Role of Conserved Threonine and Tyrosine Residues in Acetylcholine Binding and Muscarinic Receptor Activation

A STUDY WITH m3 MUSCARINIC RECEPTOR POINT MUTANTS*

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Structure-function relationship studies of the m3 muscarinic acetylcholine receptor have recently identified a series of threonine and tyrosine residues (all located within the hydrophobic receptor core) that are critically involved in acetylcholine binding (Wess, J., Gdula, D., and Brann, M. R. (1991) EMBO J. 10, 3729-3734). To gain further insight into the functional roles of these amino acids, the agonist binding properties of six rat m3 muscarinic receptor point mutants, in which the critical threonine and tyrosine residues had been individually replaced by alanine and phenylalanine, respectively, were studied in greater detail following their transient expression in COS-7 cells. The binding profiles of a series of acetylcholine derivatives suggest that the altered threonine and tyrosine residues are primarily involved in the interaction of the acetylcholine ester moiety with the receptor protein. The two m3 receptor point mutants, Thr234 → Ala and Tyr506 → Phe, which showed the most pronounced decreases in acetylcholine binding affinities (~40-80-fold as compared with the wild-type receptor), were stably expressed in CHO cells for further functional analysis. Both mutant receptors were found to be severely impaired in their ability to stimulate agonist-dependent phosphatidylinositol hydrolysis. Consistent with this observation, acetylcholine binding to the two mutant receptors was not significantly affected by addition of the GTP analog Gpp(NH)p (5'-guanylyl imidodiphosphate). Our data suggest that Thr234 and Tyr506 (located within transmembrane domains V and VI, respectively), which are conserved among all muscarinic receptors (m1-m5), may play an important role in agonist-induced muscarinic receptor activation.

Muscarnic acetylcholine (ACh) receptors are cell surface proteins that mediate their intracellular responses via coupling to guanine nucleotide regulatory proteins (G proteins) (Nathanson, 1987). Like all other members of the superfamily of G protein-linked receptors, the muscarinic receptors are predicted to conform to a generic protein fold in which seven hydrophobic transmembrane helices (TM I-VII) are joined by alternating cytoplasmic and extracellular loops.

Molecular cloning studies have revealed the existence of five distinct muscarinic receptor proteins (m1-m5) (Kubo et al., 1986; Bonner et al., 1987, 1988; Peralta et al., 1987), each of which displays distinct pharmacological and functional properties (for reviews, see Hulme et al. (1990) and Jones et al. (1992)). The availability of the cloned muscarinic receptor genes has allowed the initial mapping of receptor domains that are involved in ligand binding and G protein recognition. Studies with chimeric muscarinic receptors have shown that the N-terminal portion of the third cytoplasmic loop is the major structural determinant of G protein coupling selectivity (Wess et al., 1989, 1990; Lechleiter et al., 1990). The ligand binding site of the muscarinic receptors, consistent with findings obtained with the photoreceptor protein, bacteriorhodopsin (Findlay and Pappin, 1986; Henderson et al., 1990), and the β-adrenergic receptors (Dixon et al., 1987a, 1987b; Dohman et al., 1991), appears to be formed by the seven TM domains which enclose a well conserved ligand binding pocket (Hulme et al., 1990, 1991). Ligand binding to the muscarinic receptors appears to be initiated by ion-ion interaction between the positively charged amino head group present in all muscarinic ligands and a negatively charged Asp residue located in TM III of the receptor molecule (Asp147 in the rat m3 receptor sequence, see Fig. 1). This notion is primarily based on affinity labeling and peptide sequencing studies using the alkylation muscarinic antagonist, [3H]propylbenzilycholine mustard, as a probe (Curtis et al., 1988; Kurtenbach et al., 1990). In addition, replacement of the TM III Asp residue by Asn led to a mutant receptor that displayed drastically reduced binding affinities for muscarinic ligands (Fraser et al., 1989).

Interestingly, the TM III Asp residue is conserved among all receptors that bind biogenic amine ligands such as adrenergic, dopamine, serotonin, and histamine receptors and has been shown to serve a similar function (as found for the muscarinic receptors) in the β-adrenergic receptors (Strader et al., 1988, 1991). It seems therefore unlikely that this residue determines the specificity of a given amine ligand for a particular receptor. One may rather assume that the side chains of other receptor-specific amino acids found within the hydrophobic receptor core are involved in this process. Consistent with this notion, the pharmacological analysis of a series of m3 muscarinic receptor point mutants has identified sev-
eral Thr and Tyr residues conserved among all muscarinic receptors, which appear to play an important role in conferring binding specificity on the physiological ligand, ACh (Wess et al., 1991). In helical wheel models of the muscarinic receptor (Hulme et al., 1991), these Thr and Tyr residues, all of which are located at a similar level within different TM domains, can be arranged such that all of them face the central cavity created by the ring-like arrangement of the seven TM helices. We have therefore speculated that these amino acids may define a plane in which muscarinic agonists are bound to their target receptors (Wess et al., 1991).

This study was designed to investigate the functional role of the various conserved Thr and Tyr residues in greater molecular detail. To test the hypothesis that the hydroxyl groups present in the side chains of these amino acids are involved in hydrogen bonding with the ACh ester moiety, we have determined the binding affinities of a series of ACh derivatives for a group of mutant m3 muscarinic receptors in which the critical Thr and Tyr residues had been individually replaced by Ala and Phe, respectively. The two mutant receptors which showed the most pronounced decreases in ACh binding affinities (Thr234 → Ala and Tyr259 → Phe) were stably expressed in CHO cells for further functional analysis. Our data suggest that Thr234 and Tyr259 do not only play an important role in ACh binding but also in agonist-induced muscarinic receptor activation.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The various point mutations were introduced into an expression plasmid containing the entire coding region of the rat m3 muscarinic receptor (Rm3pCD) (Bonner et al., 1987). The mutations were created by replacing specific segments of Rm3pCD with DNA fragments containing the desired mutation prepared by the polymerase chain reaction employing mismatched oligonucleotide primers (Wess et al., 1991). The numbers given refer to amino acid positions within the m3 sequence. The aspartate residue present in TM III (D147) is conserved among all biogenic amine receptors and is thought to bind to the positively charged amino group of the amine receptor ligands. Only the membrane-proximal portions of the N-terminal receptor region (total length: 66 amino acids), the large third cytoplasmic loop (I3; total length: 240 amino acids), and the C-terminal tail (total length: 43 amino acids) are shown. In total, the rat m3 muscarinic receptor is composed of 589 amino acids (Bonner et al., 1987). The single-letter amino acid code is used.

Membrane Preparation and Binding Assays—Membrane homogenates were prepared from transfected COS-7 or CHO cells as described (Dorje et al., 1991). Binding buffer consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM MgCl2. Assays were conducted in 1 ml total volume. In the [3H]NMS saturation experiments, six to eight different concentrations of the radioligand (6.25-800 pm) were used. In the [3H]NMS displacement experiments, 10 different concentrations of the unlabelled competitors were employed. The [3H]NMS concentration in the competition binding studies was 200 pm. Nonspecific binding was determined in the presence of 1 μM atropine. Incubations were carried out at 22°C for 3 h. Data were analyzed by a nonlinear least squares curve-fitting procedure using the program DATAPLOT run on a VAX II computer as described (Dorje et al., 1991). IC50 values were converted to Ki values according to the method of Cheng and Prusoff (1973).

Protein concentrations were determined according to the method of Bradford (1976), using a Bio-Rad protein assay kit.

Statistical significance was assessed using Student’s t test; the level of significance was chosen at p < 0.05.

Creation of Stable CHO Cell Lines—CHO cells were cotransfected with Rm3pCD (or Rm3pCD mutants) and pCDneo by calcium phosphate precipitation (Chen and Okayama, 1987). Selection with the neomycin analog G 418 (600 μg/ml) was started 72 h after transfection and continued for 2-3 weeks. Clonal cell lines were obtained by single cell cloning and assayed for [3H]NMS binding capacity as described (Buckley et al., 1980).

PI Assays—CHO cells were labeled in 24-well plates (plating density, 1.5 × 105 cells/well) with 1 μCi/ml myo-[3H]inositol for 24 h. Immediately before an experiment, the cells were washed twice with phosphate-buffered saline and incubated for 15 min in Dulbecco’s modified Eagle’s medium containing 10 mM LiCl and 20 mM HEPES. The medium was then replaced by 0.25 ml of the same medium containing the experimental agents. After a 1-h incubation at room temperature, the reaction was terminated by adding 0.75 ml 10% trichloroacetic acid, followed by a 30-min incubation on ice. The trichloroacetic acid was extracted with water-saturated diethyl ether (3 X 4 ml), and the IP1 fraction was isolated by anion exchange chromatography (Berridge et al., 1988) and counted on an LKB liquid scintillation counter.

Materials—Tissue culture reagents were from Gibco. [3H]NMS (78.9 Ci/mmol) and myo-[3H]inositol (20 Ci/mmol) were purchased from Du Pont-New England Nuclear and American Radiolabeled Inc., respectively. Gpp(NH)p was from Boehringer Mannheim. The ACh derivatives 4-6 were synthesized in the Upjohn Laboratories according to published procedures (Thanei-Wyss and Waser, 1983). All other drugs used in this study were obtained from Sigma.

FIG. 1. Amino acid sequence and proposed transmembrane disposition of the rat m3 muscarinic receptor. The highlighted Tyr (Y) and Thr (T) residues are conserved among all muscarinic receptors and were individually replaced with Phe or Ala, respectively. The numbers given refer to amino acid positions within the m3 sequence. The aspartate residue present in TM III (D147) is conserved among all biogenic amine receptors and is thought to bind to the positively charged amino group of the amine receptor ligands. Only the membrane-proximal portions of the N-terminal receptor region (total length: 66 amino acids), the large third cytoplasmic loop (I3; total length: 240 amino acids), and the C-terminal tail (total length: 43 amino acids) are shown. In total, the rat m3 muscarinic receptor is composed of 589 amino acids (Bonner et al., 1987). The single-letter amino acid code is used.
RESULTS

Binding of ACh Derivatives to Mutant m3 Receptors—A series of mutant m3 muscarinic receptors, in which individual Thr and Tyr residues (Fig. 1) were replaced by Ala and Phe, respectively, were transiently expressed in COS-7 cells. The effects of these substitutions on the binding of the physiological agonist, ACh, and various synthetic ACh derivatives (compounds 2–7, Table I) were determined. The results of these experiments are summarized in Table I.

ACh (compound 1) and its carbamoyl derivative, carbachol (compound 2), displayed approximately 10–60-fold lower affinities for the six mutant receptors studied than for the wild-type m3 receptor. The two mutant receptors that showed the most pronounced affinity decrease for both agonists (25–58-fold) were Thr234→Ala and Tyr533→Phe (Table I).

Tetramethylammonium (TMA, compound 3), which can be considered the simplest muscarinic ligand, bound to the wild-type m3 receptor with about 300-fold lower affinity than ACh (Table I, Fig. 2). With the exception of Tyr533→Phe, which bound TMA with >3-fold lower affinity than the wild-type receptor, the TMA binding affinities of all other mutant receptors differed from that of the wild-type receptor by less than a factor of 2.5. The ACh derivatives 4–7, in which the ACh ester side chain has been systematically modified, bound to the wild-type m3 receptor with affinities that were intermediate between those found for ACh and TMA (Table I, Fig. 2).

Similar to the binding profile of TMA (compound 3), the binding affinities of derivatives 4–7 for the various mutant receptors (except Tyr533→Phe) were either similar to or differed by less than 4.5-fold from those found for the wild-type m3 receptor (shown for Thr234→Ala and Tyr536→Phe in Fig. 2). Tyr533→Phe again differed to some extent from the other mutant receptors in that it showed clearly reduced binding affinities for compounds 4–7 (up to 9-fold as compared with the wild-type receptor; Table I).

In summary, the agonist binding profiles of all mutant m3 receptors studied clearly differed from that of the wild-type m3 receptor. In contrast to the wild-type receptor, all mutant receptors lost the ability to bind ACh and carbachol with high affinity (shown for Thr234→Ala and Tyr536→Phe in Fig. 2). However, the various mutant receptors (with the exception of Tyr533→Phe) bound ACh derivatives that lacked the ACh ester moiety (compounds 3–7) with affinities that were in a range similar to those observed for the wild-type receptor (Fig. 2).

Functional Activity of Mutant m3 Receptors—The two mutant m3 receptors which showed the most pronounced decreases in ACh and carbachol binding affinities (Thr234→Ala and Tyr536→Phe), along with the wild-type m3 receptor, were stably expressed in CHO cells to allow their detailed functional characterization. Clonal cell lines were selected which gave comparable levels of receptor expression (Table II). As previously demonstrated in transiently transfected COS-7 cells (Wess et al., 1991), the muscarinic antagonist [1H]NMS bound to the two mutant receptors with affinities similar to that of the wild-type m3 receptor (Table II).

To assess whether Thr234→Ala and Tyr536→Phe were able to stimulate PI hydrolysis in CHO cells, their ability to mediate agonist-dependent increases in intracellular inositol monophosphate (IP) levels was determined. Untransfected CHO cells did not give any significant increase in IP, production following muscarinic agonist treatment (data not shown). Carbachol stimulation of cells expressing the wild-type m3 receptor resulted in a pronounced increase in intracellular inositol phosphate levels (maximum IP, elevation, 10.9-fold) (Table II, Fig. 3). The two mutant receptors, Thr234→Ala and Tyr536→Phe, proved to be severely impaired in their ability to mediate carbachol-induced stimulation of PI turnover, exhibiting 60–70-fold reduced carbachol potencies (Table II, Fig. 3). Moreover, the maximum IP response (measured as accumulation of IP) observed with Thr234→Ala was considerably smaller than that mediated by the wild-type receptor (~40% of the wild-type response) (Table II, Fig. 3).

Two additional ACh derivatives, TMA (compound 3) and

Table I

<table>
<thead>
<tr>
<th>Compound (CH3)N-R</th>
<th>Kapp (µM)</th>
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<tr>
<td></td>
<td>m3 (wt)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) ACh</td>
<td>1.2 (0.70)</td>
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<tr>
<td>(2) Carbachol</td>
<td>2.0 (0.71)</td>
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<tr>
<td>(3) CH3</td>
<td>1.2 (0.73)</td>
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<tr>
<td>(4) CH3CH2CH2CH3</td>
<td>3.0 (0.83)</td>
</tr>
<tr>
<td>(5) CH3CH2CH2CH3</td>
<td>3.0 (0.83)</td>
</tr>
<tr>
<td>(6) CH3CH2CH2CH3</td>
<td>3.0 (0.83)</td>
</tr>
<tr>
<td>(7) CH3SCCH3</td>
<td>1.0 (0.82)</td>
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</tbody>
</table>
Amino Acids Involved in Muscarinic Receptor Function

Effect of Gpp(NH)p on AcH Binding to Thr234 → Ala and Tyr506 → Phe—The results of the functional experiments (see previous paragraph) suggested that Thr234 → Ala and Tyr506 → Phe are impaired in their ability to efficiently interact with endogenous G proteins that mediate stimulation of PI metabolism. To further investigate this concept, we have studied the effect of the hydrolytically stable GTP analog, Gpp(NH)p (100 µM), on the binding parameters of AcH. All studies were performed in the absence of ACh esterase inhibitors, since addition of diisopropyl fluorophosphate (200 µM) proved to have no significant effect on the position and slope of the ACh competition binding curves (data not shown). All ACh inhibition binding curves were characterized by Hill coefficients significantly smaller than 1 (p < 0.05), ranging from 0.73 to 0.84 (Table III). As commonly observed with G protein-coupled receptors, Gpp(NH)p treatment of cells expressing the wild-type m3 receptor led to a small but significant (p < 0.05) rightward shift and to a steepening of the ACh inhibition binding curves (Table III, Fig. 4). In contrast, Gpp(NH)p had no significant effect on ACh binding to membrane preparations containing the mutant receptors Thr234 → Ala and Tyr506 → Phe (Table III, Fig. 4). In both cases, the slopes (nH) and the positions of the ACh competition binding curves (IC50) were not significantly different in the absence or presence of Gpp(NH)p.

DISCUSSION

Structure-function relationship studies of a vast number of ACh derivatives have shown that a positively charged ammonium head group is an essential structural requirement for muscarinic activity and that the presence of the ACh ester bond is critical for efficient receptor activation (Friedman, 1967; Cannon, 1981). Based on such studies it has been hypothesized that the ACh ammonium head group binds to an anionic site on the receptor protein and that a second point of attachment is created by hydrogen bonding between the ACh ester moiety and a receptor site located about 4–7 Å away from the anionic center (Friedman, 1967; Cannon, 1981).

The recent cloning and sequence analysis of five different muscarinic receptor genes (Kubo et al., 1986; Bonner et al., 1987, 1988; Peralta et al., 1987) has allowed the study of the molecular determinants underlying the interaction of muscarinic ligands with their target receptors in previously unthought of structural detail. Consistent with classical models of the ligand binding site, affinity labeling and peptide sequencing (Curtis et al., 1989; Kurtenbach et al., 1990) as well as mutagenesis studies (Fraser et al., 1989) suggest that ligand binding to muscarinic receptors is initiated by ionic interaction between the amine moiety of the ligand and a conserved Asp residue located in TM III (Asp445 in Fig. 1). We have recently proposed that a series of intra-membranous Thr and Tyr residues that are conserved among all muscarinic receptors may participate in the interaction of the ACh ester side chain with the receptor molecule (Wess et al., 1991). This notion is based on the observation that single point mutants of the m3 muscarinic receptor in which the hydroxyl groups (potential hydrogen bond donors) present in the side chains of these amino acids were eliminated by site-directed mutagenesis (Thr → Ala, Tyr → Phe) showed drastically reduced ACh binding affinities. In an attempt to identify specific points of contact between distinct parts of the ACh molecule and the various conserved Thr and Tyr residues, we have compared the binding affinities of a series of ACh derivatives for six m3 receptor single point mutants (Thr → Ala, Tyr → Phe) with their affinities for the wild-type m3 receptor (Table I).
### Amino Acids Involved in Muscarinic Receptor Function

#### TABLE II

<table>
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<tr>
<th>Receptor</th>
<th>[H]NMS binding</th>
<th>Carbachol</th>
<th>TMA</th>
<th>S-Acetylthiocholine</th>
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<tr>
<td></td>
<td>Kd</td>
<td>Bmax</td>
<td>EC50</td>
<td>Emax</td>
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<tr>
<td>m3 (wt)</td>
<td>38 ± 5</td>
<td>152 ± 27</td>
<td>0.0075 ± 0.0004</td>
<td>10.9 ± 1.1</td>
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<tr>
<td>Thr2AI</td>
<td>70 ± 10</td>
<td>114 ± 22</td>
<td>0.55 ± 0.12</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Tyr533F</td>
<td>72 ± 5</td>
<td>391 ± 47</td>
<td>0.47 ± 0.07</td>
<td>8.7 ± 1.1</td>
</tr>
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TMA (compound 3) in which the ACh ester side chain has been completely eliminated still retained the ability to displace specific [H]NMS binding indicating that the ACh ester side chain is not essential for muscarinic receptor binding. However, TMA bound to the wild-type m3 receptor with drastically reduced affinity (~300-fold as compared with ACh) and behaved as a weak partial agonist. In the functional experiments, consistent with classical pharmacological studies showing that the presence of the ACh ester side chain is crucial for strong agonistic activity (Friedman, 1967; Cannon, 1981). All mutant receptors (except Tyr533F → Phe, see below) bound TMA with affinities that differed from that of the wild-type receptor by less than a factor of 2.5. These data may suggest that most of the mutatedThr and Tyr residues do not play an important role in the binding of the ammonium head group to the muscarinic receptor and rule out the possibility that the reduced ACh binding affinities seen with the various mutant receptors may be based on changes in receptor conformation resulting in an impaired binding of the ammonium head group to the anionic receptor site.

The ACh derivatives 4–7 in which the ACh ester moiety has been modified by systematic substitutions of the ether and/or ester oxygen (Table I) displayed affinity profiles that were qualitatively similar to that of TMA. In contrast to ACh and carbachol which showed 10–60-fold reduced affinities for the different mutant receptors (as compared with the wild-type receptor), these compounds lacked the ability to clearly discriminate between wild-type and mutant receptors. The binding affinities of compounds 4–7 for the various mutant receptors (with the exception of Tyr533F → Phe) were similar to or differed only little (by less than a factor of 4.5) from the corresponding affinities for the wild-type receptor. The affinity profile of Tyr533F → Phe differed to some extent from that of all other mutant receptors studied here. Tyr533F → Phe did not only display clearly reduced binding affinities for ACh and carbachol (as did all mutant receptors examined), but also showed significant decreases in binding affinities for all other compounds studied (up to 9-fold as compared with the wild-type receptor).

Conspicuously, ACh and carbachol, both of which can clearly discriminate between wild-type and the individual mutant receptors, share the ester moiety (part of the carboxyl group in the case of carbachol) as a common structural element which is absent in all other “non-selective” derivatives studied. This observation supports the notion that the hydroxyl groups present in the mutated Thr and Tyr residues play an important role in the high affinity interaction of the ACh ester moiety with the muscarinic receptor. Since all mutant receptors (with the exception of Tyr533F → Phe) showed qualitatively similar affinity profiles, our data do not allow a speculation as to which particular amino acid side chain is specifically engaged in forming a hydrogen bond with a particular oxygen atom of the ACh ester moiety. However, it seems reasonable to assume that the variable Tyr and Thr residues studied here create a hydrophillic “hydroxyl group-rich” environment within the ligand binding pocket that allows the efficient interaction of the ACh ester moiety with the receptor protein. Further mutagenesis studies combined with the application of biophysical techniques (e.g. NMR and X-ray crystallography) may aid in determining which of these amino acids are directly involved in ligand binding and which only indirectly by means of stabilizing a certain receptor conformation that allows high affinity ACh binding.

As indicated above, the affinity profile of Tyr533F → Phe differed from that of all other receptors examined in that its binding affinities for compounds 3–7 were significantly lower than those determined for the wild-type and the other mutant receptors studied. Since the common structural element shared by all of these agents is a quaternary ammonium group, one may speculate that Tyr533F may play a supportive role in the binding of the ACh ammonium head group to the muscarinic receptor. Such contact could possibly be made through cation π-orbital interactions as previously suggested for the interaction of ACh with nicotinic receptors (Dennis et al., 1988; Galzi et al., 1990), ACh esterase (Sussman et al., 1991), or synthetic receptors consisting of nonconjugated aromatic rings (Dougherty and Stauffer, 1990). Additional support for this concept arises from helical wheel models (Hulme et al., 1990, 1991) in which Tyr533F is localized in close proximity to Asp417 (the primary site of attachment of the ammonium head group) and the observation that a Tyr residue analogous to Tyr533F of the m3 muscarinic receptor is found in all receptors that bind biogenic amine ligands.

A strategy similar to that described here has been successfully applied to delineate specific points of interaction between the β-adrenergic receptor and catecholamine ligands (Strader et al., 1989). Two Ser residues (Ser209 and Ser207, both located in the N-terminal portion of TM V) were identified that appear to be critically involved in ligand binding and receptor activation. The authors concluded that the interaction of catecholamine ligands with the β-adrenergic receptor involves two hydrogen bonds, one between Ser209 and the meta-hydroxyl group of the ligand and the second between...
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**FIG. 3.** Functional properties of wild-type and mutant m3 muscarinic receptors. CHO cells stably expressing wild-type m3 (●) and mutant m3 receptors, Thr<sup>234</sup> → Ala (□) and Tyr<sup>506</sup> → Phe (▲), were incubated with increasing concentrations of the muscarinic agonists carbachol (upper panel), TMA (middle panel), and S-acetylthiocholine (bottom panel). Incubations were carried out for 60 min at room temperature, and the production of IP<sub>1</sub> was determined as described under “Experimental Procedures.” The results are presented as fold increase in IP<sub>1</sub> above basal levels in the absence of agonist. Basal IP<sub>1</sub> levels (in cpm/well) were: wild-type m3 (113 ± 9), Thr<sup>234</sup> → Ala (124 ± 19), and Tyr<sup>506</sup> → Phe (90 ± 11). Curves shown are representative of two to three independent experiments, each performed in duplicate.

Ser<sup>207</sup> and the para-hydroxyl group of the ligand (Strader et al., 1989).

The mutant m3 receptors that displayed the most pronounced decreases in ACh and carbachol binding affinities were Thr<sup>234</sup> → Ala and Tyr<sup>506</sup> → Phe (25-58-fold as compared with the wild-type receptor). The functional properties of these two receptors were examined after stable expression in CHO cells. Consistent with preliminary functional studies in transiently transfected COS-7 cells (Wess et al., 1991), both mutant receptors displayed greatly reduced potencies in carbachol-induced stimulation of P1 hydrolysis. In addition, the maximum functional response seen with Thr<sup>234</sup> → Ala was

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**TABLE III**

Effect of Gpp(NH)p on ACh binding to wild-type and mutant m3 muscarinic receptors stably expressed in CHO cells

ACh binding parameters were determined in competition binding assays using [<sup>3</sup>H]NMS (200 nM) as a radioligand. Membrane homogenates were prepared from stably transfected CHO cell lines and were assayed in the absence (control) or presence of Gpp(NH)p (100 µM). IC<sub>50</sub> values and Hill coefficients (nH) were obtained by computer fit, as described (Dörje et al., 1991). Data are given as means ± S.E. of two to four independent experiments, each performed in duplicate.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Control</th>
<th>+Gpp(NH)p</th>
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<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>nH</td>
</tr>
<tr>
<td>m3 (wt)</td>
<td>7.5 ± 0.6</td>
<td>0.73 ± 0.02</td>
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<tr>
<td>Thr&lt;sup&gt;234&lt;/sup&gt; → Ala</td>
<td>271 ± 22</td>
<td>0.78 ± 0.03</td>
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<tr>
<td>Tyr&lt;sup&gt;506&lt;/sup&gt; → Phe</td>
<td>192 ± 9</td>
<td>0.75 ± 0.02</td>
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</table>

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**FIG. 4.** Effects of Gpp(NH)p on ACh binding to wild-type and mutant m3 muscarinic receptors. Competition binding studies were carried out with membranes prepared from stably transformed CHO cell lines as described under “Experimental Procedures,” using [<sup>3</sup>H]NMS as radioligand (200 nM). Assays were performed in the absence (●) or presence (□) of 100 µM Gpp(NH)p. The ACh binding properties of the following receptors were studied: wild-type m3 receptor (top panel), Thr<sup>234</sup> → Ala (middle panel), and Tyr<sup>506</sup> → Phe (bottom panel). Curves were generated by computer fit as described (Dörje et al., 1991). Curves shown are representative of two to four independent experiments, each performed in duplicate. Computer-fitted binding parameters are summarized in Table III.
only about 40% of that of the wild-type receptor. In order to exclude the possibility that the decrease in carbachol potencies merely reflects the reduction in carbachol binding affinities seen with these mutant receptors, two partial agonists, TMA (compound 3) and S-acetylthiocholine (compound 7), which showed similar binding affinities for the wild-type and the two mutant receptors were also included in the functional studies. Interestingly, no PI response (Thr<sup>234</sup> → Ala) or only residual functional activity (Tyr<sup>506</sup> → Phe) were observed after incubation of the two mutant receptors with these two agents. In agreement with this observation, ACh binding parameters for Thr<sup>234</sup> → Ala and Tyr<sup>506</sup> → Phe were not significantly affected by the GTP analog, Gpp(NH)p, indicating that receptor-G protein coupling is severely impaired in the case of both mutant receptors.

Thr<sup>234</sup> and Tyr<sup>506</sup> are located on TM V and VI, respectively, within the outer leaflet of the plasma membrane, on the same level as Asp<sup>137</sup> in TM III (Fig. 1). The former two TM helices are connected by a large cytoplasmic loop (Fig. 1, i3), which has been shown to play a pivotal role in G protein recognition and activation (Kubo et al., 1988; Wess et al., 1989, 1990; Lechleiter et al., 1990). One may therefore speculate that Thr<sup>234</sup> and Tyr<sup>506</sup> participate in the agonist-induced changes in receptor conformation that trigger the functional activation of the i3 domain and the interaction with specific G proteins.

In conclusion, we have demonstrated that a series of Thr and Tyr residues which are conserved among all muscarinic receptors play important roles in agonist binding and receptor activation. The elucidation of the precise molecular mechanisms involved in ligand binding and receptor function may eventually lead to a more rational design of novel, therapeutically useful muscarinic drugs.

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