Homologous Mutations Near the Junction of the Sixth Transmembrane Domain and the Third Extracellular Loop Lead to Constitutive Activity and Enhanced Agonist Affinity at all Muscarinic Receptor Subtypes

DIANE J. FORD, ANTHONY ESSEX, TRACY A. SPALDING, ETHAN S. BURSTEIN, and JOHN ELLIS

Department of Pharmacology (D.J.F., J.E.) and Department of Psychiatry (J.E.), the Pennsylvania State University College of Medicine, Hershey, Pennsylvania; and ACADIA Pharmaceuticals (A.E., T.A.S., E.S.B.), San Diego, California

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ABSTRACT

Previous studies have found that a mutation near the junction of the sixth transmembrane domain (TM6) and the third extracellular loop of the M5 muscarinic receptor leads to constitutive activation and enhanced agonist affinity for the mutated receptor. These results were consistent with the extended ternary complex model, which predicts a correlation between agonist affinity and constitutive activity. We have introduced the homologous mutation into all five subtypes of the highly conserved muscarinic receptor family; SerThr→ TyrPro was introduced into M1, M3, and M5, and AsnThr→ TyrPro was introduced into M2, M3, and M4. In binding assays, these mutations produced increases in affinities toward acetylcholine and carbachol that ranged from 5-fold at the M2 receptor to 15- to 20-fold at M1, M3, and M5, to 40-fold at M5. In functional assays, all five mutant receptors exhibited constitutive activity, at levels ranging between 30 and 80% of the maximal response elicited by carbachol. In every case, the muscarinic antagonist atropine inhibited this constitutive activity with high affinity. Thus, despite differences in effector coupling and in wild-type sequence at the mutation site, all five subtypes were activated by this mutation at the top of TM6. Previous studies of the M5 subtype have indicated that TM6 is a ligand-dependent switch that sets the activation state of the receptor. Based on the results of the present study, it is possible that TM6 represents a general switch for the activation of the muscarinic receptor family.

Muscarinic acetylcholine receptors consist of five subtypes, M1-M5, all of which have been cloned (Kubo et al., 1986; Bonner et al., 1987, 1988; Peralta et al., 1987). All five subtypes are seven-transmembrane, G-protein-coupled receptors. M1, M3, and M5 preferentially couple to Gq and stimulate phosphoinositide hydrolysis (Peralta et al., 1987; Bonner et al., 1988; Conklin et al., 1988; Liao et al., 1989). M2 and M4 preferentially couple to Gi and inhibit adenylyl cyclase activity (Jones et al., 1988; Peralta et al., 1988). Although the subtypes differ in respect to which G-proteins they preferentially couple, the amino acid sequences among all five subtypes are highly conserved, especially in the transmembrane regions. For example, the five muscarinic receptors are significantly more closely related to each other than are the α1 and α2 adrenergic receptors (Schwartz et al., 1997). This highly conserved nature of the muscarinic family is believed to be the reason for the difficulty encountered in developing highly selective agents for each of the muscarinic receptor subtypes.

Naturally occurring mutations in G-protein-coupled receptors underlie certain pathologies. In particular, some mutations have been shown to result in constitutive activity or activity in the absence of an agonist. Constitutively activating mutations in the thyrotropin receptor, parathyroid hormone receptor, and luteinizing hormone receptor have been shown to cause hyperfunctioning thyroid adenomas (Parma et al., 1995; Kopp et al., 1997), Jansen-type metaphyseal chondrodysplasia (Schipani et al., 1995), and familial male precocious puberty (Laue and Cutler 1994; Kosugi et al., 1995), respectively. Mutations in recombinant receptors can also elicit constitutive activity. Among these recombinantly expressed receptors are the α1B-adrenergic receptor (Cotecchia et al., 1990), β2-adrenergic receptor (Samama et al., 1993), and the dopamine D1 receptor (Cho et al., 1996). Similarly, the M1, M3, and M5 muscarinic acetylcholine receptors have been shown to be constitutively active as a result of a mutation or series of mutations (Bluml et al., 1994; Hogger et al., 1995; Spalding et al., 1995, 1997, 1998; Lu and Hulme, 1999). These constitutively active recombinant receptors usually exhibit increased affinity toward agonists, in

ABBREVIATIONS: NMS, N-methylscopolamine; TM6, transmembrane domain 6; PBS, phosphate-buffered saline.
comparison with the respective wild-type receptors. Exceptions to this rule have included amino acids whose mutation led to constitutive activity, but that were considered to be directly involved in the binding of agonist. Thus, there was not a clear correlation between agonist affinity and constitutive activity in these cases (Spalding et al., 1998). No examples of constitutively activating mutations have yet been reported for the M2 and M4 muscarinic receptor subtypes.

In the M5 receptor, it was shown that when a SerThr sequence near the junction of TM6 and the third extracellular loop was mutated to TyrPro, the receptor was constitutively activated. In addition, a 40-fold increase in affinity for carbachol was observed as a result of the mutation (Spalding et al., 1995). When the same mutation was introduced at the homologous position in the M1 receptor and expressed in A9L cells, it also resulted in similar enhancements of agonist affinity (Huang et al., 1998).

Because the M1 and M5 sequences are identical at these positions and preferentially couple to the same G-protein family, it is perhaps not surprising that the same mutation in these two subtypes leads to similar results. The M5 subtype differs in its sequence at this position, consisting of AsnThr in place of the SerThr of M1 and M5. The M2 and M4 receptors not only share the AsnThr of M3 in this position, but also differ from the M1, M3, and M5 receptors in that they preferentially couple to G1. In view of these specific differences within a background of a high degree of sequence conservation, we were interested to determine whether the M1, M2, M3, and M4 receptors would be sensitive to the TyrPro mutation that activates the M5 receptor. We have found that the introduction of this TyrPro mutation results in constitutive activity and a significantly increased affinity toward acetylcholine and carbachol in all five muscarinic receptor subtypes.

**Experimental Procedures**

**Materials.** Acetylcholine chloride, atropine sulfate, carbamylcholine chloride, Dulbecco’s modified Eagle’s medium, o-nitrophenyl-β-d-galactopyranoside, and Nonidet P-40 were obtained from Sigma (St. Louis, MO). pSII-β-galactosidase was obtained from Promega (Madison, WI). Tritiated N-methylscopolamine chloride was purchased from New England Nuclear (Boston, MA).

**Mutagenesis and Expression.** Mutant receptors were created using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides of approximately 35 bases in length were designed with the desired base substitutions. The oligonucleotides were annealed to the heat-denatured double-stranded plasmid containing the gene for the receptor and a high fidelity DNA polymerase was used to extend the oligonucleotides. The parental DNA was then digested by a methylation-specific nuclease, resulting in a population of plasmids enriched with the mutated receptor gene. The plasmids were then purified from transformed bacteria using the Maxi Prep kit (QIAGEN, Valencia, CA) and mutations were confirmed by sequencing. COS-7 cells were transfected with either the mutant or wild-type human muscarinic receptor plasmids via the calcium phosphate precipitation method. After 72 h, the cells were harvested, homogenized, and centrifuged at 50,000g for 30 min to obtain membranes. The membranes were frozen as aliquots in 5 mM phosphate buffer, pH 7.4, and stored at −70°C until use in binding assays.

**Membrane Binding Assays.** The affinity of NMS for wild-type or mutant receptors was determined using membranes from COS-7 cells. Membranes were incubated with concentrations from 1 to 100 pM [3H]NMS in 5 mM phosphate buffer at 25°C for 2 h. Nonspecific binding was determined in the presence of 3 μM atropine. The incubations were terminated by filtration through S&H no. 32 glass fiber filters (Schleicher and Schuell, Keene, NH), followed by three rinses with 0°C 40 mM phosphate buffer. Bound radioactivity was determined by scintillation counting. Data were expressed as specific [3H]NMS binding and fitted to the following equation:

\[
B = \frac{B_{\text{max}} \cdot L}{L + K_D}
\]

where \(B_{\text{max}}\) is the total number of receptor binding sites, \(L\) is the concentration of unbound [3H]NMS, and \(K_D\) is the equilibrium dissociation constant.

**Agonist binding potencies were determined in competition with [3H]NMS. Membranes from COS-7 cells containing wild-type or mutant receptors were incubated with 0.1 nM [3H]NMS and concentrations from 10 pM to 4 mM either acetylcholine or carbachol in 5 mM phosphate buffer at 25°C for 3 h.** The assays were terminated by filtration, as described above. Data were expressed as the percent inhibition of specific [3H]NMS binding and fitted to the following equation:

\[
Y = R + \frac{(L - R)}{1 + (X/C)^d}
\]

where \(R\) is the right plateau, \(L\) is the left plateau, \(X\) is the concentration of the inhibitor, \(C\) is the IC_{50}, and \(d\) is related to the slope of the curve. Corrected IC_{50} values were calculated according to the equation: Corrected IC_{50} = IC_{50}/1 + ([3H]NMS/K_D), as previously described (Cheng and Prusoff 1973).

**Functional Assays.** Receptor selection and amplification technology (R-SAT) assays were performed as follows: NIH-3T3 cells were plated 1 day before transfection using 7.5 × 10^4 cells in 0.1 ml of media per well of a 96-well plate. Cells were transfected using 0.6 μl per well Superfect (QIAGEN) according to the manufacturers instruction using 1.5 to 50 ng per well receptor DNA, 4 ng of G_{aq5} DNA per well (M2 and M4 only) and 40 ng of pSII-β-galactosidase per well (Burstein et al., 1997). One day after transfection, media was changed, and cells were combined with ligands in Dulbecco’s modified Eagle’s medium supplemented with 2% cyto-SF3 synthetic supplement (Kemp Laboratories, Frederick, MD) instead of calf serum to a final volume of 200 μl/well. After 5 days in culture, β-galactosidase levels were measured as described (Lim and Chae, 1989). The media was removed and β-galactosidase activity was determined by adding 200 μl of PBS with 3.5 mM o-nitrophenyl-β-d-galactopyranoside and 0.5% Nonidet P-40 to each well. The 96-well plates were incubated at room temperature and after 3 h the plates were read at 420 nm on a plate reader (EL 310, Bio-Tek Instruments, Winooski, VT; Molecular Devices, Menlo Park, CA). Absorbance data were fitted to the 4-parameter equation described above for agonist binding.

To estimate receptor expression levels, scaled-up transfections were also carried out in 12-well plates. Corresponding to the 10-fold larger area of the wells, 10 times the amounts of cells, pSII-β-galactosidase, receptor DNA, and Superfect were employed. The binding of a saturating level of [3H]NMS was determined 48 h after transfection. Cells were rinsed with PBS (containing calcium and magnesium), incubated with 1 nM [3H]NMS in PBS for 60 min at room temperature, and then quickly rinsed twice with ice-cold PBS. Subsequently, the contents of each well were solubilized in 0.5 ml of 1% sodium dodecyl sulfate and added to 5 ml of scintillation fluid. Nonspecific binding was determined as the binding to cells that did not receive any receptor DNA. The total protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Under these conditions, we found approximately 20 μg of protein per well of a 12-well plate.
Results

Figure 1 shows the location of the mutations in each of the receptors, near the junction of TM6 and the third extracellular loop. The high degree of conservation among the muscarinic subtypes in this region can also be appreciated in this figure. Radioligand binding studies were performed on membranes prepared from COS-7 cells transiently transfected with either wild-type or mutant receptors. The $K_d$ values for NMS at the wild-type and mutant receptors ranged from about 0.02 to about 0.05 nM and are shown in Table 1. The mutation caused less than 1.3-fold effect on NMS affinity at the M3 subtype and negligible effects at the other subtypes.

Table 1: Antagonist binding properties of wild-type and mutant receptors

<table>
<thead>
<tr>
<th>Subtype</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.015 ± 0.006 (3)</td>
</tr>
<tr>
<td>M1-YP</td>
<td>0.020 ± 0.007 (3)</td>
</tr>
<tr>
<td>M2</td>
<td>0.0440 ± 0.0005 (2)</td>
</tr>
<tr>
<td>M2-YP</td>
<td>0.056 ± 0.002 (2)</td>
</tr>
<tr>
<td>M3</td>
<td>0.0180 ± 0.0025 (2)</td>
</tr>
<tr>
<td>M3-YP</td>
<td>0.0190 ± 0.0005 (2)</td>
</tr>
<tr>
<td>M4</td>
<td>0.021 ± 0.005 (3)</td>
</tr>
<tr>
<td>M4-YP</td>
<td>0.021 ± 0.005 (3)</td>
</tr>
<tr>
<td>M5</td>
<td>0.020 ± 0.002 (3)</td>
</tr>
<tr>
<td>M5-YP</td>
<td>0.019 ± 0.003 (3)</td>
</tr>
</tbody>
</table>

The major finding of this study was that homologous mutations produced similar and dramatic effects in each of the subtypes and negligible effects at the other subtypes. About 0.02 to about 0.05 nM and are shown in Table 1. The mutation caused less than 1.3-fold effect on NMS affinity at the M3 subtype and negligible effects at the other subtypes. In Fig. 2, the effects of TyrPro mutations in M1, M3, and M5 on agonist binding affinities are shown for representative experiments. The IC$_{50}$ for acetylcholine was reduced from about 10 μM at M1 to about 0.4 μM at M1-YP, from about 2 μM at M3 to about 0.07 μM at M3-YP, and from about 1.5 μM at M5 to about 0.04 μM at M5-YP. Figure 3 shows the effect of TyrPro mutations on the M2 and M4 receptors. The IC$_{50}$ for carbachol was reduced from about 0.25 μM at M2 to about 0.04 μM at M2-YP and from about 7 μM at M4 to about 0.5 μM at M4-YP.

The results from multiple experiments conducted as in Figs. 2 and 3 are summarized in Table 2, with the IC$_{50}$ values for acetylcholine and carbachol corrected for the presence and affinity of [3H]NMS in the assays. The average enhancements of agonist affinity induced by the mutation ranged from about 5-fold at the M2 receptor, to 15- to 20-fold at the M1, M3, and M4 receptors, to about 40-fold at the M5 receptor.

Functional (R-SAT) assays were carried out using NIH-3T3 cells that were transiently transfected with the indicated amounts of receptor DNA (see under Experimental Procedures for assay details). For the M1, M3, and M5 receptors, 1.5 to 50 ng of DNA was used per well of a 96-well plate.

As shown in Fig. 4, the mutant M1, M3, and M5 receptors exhibited enhanced basal activity at every transfection level. This enhanced basal activity was inhibited by atropine but could also be further increased by carbachol. Each curve of each figure was fitted to the equation given under Experimental Procedures and from this curve-fitting we were able to estimate basal response, maximally stimulated or inhibited response)/(maximally stimulated response/H11002).

Response data for the M2 and M4 receptors are shown in Fig. 5 and the derived parameters are presented in Table 3. For those experiments, 50 ng of receptor DNA was cotransfected with the chimeric G-protein Gqi5 to allow coupling of these Gi-preferring receptors to the R-SAT response. Similar to the results seen with M1, M3, and M5, the mutation led to a significant degree of constitutive activity at M4, along with a significant increase in the potency of carbachol. The M2 mutant displayed even more robust constitutive activity, but the data did not permit a determination of carbachol's potency in this case.

Discussion

The major finding of this study was that homologous mutations produced similar and dramatic effects in each of the...
five muscarinic receptor subtypes. Substitutions with the sequence tyrosine-proline near the junction of TM6 and the third outer loop led to constitutive activation and to enhanced agonist affinity in binding and response assays. The importance of these residues in these processes was not dependent on the wild-type sequences at the mutation site or on the G-protein-coupling preferences of the subtypes (e.g., compare M1 and M4). This is the first report of a constitutively activating mutation at the M2 or M4 muscarinic receptor subtype.

Heptahelical receptors have been found to be sensitive to constitutively activating mutations at many sites within TM and intracellular regions (Pauwels and Wurch, 1998). Most of these sites lie between the middle of the TM regions and the membrane-adjacent regions of the second and third intracellular loops. In particular, mutations of residues within or adjacent to the "BBXXB" motif found just below TM6, in the C-terminal portion of the third intracellular loop, lead to activation of many receptors. This motif of basic (B) and nonbasic (X) residues has been shown to be a crucial element of G-protein-activating peptides (Okamoto and Nishimoto, 1992). However, Spalding et al. (1995) used a random saturation approach to construct and screen a library of mutants of the M1 receptor and isolated a strongly activating mutation at the other end of TM6, adjacent to the third outer loop. This mutant (Ser465Thr4663TyrPro), which served as the template for the homologous mutations investigated in the present study, also exhibited a large increase in affinity toward carbachol, in agreement with the predictions of the extended ternary complex model (Samama et al., 1993).

It was later found that the mutation of Ser465 alone to a number of different amino acids led to constitutive activity in the M5 receptor (Spalding et al., 1997). When the serine was mutated to a large or basic residue, a higher degree of constitutive activity was observed, whereas mutation to a small or acidic residue produced no significant constitutive activity. Agonist affinity correlated with the degree of constitutive activity. Strikingly, the body of functional data from the wild-type receptor and 13 mutants could be reconciled by simply postulating that the mutations altered the isomerization constant (J), which describes the equilibrium between inactive and active forms of the receptor in the absence of ligand. Subsequent studies found that the homologous mutation in the M1 receptor (STYP) also led to enhanced agonist affinity and, again, that the serine was the key amino acid in the mutation (Huang et al., 1998, 1999). Based on these results, it seems most likely that it is the change of AsnTyr in the M2, M3, and M4 receptors that leads to the constitutive activity and to the increase in agonist affinity. Interestingly, Asn and Gln were two of the few substitutions that were not investigated at Ser465 in the M2 subtype (Spalding et al., 1997). From the similarity in the effect of our mutation at M2, M3, and M4, we would also expect that the mutation Ser 243 Asn at this position would have little effect on any of the five subtypes.

Fig. 2. Agonist binding studies on wild-type and mutant M1, M3, and M5 receptors. Acetylcholine inhibition binding curves were generated in the presence of 0.1 nM [3H]NMS. Assays were performed with membranes from COS-7 cells transfected with wild-type or mutant receptors, as indicated. Data represent one of at least three separate experiments. Summary data for acetylcholine and carbachol are shown in Table 2.
the constitutive activity is primarily due to the mutation, for a significant amount of constitutive activity was observed in the mutated receptors even when the lowest amounts of DNA were transfected (1.5 ng per well) and the lowest receptor expression was observed. Conversely, significant constitutive activity was not observed in the wild-type receptors even at the highest levels of DNA, where much higher expression levels were found. Nonetheless, there was an effect of transfection level on agonist potency at both wild-type and mutated receptors, except for \( M_1 \)-YP. The enhancement of carbachol’s affinity with increasing levels of expression would be consistent with the presence of a receptor reserve. This apparent spareness is not inconsistent with the increased constitutive activity observed when receptor levels are increased (discussed above; for a theoretical discussion, see Chen et al. 1999, 2000). It is not clear why this effect does not occur at \( M_1 \)-YP. A couple of factors could account for the different behavior of \( M_1 \)-YP. First, possibly because of protein structure, both wild-type and mutant \( M_1 \) receptors may inherently have fewer propensities to adopt an active configuration. Indeed for all parameters measured, both wild-type and mutant forms of \( M_1 \) appear to be less easily activated than the corresponding \( M_3 \) or \( M_5 \) receptors. Second, there could be less receptor spareness for \( M_1 \) due to lower expression, but we did not find this to be the case. The levels of expression of \( M_1/M_1 \)-YP were very similar to those of \( M_2/M_2 \)-YP and considerably greater than those of \( M_5/M_5 \)-YP. Finally, given that G-protein-coupled receptors are now known to couple to a much wider array of G-protein-dependent and -independent effectors (Heuss and Gerber, 2000), and that the \( R \)-SAT response integrates a complex combination of signaling events, one cannot assume that \( M_1 \)-induced responses are completely equivalent to \( M_3 \) or \( M_5 \), even though they are highly related receptors. These differences could account for the lower maximal achievable responses for \( M_1 \).

In the extended ternary complex model, antagonists can be defined as neutral or negative, according to their ability to affect the equilibrium between \( R \) and \( R^* \). A neutral antagonist does not affect the equilibrium between \( R \) and \( R^* \), but only prevents the binding of agonist to the receptor, thus producing competitive antagonism. A negative antagonist

![Fig. 3. Agonist binding studies on wild-type and mutant \( M_2 \) and \( M_4 \) receptors.](image)

### Table 2
Agonist binding properties of wild-type and mutant receptors

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Acetylcholine</th>
<th>Carbachol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corrected IC(_{50}) ((\pm) S.E.M.)</td>
<td>Fold Change</td>
</tr>
<tr>
<td>( M_1 )</td>
<td>1.31 ± 0.12 (5)</td>
<td>20</td>
</tr>
<tr>
<td>( M_1 )-YP</td>
<td>0.064 ± 0.010 (5)</td>
<td></td>
</tr>
<tr>
<td>( M_2 )</td>
<td>0.020 ± 0.001 (5)</td>
<td>5</td>
</tr>
<tr>
<td>( M_2 )-YP</td>
<td>0.004 ± 0.001 (4)</td>
<td></td>
</tr>
<tr>
<td>( M_3 )</td>
<td>0.34 ± 0.09 (3)</td>
<td>15</td>
</tr>
<tr>
<td>( M_3 )-YP</td>
<td>0.023 ± 0.006 (4)</td>
<td></td>
</tr>
<tr>
<td>( M_4 )</td>
<td>0.31 ± 0.06 (4)</td>
<td>12</td>
</tr>
<tr>
<td>( M_4 )-YP</td>
<td>0.026 ± 0.008 (4)</td>
<td></td>
</tr>
<tr>
<td>( M_5 )</td>
<td>0.23 ± 0.09 (4)</td>
<td>38</td>
</tr>
<tr>
<td>( M_5 )-YP</td>
<td>0.006 ± 0.002 (4)</td>
<td></td>
</tr>
</tbody>
</table>
actually shifts the equilibrium toward R; by virtue of atropine’s ability to inhibit the constitutive activity in the present study, it is a negative antagonist. Because the basal states of wild-type receptors are usually strongly shifted toward R, small changes in the isomerization constant $J$ are expected to produce larger changes in agonist affinities than in antagonist affinities (Spalding et al., 1997). This is exactly the effect that we observed in our binding assays, in which agonist affinities were significantly enhanced by the mutation (Table 2), whereas antagonist affinity was reduced modestly, if at all (Table 1). Carbachol’s affinities in the R-SAT assays were also much higher at the mutant receptors than at the wild-

Fig. 4. Pharmacology of wild-type and mutant $M_1$, $M_3$, and $M_5$ muscarinic receptors in functional assays. The R-SAT assay was performed as described under Experimental Procedures, in the presence of the indicated concentrations of atropine or carbachol. Cells were transfected with the amounts of receptor DNA indicated in parentheses in the graphs (in ng per well). Curves represent the best fits to the model given under Experimental Procedures and the resulting parameters are listed in Table 3. ■, carbachol, wild-type; □, atropine, wild-type; ▲, carbachol, $M_5$-YP; △, atropine, $M_5$-YP. Data represent two experiments, each performed in duplicate.

Fig. 5. Pharmacology of wild-type and mutant $M_2$ and $M_4$ muscarinic receptors. Assays were performed and analyzed as in Fig. 4. Cells were transfected with 50 ng of receptor DNA and 4 ng of $G_{q,5}$ DNA per well. ■, carbachol, wild-type; □, atropine, wild-type; ▲, carbachol, $M_5$-YP; △, atropine, $M_5$-YP. Data represent two experiments, performed in duplicate.
constitutively active wild-type and mutated receptors. The M5 subtype have suggested that interactions between TM3 and TM6 acts as a ligand-dependent switch (Spalding et al., 1991). In light of the similar effects of identical mutations at TM6 on constitutive activation and agonist binding in all five subtypes, TM6 may be a general switch for all of the muscarinic receptors.

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References


Address correspondence to: Dr. John Ellis, Department of Psychiatry H073, Milton S. Hershey Medical Center, Penn State University, Hershey, PA 17033. E-mail: jxe11@psu.edu