, position 414.5 indicates a receptor that is M_5 sequence everywhere, except that the Asp-Lys residues in o3 have been replaced by Ala–Pro.

```
        <==TM-VI  -----o3-----  TM-VII==>
m4   PYNVMVLV NTFCQSCIPDTV WSIGYWLCY
m1   PYNIMVLV STFCKDCVPETL WELGYWLCY

m2   PYNVMVLI NTFCAPCIPNTV WTIGYWLCY

m3   PYNIMVLV NTFCDSCIPKTF WNLGYWLCY
m5   PYNIMVLV STFCDKCVPVTL WHLGYWLCY
```

Fig. 4. Muscarinic receptor sequences of o3 and adjacent TMs. The positions of the shaded residues are homologous to the asparagine located at position 419 in the M_2 receptor.

However, it is worth noting that many mutations at this residue in M_5 have been reported to induce constitutive activity, although asparagine was one of the few amino acids that was not tested in that study (Spalding et al., 1997). Nonetheless, induction of receptor activity undoubtedly entails conformational changes quite remote from the top of

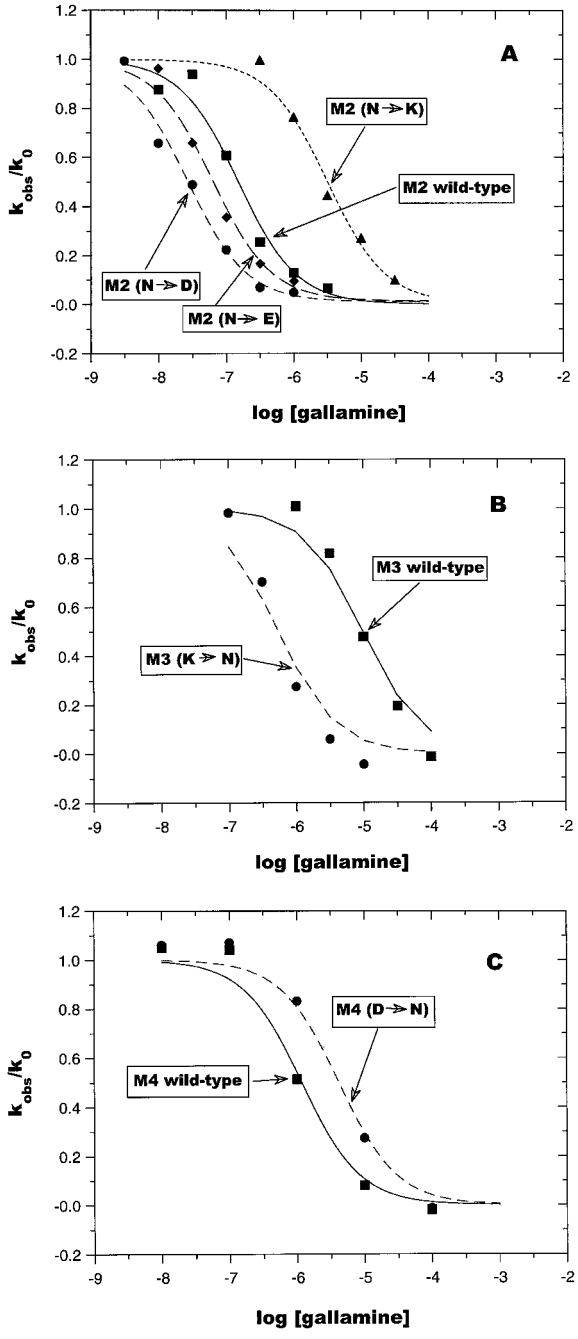

Fig. 5. Mutations at homologous positions in the o3 regions of muscarinic receptor subtypes yield consistent effects on gallamine's affinities. Mutations were created at the position shaded in Fig. 4. Data are representative of at least three similar experiments. A, gallamine's affinity for the M_2 receptor is markedly reduced when the asparagine at position 419 is replaced by its M_3 homolog (lysine), but somewhat enhanced when an aspartate or glutamate is present, as in M_4 and M_1, respectively. B, gallamine's affinity for the M_3 receptor is markedly enhanced by the opposite mutation of that shown above, i.e., replacement of the M_3 lysine by asparagine. C, gallamine's affinity for the M_4 receptor is somewhat reduced by the replacement of aspartate by asparagine.

TM6, and mutations at this position might be especially capable of interfering indirectly with gallamine's binding.

The replacement of lysine by proline in the o3 region of M_5 resulted in a marked increase in affinity toward gallamine. Because proline can sometimes have unique effects on protein structure (MacArthur and Thornton, 1991), it seemed possible that this proline might play an important organizing role in o3. However, a more direct explanation is available. In M_2, the sequence at 414 to 415 in o3 is Ala-Pro, whereas the corresponding residues in M_5 are Asp-Lys. Mutation of the M_5 aspartate to alanine has no effect on gallamine binding by itself, but prevents the effect of the lysine to proline mutation (Fig. 3). Thus, it appears that the insertion of the proline into M_5 serves to make the adjacent aspartate available to gallamine, and that the mutations at these positions cannot be relevant to the binding of gallamine to either wild-type receptor. It is worth noting that mutations of the pair of residues corresponding to $M_2^{409\text{-}410}$ do not interact in this way. The substitution of asparagine for serine (in M_5) has been described (above) to reduce affinity toward gallamine. Substitution of isoleucine for valine at the adjacent position had no effect by itself and did not interfere with the effect of the serine to asparagine mutation (Fig. 3).

Only one other mutation in M_5 produced a marked increase in affinity toward gallamine, namely, the substitution of asparagine for valine near the end of o3, just above TM7. We have shown previously that swapping this region of the receptor between M_2 and M_3 produces predictable and converse effects on affinity toward gallamine (Ellis et al., 1993). Point

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   <=TM-IV ----------o2---------- TM-V==>
m4 APAILFW QFVVGKRTVPDNQCFIQFLSNP AVTFGTA
m1 APAILFW QYLVGERTVLAGQCYIQFLSQP IITFGTA

m2 APAILFW QFIVGVRTVEDGECYIQFFSNA AVTFGTA

m5 APAILCW QYLVGKRTVPLDECQIQFLSEP TITFGTA
m3 APAILFW QYFVGKRTVPPGECFIQFLSEP TITFGTA
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Fig. 6. Muscarinic receptor sequences of o2 and adjacent TM regions. As in Fig. 4, the shaded regions lie in homologous positions.

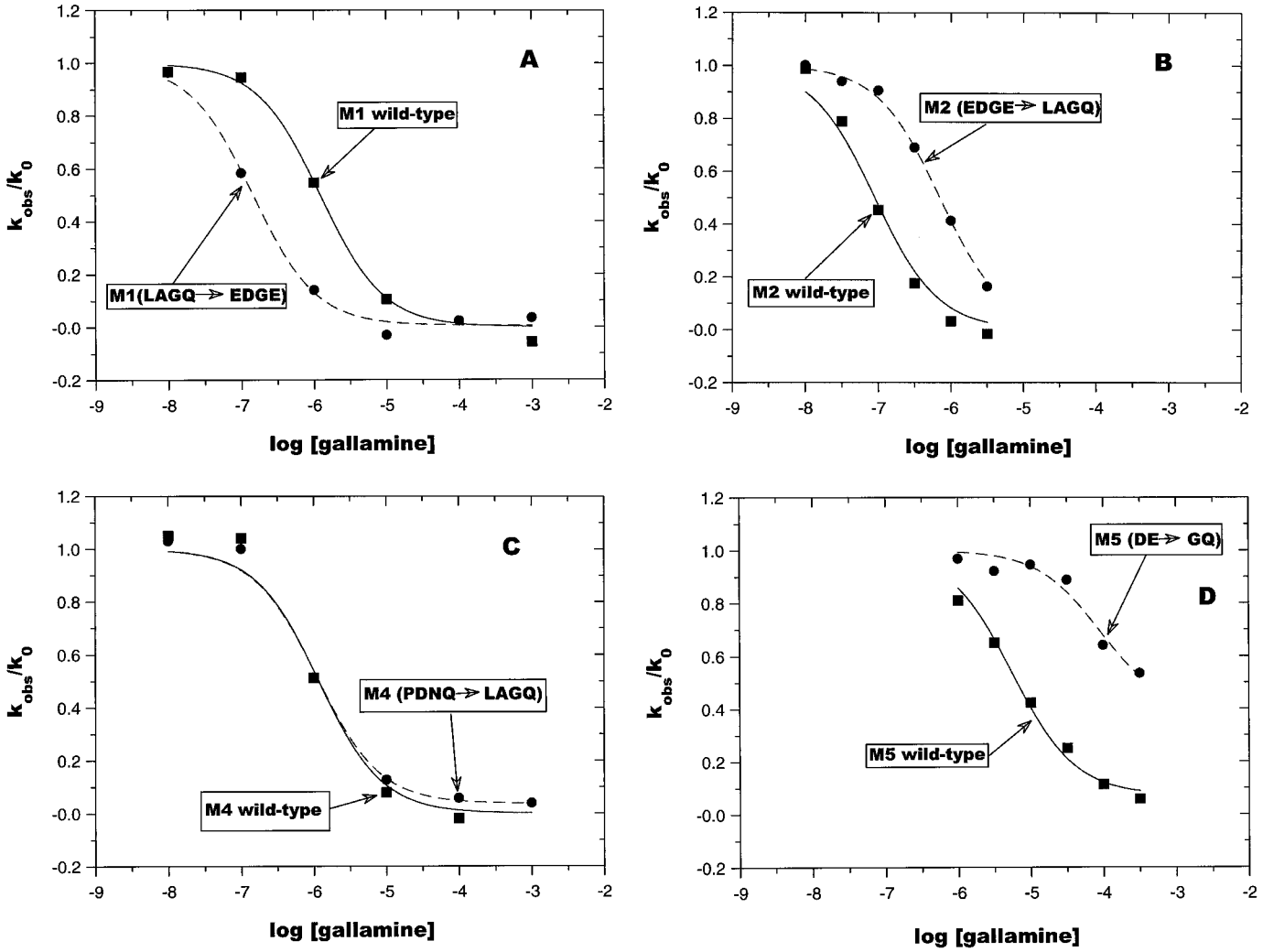

Fig. 7. Mutations in o2 regions of muscarinic receptor subtypes yield consistent effects on gallamine's affinities, with a different pattern from that seen in o3. Mutations were created within the positions shaded in Fig. 6. Data shown are representative of at least three similar experiments. A, insertion of the acidic sequence (Glu–Asp–Gly–Glu) from M_2 into the M_1 subtype leads to an increase in affinity toward gallamine. B, conversely to A, removal of the acidic sequence leads to a reduction in affinity toward gallamine. C, unlike the case in B, replacement of the M_4 sequence by M_1 sequence has no effect on affinity toward gallamine. D, replacement of the two acidic residues in this region of the M_5 receptor (Asp–Glu) by the corresponding M_1 sequence (Gly–Gln) markedly reduces the affinity toward gallamine.

mutations at this position mimicked the effects seen in the chimeric receptors; i.e., replacing lysine with asparagine at that position in the M_3 receptor markedly increased affinity toward gallamine, whereas replacing asparagine with lysine in M_2 reduced affinity toward gallamine (see Figs. 4 and 5). Interestingly, an asparagine residue in TM7 of β-adrenergic and some 5-hydroxytryptamine receptors, as well as mutant α_2-adrenergic receptors and 5-hydroxytryptamine receptors, has been implicated in interactions with β-adrenergic antagonists (Suryanarayana et al., 1991; Guan et al., 1992; Adham et al., 1994). These studies have suggested the importance of an interaction between asparagine and the oxygen atom of an alkoxy group that links to an aromatic ring (Suryanarayana et al., 1991). Gallamine and many other muscarinic allosteric ligands possess just such alkoxy groups. Glennon et al. (1996) have used mutagenesis and a series of propranolol analogs to conclude that propranolol forms two hydrogen bonds with that asparagine. The spacing between the ether oxygen and the hydroxyl group of propranolol is similar to that between the ether oxygen and the quaternary nitrogen of gallamine, but it remains to be determined whether two interactions can occur between gallamine and the asparagine at M_2^{419}.

The M_1 and M_4 subtypes have acidic residues at this position (Fig. 4). Replacing the aspartate of M_4 with asparagine leads to a small reduction in affinity toward gallamine; replacing the asparagine of M_2 with aspartate or glutamate leads to similar small increases in affinity toward gallamine (Fig. 5). This set of mutations suggests that the interaction between aspartate or glutamate and gallamine's quaternary nitrogen is somewhat stronger than the interaction(s) with asparagine.

We have previously suggested that the epitope in o2 was not apparent with the M_2/M_5 chimeric receptors because M_5 also possesses that binding component (Ellis, 1997). Supporting that idea, the replacement of the acidic amino acids in that region of M_5 (Asp-Glu) with the corresponding M_1 residues (Gly-Gln) dramatically reduced the already low affinity toward gallamine. However, replacement of the M_4 sequence in this region by M_1 sequence has no effect on affinity toward gallamine, in spite of the presence of an acidic amino acid (at a different position) in M_4 (see Figs. 6 and 7).

Our findings in o2 are in agreement with the conclusion of Leppik et al. (1994) that the $M_2^{172\text{-}175}$ sequence presents an important epitope for gallamine that is lacking in M_1. Furthermore, our data suggest that the M_5 subtype also possesses this epitope, whereas M_4 does not. The locations of acidic residues in M_2, M_4, and M_5 in this region point most strongly to the glutamate at M_2^{175}, although that remains to be confirmed. If so, M_3 would also be expected to derive affinity toward gallamine from this residue (Fig. 6). In the o3 region, the residue involved in gallamine's affinity corresponds to the asparagine at M_2^{419} (Fig. 4). The presence of valine (M_5) or lysine (M_3) appears to be associated with lower affinity for gallamine, whereas aspartate (M_4) or glutamate (M_1) is somewhat more favorable toward gallamine binding. Thus, in both regions the findings are in agreement with the grouping of affinities toward gallamine among the muscarinic subtypes, because M_1 and M_4 have similar affinities, whereas M_3 is similar to M_5 (Ellis et al., 1991, 1993).

Synthetic peptides based on the o2 region of the M_2 muscarinic receptor have been found to bind to autoantibodies from patients with dilated cardiomyopathy. These autoantibodies inhibit radioligand binding to M_2 receptors in an atropine-sensitive, but apparently noncompetitive, manner. The autoantibodies also appear to activate the receptor, again in an atropine-sensitive manner (Matsui and Fu, 1998). Interactions between these antibodies and allosteric muscarinic ligands do not appear to have been investigated. We have found that many allosteric ligands are sensitive to our M_2/M_5 chimeric receptors in which o2 has been swapped

TABLE 1

Summary data for all receptor constructs

Receptors are described as the parent receptor and the amino acids substituted. The location of the substitution is indicated either as the residue number in the M_2 numbering scheme or as o2, meaning that the mutation was within the o2 region of the receptor. Additional detail about the locations of the mutations is presented in Figs. 1, 4, and 6. Data represent the mean (± S.E.) from three or more experiments. wt indicates wild-type; k_0, rate of dissociation of [^{3}H]NMS from the receptor in the absence of gallamine; m and K_{app} are as described in *Experimental Procedures*.

Receptor	Mutation	Location	k_0 (min^{-1})	m	pK_{app}
M_1	wt		0.0316 (0.0009)	0.863 (0.105)	6.02 (0.14)
M_1	LAGQ ⇒ EDGE	o2	0.0382 (0.0068)	0.994 (0.003)	6.74 (0.13)
M_2	wt		0.166 (0.010)	0.984 (0.013)	6.79 (0.06)
M_2	N ⇒ D	419	0.161 (0.027)	1.00[a]	7.43 (0.09)
M_2	N ⇒ E	419	0.184 (0.014)	1.00[a]	7.20 (0.04)
M_2	N ⇒ K	419	0.0815 (0.0082)	0.881 (0.104)	5.84 (0.21)
M_2	EDGE ⇒ LAGQ	o2	0.110 (0.011)	1.00[a]	6.04 (0.03)
M_3	wt		0.0198 (0.0015)	1.00[a]	5.03 (0.05)
M_3	K ⇒ N	419	0.0418 (0.0035)	0.996 (0.004)	6.23 (0.04)
M_4	wt		0.0280 (0.0027)	1.00[a]	5.93 (0.17)
M_4	PDNQ ⇒ LAGQ	o2	0.0235 (0.0034)	0.963 (0.022)	5.96 (0.13)
M_4	D ⇒ N	419	0.0158 (0.0019)	1.00[a]	5.38 (0.08)
M_5	wt		0.00867 (0.00060)	0.720 (0.035)	4.79 (0.09)
M_5	T ⇒ A	401	0.0137 (0.0009)	0.658 (0.047)	4.71 (0.10)
M_5	I ⇒ V	405	0.00856 (0.00076)	0.746 (0.054)	4.80 (0.20)
M_5	V ⇒ I	409	0.00925 (0.00023)	0.853 (0.127)	4.54 (0.27)
M_5	S ⇒ N	410	0.00807 (0.00130)	0.434 (0.076)	3.98 (0.27)
M_5	VS ⇒ IN	409–410	0.00617 (0.00033)	0.468 (0.019)	4.07 (0.21)
M_5	D ⇒ A	414	0.00960 (0.00172)	0.759 (0.055)	5.00 (0.17)
M_5	K ⇒ P	415	0.0106 (0.0008)	0.803 (0.056)	5.72 (0.08)
M_5	DK ⇒ AP	414–415	0.0104 (0.0014)	0.681 (0.125)	4.88 (0.13)
M_5	V ⇒ N	419	0.00865 (0.00065)	0.920 (0.025)	5.53 (0.06)
M_5	L ⇒ V	421	0.00952 (0.00086)	0.742 (0.058)	4.91 (0.09)
M_5	DE ⇒ GQ	o2	0.00439 (.00036)	0.689 (0.050)	4.08 (0.06)

[a] Mean value is the upper constraint on m; no S.E. could be calculated.

(J. Ellis and M. Seidenberg, in preparation). However, this almost certainly implicates a different portion of the o2 loop, because gallamine was insensitive to this same chimeric receptor. Additionally, no allosteric ligand other than gallamine has demonstrated a dramatic and selective sensitivity to the o3 chimera (i.e., CR4). This is probably related to the structural diversity of presently known muscarinic allosteric ligands. Future studies should reveal whether structurally related allosteric ligands (Gharagozloo et al., 1999; Nassif-Makki et al., 1999) share common epitopes.

Values for k_0, m, and K_{app} were compiled and presented in Table 1. The effects of mutations on pK_{app} have already been discussed extensively above. The effects of the mutations on k_0 and m were found to be less dramatic and less consistent, and are more difficult to interpret for several reasons. One of the largest effects was the N ⇒ K mutation in M_2, which slowed k_0 by a factor of ~2. The reverse substitution, K ⇒ N in M_3, accelerated k_0 by approximately the same amount, but the V ⇒ N mutation in M_5 had no effect at all. Furthermore, whereas the asparagine speeds dissociation in M_3, the D ⇒ N mutation in M_4 slows k_0 by almost as much, and the converse N ⇒ D mutation in M_2 has no effect on k_0. The kinetics of [^{3}H]NMS binding must be sensitive to the overall structure of the binding pocket; it does not seem surprising that even slight kinks in that pocket might have small effects that are difficult to predict or interpret. Because m combines with k_0 to define the dissociation of [^{3}H]NMS from the ternary complex, it suffers from much the same difficulties. The most obvious effect on m arises from the S ⇒ N mutation in M_5 near the top of TM6 (Table 1; Fig. 1). As noted above, this mutation was the only one in the M_5 TM6–o3 series to reduce affinity toward gallamine, and many mutations at this serine have been reported to activate the receptor (Spalding et al., 1997).

All of the present studies have been performed in a hypotonic buffer, which has been shown by a number of laboratories to enhance the affinities of many allosteric ligands, especially gallamine (Ellis et al., 1991; Waelbroeck, 1994; Trankle et al., 1996). Thus, it is not inconceivable that gallamine's allosteric affinity might be affected by different residues in a more physiological buffer. However, we do not think this is likely, based on the available evidence. For example, in a more nearly isotonic buffer (50 mM PB), Leppik et al. (1994) observed a very similar change in affinity attributable to the M_2 EDGE ⇒ LAGQ mutation, as well as a similar degree of slowing of the dissociation of [^{3}H]NMS. Also, we have found the affinity of the allosteric ligand alcuronium to be sensitive to a unique portion of the receptor, whether assayed in hypotonic or physiological buffers (JE and MS, unpublished observations).

It should be noted that this study has used dissociation assays exclusively. The great advantage of this assay is that it ensures that only allosteric effects will be observed. The disadvantage is that the affinity of gallamine for the unliganded receptor cannot be extracted from these data. However, to extract that affinity requires confidence that the simple allosteric model applies, and thus far this model has only been tested rigorously at the wild-type M_2 receptor (Waelbroeck, 1994; Ellis, 1997; Ellis and Seidenberg, 1999).

In summary, we have investigated two epitopes involved in gallamine's allosteric subtype specificity at muscarinic receptors. Our results suggest that gallamine adopts a similar orientation at the different subtypes and continue to support the concept that muscarinic allosteric ligands bind to the outermost portions of these receptors. The M_1 and M_4 subtypes appear to derive affinity toward gallamine from acidic residues in the o3 region, whereas the M_3 and M_5 receptors seem to derive affinity from acidic residues in o2. Gallamine binds to the M_2 subtype with the highest affinity, and both o2 and o3 appear to be involved in this interaction.

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