Molecular Mechanisms Involved in Muscarinic Acetylcholine Receptor-mediated G Protein Activation Studied by Insertion Mutagenesis*

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We have recently shown that a four-amino acid epitope (VTIL) on the m2 muscarinic receptor (corresponding to Val385, Thr386, Ile389, and Leu390) is essential for G<sub>i/o</sub> coupling specificity and G<sub>i/o</sub> activation (Liu, J., Conklin, B. R., Blin, N., Yun, J., and Wess, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11642–11646). Because this sequence element is thought to be located at the junction between the third intracellular loop and the sixth transmembrane helix (TM VI), we speculated that agonist binding to the m2 receptor protein results in conformational changes that enable the VTIL motif to interact with G<sub>i/o</sub> proteins. To test the hypothesis that such structural changes might involve a relative movement of TM VI toward the cytoplasm, we created a series of mutant m2 muscarinic receptors in which one to four extra Ala residues were inserted into TM VI immediately after Leu390. Based on the geometry of an α-helix, such mutations are predicted to “push” the VTIL sequence away from the lipid bilayer. Consistent with our working hypothesis, second messenger assays with transfected COS-7 cells showed that all mutant m2 receptors containing extra Ala residues C-terminal of Leu390 could activate the proper G proteins even in the absence of agonist. However, replacement of the VTIL motif in such constitutively active m2 receptors with the corresponding m3 muscarinic receptor sequence (AALS) or deletion of Ala<sup>391</sup> from the wild type m2 receptor completely abolished G protein coupling. Interestingly, introduction of extra Ala residues C-terminal of the AALS motif in the m3 muscarinic receptor completely abolished functional activity. Mutant m2 and m3 receptors that contained extra Ala residues immediately N-terminal of the VTIL and AALS motif, respectively, displayed wild type-like coupling properties. Our data are consistent with a model in which agonist binding to the m2 muscarinic receptor leads to a relative movement of TM VI toward the cytoplasm, thus enabling the adjacent VTIL sequence to interact with the C terminus of G<sub>i/o</sub> subunits.

All members of the superfamily of G protein-coupled receptors are predicted to share a similar molecular architecture consisting of seven α-helically arranged transmembrane domains (TM I–VII)<sup>1</sup> connected by three extracellular and three intracellular loops (i1–i3). Binding of an agonist to the receptor protein (which involves residues in the extracellular receptor domains and/or the TM helices) is predicted to cause conformational changes in the TM receptor core that are propagated to the intracellular receptor surface where the interaction with specific classes of G proteins is thought to occur (Dohlman et al., 1991; Savarese and Fraser, 1992; Hedin et al., 1993; Wess, 1993; Strader et al., 1994). The molecular nature of these agonist-induced conformational changes remains unknown at present.

We have used the m2 and m3 muscarinic acetylcholine receptors as model systems to study the molecular basis of receptor/G protein coupling selectivity and receptor-mediated G protein activation (Wess, 1996). Whereas the m2 receptor is selectively linked to G proteins of the G<sub>i/o</sub> class (primary biochemical response: inhibition of adenylyl cyclase), the m3 receptor is preferentially coupled to G proteins of the G<sub>q/11</sub> family (primary biochemical response: stimulation of phosphatidylinositol (PI) hydrolysis via activation of phospholipase C<sub>β</sub>).

In a recent study (Liu et al., 1995a), we identified a four-amino acid epitope on the m2 muscarinic receptor (VTIL), corresponding to Val<sup>385</sup>, Thr<sup>386</sup>, Ile<sup>389</sup>, and Leu<sup>390</sup>; see Fig. 1) that is essential for G<sub>i/o</sub> coupling specificity and G<sub>i/o</sub> activation. In agreement with this notion, substitution of this structural motif into the wild type m3 muscarinic receptor resulted in a mutant receptor that gained the ability to mediate inhibition of adenylyl cyclase (Liu et al., 1995a). Moreover, coexpression studies with hybrid m2/m3 muscarinic receptors and C-terminally modified mutant G protein α<sub>q</sub> (G<sub>αq</sub>) subunits suggested that the VTIL epitope (corresponding sequence in the m3 receptor: AALS; see Fig. 1) can functionally interact with the C-terminal five amino acids of G<sub>αq</sub> subunits of the G<sub>αq</sub> family. Consistent with this notion, only those mutant receptors that contained the VTIL motif were able to activate mutant G<sub>αq</sub> subunits in which the last five amino acids of G<sub>αq</sub> were replaced with the corresponding α<sub>q</sub> (q5) or α<sub>αq</sub> (q05) sequence (Liu et al., 1995a).

Whereas Val<sup>385</sup> and Thr<sup>386</sup> are predicted to be located at the C terminus of the i3 loop of the m2 muscarinic receptor, Ile<sup>389</sup> and Leu<sup>390</sup> are thought to be contained within the N terminus of TM VI (see Fig. 1); Bonner et al., 1987; Hulme et al., 1990). Computational approaches suggest that the region at the i3

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1 The abbreviations used are: TM I–VII, the seven transmembrane domains of G protein-coupled receptors; AVP, [Arg<sup>8</sup>]vasopressin; i1–i3, the three intracellular loops of G protein-coupled receptors; NMS, N-methylscopolamine; PI, phosphatidylinositol.
loop/TM VI junction in muscarinic and other G protein-coupled receptors is \(\alpha\)-helically arranged (Strader et al., 1989). Based on this notion, Val\(^{385}\), Thr\(^{386}\), Ile\(^{389}\), and Leu\(^{390}\) are predicted to be located on one side of an \(\alpha\)-helix and may thus form a contiguous hydrophobic surface that can interact with the C terminus of G\(\alpha\) subunits. We therefore hypothesized that this receptor surface becomes available for interaction with G\(\alpha\) subunits only in the agonist-bound receptor conformation, resulting perhaps from an agonist-induced rotation or movement toward the cytoplasm of the N-terminal portion of TM VI and the adjacent loop sequence.

To test this hypothesis, we speculated that such structural changes might be mimicked (at least partially) by the insertion of one or more extra Ala residues into the N-terminal segment of TM VI of the m2 muscarinic receptor, immediately after Leu\(^{390}\) (see Fig. 1). Consequently, a series of mutant m2 receptors containing one or more additional Ala residues C-terminal of Leu\(^{390}\) were created and studied for their ability to mediate inhibition of adenylyl cyclase and to functionally interact with C-terminally modified mutant G\(\alpha\) subunits such as Q556 or Q56. For comparison, the functional effects of inserting one or two additional Ala residues immediately N-terminal of Val\(^{385}\) were also examined. Moreover, the m3 muscarinic receptor was structurally modified in a fashion analogous to that described for the m2 receptor to study the effects of such mutations on the function of a G\(\alpha_{q}\)–G\(\alpha_{q}\)–coupled receptor.

Consistent with our working hypothesis, we show in this study that mutant m2 muscarinic receptors containing one or more additional Ala residues after Leu\(^{390}\) are able to activate G proteins even in the absence of agonist. In contrast, similarly modified mutant m3 muscarinic receptors are functionally completely inactive, suggesting that the molecular mode of receptor-mediated G protein activation may differ between the G\(\alpha_{q}\)–G\(\alpha_{q}\)–coupled muscarinic receptors.

**EXPERIMENTAL PROCEDURES**

Creation of Mutant Muscarinic Receptor Genes—All mutations were introduced into Hm2pcD and Rm3pcD, two mammalian expression plasmids coding for the human m2 and rat m3 muscarinic receptor, respectively (Bonner et al., 1987). To facilitate the construction of mutant m2 receptors, an Nhe I site was introduced into Hm2pcD (at codons Leu\(^{395}\)-Ala\(^{396}\)) by oligonucleotide-directed mutagenesis without changing the amino acid sequence (Wess et al., 1989). The 38-base-pair Smal–Nhe I fragment was cut out from the resulting plasmid and replaced in a plasmid containing the wild-type muscarinic receptor constructs (an expression plasmid coding for the human V2 vasopressin receptor; Liu et al., 1995b), and 1 \(\mu\)g of wild type G\(\alpha_{q\delta}\). Approximately 24 h after transfections, cells were transferred into 6-well plates (\(0.75 \times 10^{5}\) cells/well), and 2 \(\mu\)g/ml of [\(^{3}H\)]adenosine (15 Ci/mm, American Radiolabeled Chemicals Inc.) was added to the growth medium. After a 24-h labeling period, cells were preincubated for 15 min in Hanks’ balanced salt solution containing 20 mM HEPES and 10 mM LiCl. Cells were then stimulated, in the same buffer, with the muscarinic agonist carbachol (1 mM) for 1 h at 37°C. After removal of the medium, the reaction was stopped by the addition of 10 mM formic acid. Cell extracts were collected after a 30-min incubation period at 4°C and neutralized with 0.4 ml of 1 M ammonium hydroxide. The inositol monophosphate fraction was then isolated by anion exchange chromatography as described (Berridge et al., 1983).

**cAMP Assays**—For cAMP assays, COS-7 cells were cotransfected in 12-well dishes with 4 \(\mu\)g of muscarinic receptor DNA, 1 \(\mu\)g of V2-pcD–PS (a 699-base pair Nhe I–XI–Nhe I restriction fragment was removed from the resulting plasmid and reinserted into the V2-pcD–PS plasmid, thus generating a full-length wild-type V2 vasopressin receptor construct). After a 24-h labeling period, cells were preincubated for 15 min in Hanks’ balanced salt solution containing 20 mM HEPES and 1 mM 3-isobutyl-1-methylxanthine. Cells were then stimulated for 30 min at 37°C with 0.5 mM of [Arg\(^{8}\)]vasopressin (AVP) in the absence or the presence of carbachol (0.1 mM). Maximum cAMP production (set at 100%) induced by 0.5 mM AVP was determined in cotransfected cells incubated with pertussis toxin (500 ng/ml) for the last 16–18 h of culture. The reaction was terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloroacetic acid containing 1 mM ATP and 1 mM CAMP. [\(^{3}H\)]cAMP was determined by anion exchange chromatography as described (Salomon et al., 1974; Liu et al., 1995b).

Western Blotting—All mutant G\(\alpha_{q}\) subunits were tagged with the influenza virus hemagglutinin epitope sequence DVEPDY A as described (Wedegaertner et al., 1993). The presence of the epitope tag that replaced G\(\alpha_{q}\) residues 125–130 did not affect the receptor and effector coupling properties of wild type G\(\alpha_{q}\) (Wedegaertner et al., 1993) and the various mutant G\(\alpha_{q}\) subunits (data not shown). Samples containing 20 \(\mu\)g of crude membrane protein prepared from transfected COS-7 cells were resolved by SDS-polyacrylamide gel electrophoresis (12%), electrotransferred onto nitrocellulose, and probed with the monoclonal antibody 12CA5 (Böhringer Mannheim). Immunoblotting was performed by detection with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Corp.) and visualized using an enhanced chemiluminescence system (Amersham Corp.).

**RESULTS**

The functional properties of all wild type and mutant m2 and m3 muscarinic receptors were examined after their transient expression in COS-7 cells. Mutant muscarinic receptors containing extra Ala residues were denoted as m2(x + yA) or m3(x + yA) where x indicates the position of the amino acid in the human m2 and rat m3 receptor (Bonner et al., 1987), respectively, after which y extra Ala residues were inserted (Fig. 1).

**Functional Properties of the m2(390 + 1A) Mutant Receptor**—In a previous study (Liu et al., 1995a), we identified a four-amino acid motif in the m2 muscarinic receptor (VTIL, corresponding to Val\(^{385}\), Thr\(^{386}\), Ile\(^{389}\), and Leu\(^{390}\), Fig. 1), located at the i3 loop/TM VI junction, which plays a key role in recognition and activation of G proteins of the G\(\beta\)\(\gamma\) family. Initially, we created a mutant m2 receptor, m2(390 + 1A), that contained one extra Ala residue immediately C-terminal of Leu\(^{390}\). We speculated that this mutation might mimic (at least partially) the conformational changes in the receptor protein...
Physiologically induced by agonist binding.

We first examined the ability of the m2(390 + 1A) mutant receptor to functionally interact with Gαq subunits in which the last five amino acids were replaced with the corresponding α2 (q5) or α1 (q05) sequences. Consistent with published results (Liu et al., 1995a), the wild type m2 receptor, when coexpressed with q05 or q5 and challenged with the muscarinic agonist carbachol (1 mM), was able to induce a pronounced increase in phospholipase C activity (4–7-fold increase in inositol phosphate levels above basal; Fig. 2). This effect was not observed upon coexpression of the wild type m2 receptor with wild type Gαq (qwt) or a mutant Gαq subunit (q5) in which the last five amino acids of qwt were replaced with the corresponding α1 sequence (Fig. 2; Liu et al., 1995a). As shown in Fig. 2, the m2(390 + 1A) mutant receptor displayed a G protein coupling pattern very similar to that of the wild type m2 receptor (no or poor coupling to qwt and q5 but efficient coupling to q05 and q5). However, in contrast to the wild type receptor, m2(390 + 1A) was able to activate q05 and q5 even in the absence of agonist (3–4-fold stimulation in phospholipase C activity above basal; Fig. 2). The addition of carbachol led to a significant increase in the magnitude of this response. Consistent with the results of the PI assays, the m2(390 + 1A) mutant receptor also gained the ability to inhibit AVP-stimulated cAMP production (when coexpressed with the wild type V2 vasopressin receptor and wild type Gα12) in an agonist-independent fashion (20–25% inhibition; Fig. 3). The addition of carbachol (0.1 mM) did not lead to a significant further increase in the magnitude of this response. In contrast, efficient inhibition of adenylyl cyclase activity (40–45%) by the wild type m2 receptor was observed only in the presence of carbachol (Fig. 3).

Diagram:  
![Diagram of muscarinic receptors]

**Fig. 1.** Amino acid sequences of the m2 and m3 muscarinic receptors at the junction between the i3 loop and TM VI. Numbers refer to amino acid positions in the human m2 and the rat m3 muscarinic receptors, respectively (Bonner et al., 1987). Mutant muscarinic receptors were created by inserting one or more alanine residues at the indicated positions (arrows). The abbreviations for the amino acids residues are as follows: A, Ala; I, Ile; K, Lys; L, Leu; Q, Gin; R, Arg; S, Ser; T, Thr; V, Val. Residues known to be important for proper G protein recognition are highlighted in black (Blin et al., 1995; Liu et al., 1995a).

**Fig. 2.** Stimulation of PI hydrolysis mediated by the wild type m2 and the m2(390 + 1A) mutant muscarinic receptor after coexpression with mutant Gαq subunits. A, COS-7 cells were cotransfected with expression plasmids coding for the wild type m2 (top) or the m2(390 + 1A) mutant receptor (bottom) and the indicated Gαq subunits. In qo5, q5, and q5s, the last five amino acids of wild type Gαq (qwt; EYNLV) were replaced with the corresponding sequences derived from α2 (GCGLY), α1 (DCGLF), or α1 (QYELL), respectively. About 48 h after transfections, cells were incubated for 1h at 37°C in the presence of the agonist carbachol (1 mM) or the antagonist atropine (5 μM) or in the absence of drugs (basal). The resulting increases in intracellular inositol monophosphate levels were determined as described under “Experimental Procedures.” The data are expressed as means ± S.E. and are representative of a single experiment carried out in triplicate; one or two additional experiments gave similar results. B, immunoblot analysis showing similar levels of expression for wild type and mutant Gαq subunits in COS-7 cells cotransfected with the wild type m2 receptor. Gαq subunits were detected by Western blotting using the 12CA5 antibody as described under “Experimental Procedures” (Wedegaertner et al., 1993).
of control experiments, one or two additional Ala residues were inserted into the wild type m2 receptor immediately N-terminal of the VTIL motif (after Lys

![FIG. 3.](http://www.jbc.org/)

**Inhibition of adenyl cyclase by wild type and mutant m2 muscarinic receptors.** The structures of the various m2 insertion mutants are given in Fig. 1. COS-7 cells cotransfected with muscarinic receptor DNA and plasmids coding for the V2 vasopressin receptor and wild type Gαi2 were studied for their ability to mediate carbachol-induced (0.1 mM) inhibition of AVP-stimulated cAMP levels (Liu et al., 1995a). Basal CAMP levels (no drug added) were not significantly different between cells expressing the wild type or the various mutant receptors (wild type m2 receptor: 1780 ± 220 cpm/well). The data are expressed as the percentage of inhibition of maximum cAMP production induced by 0.5 nM AVP (100% = 3-fold above basal levels), determined in the presence of pertussis toxin (500 ng/ml). The data are given as means ± S.E. of triplicate determinations in a single experiment; a separate experiment gave similar results.

![FIG. 4.](http://www.jbc.org/)

**Stimulation of PI hydrolysis mediated by wild type and mutant m2 muscarinic receptors coexpressed with wild type Gαo (q(wt)) or the mutant Gαo subunit (qo5).** The structures of the various m2 insertion mutants are given in Fig. 1. In the m2(390 + 1A/VTIL->AALS) and m2(390 + 2A/VTIL->AALS) mutant receptors, Val

![Text content...](http://www.jbc.org/)

the mutant Gαq subunit, qo5 (in the absence or the presence of agonist). Similar results were obtained when Ala

![Text content...](http://www.jbc.org/)
Consistent with our working hypothesis, we found that all mutant m2 receptors that contained additional (one to four) Ala residues after Leu390 were constitutively active. Even in the absence of agonist, all four mutant receptors were able to mediate inhibition of adenylyl cyclase (via activation of G\textsubscript{i}) and to efficiently stimulate phospholipase C activity when coexpressed with a mutant Go\textsubscript{q,sub} subunit containing \( \alpha \) (or \( \alpha \)) sequence at its C terminus. The addition of agonist had no or little effect on the magnitude of these responses (\( E_{\text{max}} \approx 50\% \) of wild type m2), which, however, could be completely prevented by incubation with atropine (5 \( \mu M \)). According to the recently proposed “allosteric ternary complex model” of ligand/receptor/G protein interactions (see below; Lefkowitz et al., 1993), atropine can therefore act as an inverse agonist (see also Blüml et al., 1994; Högger et al., 1995).

Whereas insertion of extra Ala residues C-terminal of Leu390 rendered the resulting m2 mutant receptors constitutively active, deletion of Ala\textsubscript{391} from the wild type m2 receptor resulted in a mutant receptor (m2(\( \Delta \text{A391} \))) that was unable to interact with G proteins, either in the absence or the presence of agonist. Taken together, these results suggest a model of agonist-induced m2 receptor activation in which agonist binding induces a movement of TM VI toward the cytoplasm, thus enabling the VTIL motif to interact with the C terminus of Go\textsubscript{q,sub} subunits.

Based on computational approaches (Strader et al., 1989) and recent mutagenesis data (Liu et al., 1995a), we speculated that the residues forming the VTIL motif are located on one side of an \( \alpha \)-helical receptor segment. If this is correct, one would expect (due to changes in helix register) that the stepwise insertion of extra Ala residues C-terminal of this sequence element should lead to a progressive rotation (in 100 \( ^\circ \) increments) of the VTIL surface. Because the degree of constitutive receptor activity was found to be virtually independent of the number of inserted Ala residues, one might conclude that the C terminus of Go\textsubscript{q,sub} subunits can interact with the VTIL site independent of its precise spatial orientation. However, such a mechanism does not appear very likely, because many studies suggest that proper receptor/G protein coupling involves coordinated interactions between several intracellular receptor domains (Dohlman et al., 1991; Savarese and Fraser, 1992; Hedin et al., 1993; Strader et al., 1994) and at least three sites on the Go subunits (including the C terminus; Conklin and Bourne, 1993; Rens-Domiano and Hamm, 1995). One may therefore speculate that the insertion of multiple Ala residues between Leu390 and Ala\textsubscript{391} (m2 receptor) does not lead to a progressive register shift (involving the residues N-terminal of the insertion point) but rather results in a local disruption of the TM VI helix. Structural studies with various insertion mutants of T4 lysozyme have shown, for example, that extra Ala residues can be accommodated within an \( \alpha \)-helical protein domains by “looping out” of the inserted amino acids (Matthews, 1995). However, the possibility can also not be excluded that the receptor segment in which the VTIL motif is located is not \( \alpha \)-helically arranged (in contrast to predictions made based on previous results by Liu et al. (1995a)) but is perhaps relatively disordered. To distinguish between these possibilities, high resolution structural information (obtained, e.g. by NMR or x-ray crystallography) would be required.

Pioneering work by Lefkowitz and co-workers has shown that mutational modification of the C-terminal portion of the i3 loop of several adrenergic receptor subtypes also leads to constitutive receptor activity (Kjelsberg et al., 1992; Ren et al., 1993; Samama et al., 1993). It could be demonstrated, for example, that replacement of Thr\textsuperscript{346} (initially erroneously referred to as Thr\textsuperscript{345}) in the \( \alpha \text{2A}- \) adrenergic receptor (correspond-
ing to Thr<sup>386</sup> in the m2 receptor; Fig. 1) with five different amino acids (Ren et al., 1993) or substitution of Ala<sup>293</sup> in the α<sub>1A</sub>-adrenergic receptor (corresponding to Ala<sup>489</sup> in the m3 receptor; Fig. 1) with all 19 possible amino acids (Kjelsberg et al., 1992) resulted in mutant receptors that could activate G proteins in an agonist-independent fashion (note, however, that introduction of structurally homologous mutations into the m2, m3, and m5 muscarinic receptors does not result in constitutive receptor activity) (Burstein et al., 1995). Based on the functional properties of such constitutively active adrenergic receptors, it was proposed that residues in the C terminus of the i3 loop play a role in constraining the adrenergic receptors in an inactive conformation and that replacement of these residues removes this constraining function allowing the receptor to "relax" into an active conformation (Lefkowitz et al., 1993).

In the light of these findings, one may argue that the agonist-independent activity displayed by the four m2 receptor insertion mutants described in this study could also simply be due to the loss of a constraining interaction involving residues at the i3 loop/TM VI junction. We could show, however, that replacement of the VTIL motif in such constitutively active m2 receptors with the corresponding m3 receptor residues (AALS) completely abolished agonist-independent (as well as agonist-dependent) signaling. This observation is in agreement with previous results suggesting that the VTIL site is directly involved in G protein recognition and activation (Liu et al., 1995a). This notion is further supported by the finding that a 19-amino acid synthetic peptide (including residues at the i3 loop/TM VI junction) corresponding to the C-terminal portion of the i3 loop of the G<sub>o</sub>-coupled m4 muscarinic receptor can activate G<sub>o</sub> proteins at nanomolar concentrations in a reconstituted system (Okamoto and Nishimoto, 1992). Moreover, a short synthetic peptide derived from the i3 loop/TM VI junction of the G<sub>o</sub>-coupled α<sub>2A</sub>-adrenergic receptor could be chemically cross-linked to G<sub>o</sub> and β subunits in vitro (Taylor et al., 1994).

As an extension of the model of receptor activation proposed by Lefkowitz et al. (1993), these data strongly suggest that agonist binding to G<sub>o</sub>-coupled (muscarinic) receptors leads to structural changes at the i3 loop/TM VI junction, allowing distinct residues located in this region to interact with specific sites on the G protein(s).

The constitutively active mutant m2 receptors described in this study displayed agonist binding properties similar to those of the wild type m2 receptor. Unchanged agonist binding affinities have also been reported for several constitutively active glycoprotein hormone receptors (Kosugi et al., 1995; Kopp et al., 1995). In contrast, virtually all known mutant adrenergic receptors capable of agonist-independent signaling (as well as a recently described constitutively active m1 (Glu<sup>460</sup> → Ala) mutant muscarinic receptor) (Höger et al., 1995) show considerably higher agonist affinities than the corresponding wild type receptors (Kjelsberg et al., 1992; Ren et al., 1993; Samama et al., 1993). Based on this finding, together with the observation that the extent of this affinity increase is related to agonist activity (Samama et al., 1993), an allosteric ternary complex model (as an extension of the "classical ternary receptor model") (De Lean et al., 1980) of ligand/receptor/G protein interactions was proposed (Samama et al., 1993; Lefkowitz et al., 1993). This model predicts that the receptor exists in an equilibrium (characterized by the equilibrium constant J) between an inactive (R) and an active conformation (R<sup>*</sup>) and that agonists, by preferentially binding to the R<sup>*</sup> form, shift this equilibrium to the active receptor conformation. According to this model, a similar shift in the equilibrium toward R<sup>*</sup> can also result from mutations resulting in constitutive receptor activity. The lack of increased agonist binding affinity observed with the constitutively active mutant receptors described here may therefore be explained by assuming that the proportion of mutant receptors that are present in the R<sup>*</sup> state is too small to be detected in radioligand binding assays. Alternatively, because the allosteric ternary complex model predicts that an increase in J causes an increase in agonist affinity, it is also possible that the activating mutations described here primarily

### Table 1

Ligand binding properties of wild type and mutant m2 and m3 muscarinic receptors

| Receptor                        | K<sub>D</sub> (μM) | B<sub>max</sub> (pmol/mg) | IC<sub>50</sub> (μM) | n<sub>H</sub><sup>a</sup> |
|---------------------------------|-------------------|--------------------------|----------------------|------------------------|
| **Wild type and mutant m2 receptors** |                   |                          |                      |                        |
| m2(wt)                          | 72 ± 8            | 562 ± 65                 | 16.0 ± 4.1           | 0.69 ± 0.06            |
| m2(390 + 1A)                    | 84 ± 6            | 508 ± 79                 | 13.2 ± 1.4           | 0.72 ± 0.05            |
| m2(390 + 2A)                    | 80 ± 10           | 489 ± 48                 | 17.8 ± 1.7           | 0.67 ± 0.04            |
| m2(390 + 3A)                    | 73 ± 11           | 273 ± 52                 | 12.4 ± 3.2           | 0.70 ± 0.07            |
| m2(390 + 4A)                    | 87 ± 13           | 214 ± 43                 | 14.9 ± 1.5           | 0.59 ± 0.08            |
| m2(390 + 1A/VTIL → AALS)        | 67 ± 4            | 519 ± 59                 | 18.8 ± 2.1           | 0.65 ± 0.05            |
| m2(390 + 2A/VTIL → AALS)        | 92 ± 13           | 455 ± 64                 | 15.2 ± 1.9           | 0.58 ± 0.06            |
| m2(3A391)                       | 97 ± 8            | 345 ± 60                 | 11.5 ± 1.8           | 0.62 ± 0.05            |
| m2(384 + 1A)                    | 65 ± 7            | 521 ± 28                 | 19.2 ± 3.5           | 0.52 ± 0.10            |
| m2(384 + 2A)                    | 57 ± 8            | 547 ± 49                 | 23.6 ± 2.5           | 0.65 ± 0.06            |
| **Wild type and mutant m3 receptors** |                   |                          |                      |                        |
| m3(wt)                          | 32 ± 5            | 684 ± 72                 | 89 ± 8               | 0.70 ± 0.06            |
| m3(493 + 1A)                    | 28 ± 6            | 614 ± 84                 | 162 ± 10             | 0.58 ± 0.04            |
| m3(493 + 2A)                    | 29 ± 6            | 546 ± 67                 | 182 ± 7              | 0.63 ± 0.03            |
| m3(493 + 3A)                    | 39 ± 8            | 482 ± 71                 | 155 ± 10             | 0.52 ± 0.06            |
| m3(493 + 4A)                    | 48 ± 4            | 348 ± 51                 | 194 ± 10             | 0.57 ± 0.03            |
| m3(487 + 1A)                    | 42 ± 3            | 658 ± 44                 | 81 ± 10              | 0.68 ± 0.07            |
| m3(487 + 2A)                    | 33 ± 5            | 609 ± 69                 | 97 ± 9               | 0.74 ± 0.06            |

<sup>a</sup>Hill coefficient.

<sup>2</sup>J. Liu, Z. Vogel, S. Gutkind, and J. Wess, unpublished results.
affect the affinity of R* for the G protein. The notion that mutations can activate receptors by different molecular mechanisms is also supported by the finding that the activity of the constitutively active m2 mutant receptors described here, in contrast to the functional properties of previously published mutationally activated receptors (Lefkowitz et al., 1993), was not significantly increased by the addition of agonist. Such a potential heterogeneity of receptor activation mechanisms would also be consistent with the observation that constitutively active G protein-coupled receptors can result from mutations in various different receptor regions including the TM helices and various extracellular and intracellular regions (for recent reviews, see Coughlin (1994) and Shenker (1995)).

Interestingly, when extra Ala residues were introduced after Ser^493 into the G9R1-coupled m3 muscarinic receptor, the resulting mutant receptors, in contrast to the structurally homologous m2 receptor mutants, did not display constitutive activity (even in the presence of coexpressed wild type Gq; data not shown) but were functionally completely inactive. Given the high degree of sequence homology found among different muscarinic receptor subtypes (Bonner et al., 1987; Hulme et al., 1990), this finding may indicate that the G_{i0} and G9R1-coupled muscarinic receptors interact with their cognate G proteins in a somewhat different fashion. Such a notion would also be consistent with the observation that the four m3 receptor residues (AALS, Ala^488, Ala^489, Leu^492, and Ser^493) corresponding to the VTIL motif in the m2 receptor are not essential for G_{i0} coupling (in contrast to the functional role of the VTIL motif in the m2 receptor) (Liu et al., 1995a), although they contribute to the efficiency of receptor-mediated G_{i0} activation (Blin et al., 1995). Moreover, substitution of the AALS motif (by itself) into the wild type m2 muscarinic receptor failed to establish coupling to G_{i0} (Blin et al., 1995). However, consistent with previous mutagenesis studies (Kunkel and Peralta, 1993; Höger et al., 1995), the complete lack of agonist-dependent signaling observed with the mutant m3 receptors containing additional Ala residues after Ser^493 suggests that the structural integrity of the C terminus of the i3 loop is critical for proper receptor-G protein interactions.

In a set of control experiments, one or two extra Ala residues were also inserted into the wild type m2 and m3 muscarinic receptors immediately N-terminal of the VTIL and AALS motif, respectively. Second messenger assays showed that these modifications had little effect on the magnitude of the m2 and m3 receptor-mediated functional responses. Interestingly, the VTIL (or AALS) motif is preceded by a four-amino acid sequence element (B-B-Glu-B, where B is a basic amino acid) that is conserved among virtually all G protein-coupled receptors that bind biogenic amine ligands (Watson and Arkinstall, 1994). Loss-of-function mutagenesis studies have shown that one or more of these charged residues are generally important for efficient G protein coupling (Kunkel and Peralta, 1993; Höger et al., 1995). Our data therefore suggest that the precise spatial orientation of this highly charged sequence motif (such as its direct proximity to residues at the i3 loop/TM VI junction) is not essential for receptor-mediated G protein activation.

In conclusion, we have demonstrated that insertion mutagenesis can serve as a useful tool to study the molecular mechanisms involved in receptor/G protein recognition and receptor-mediated G protein activation. It should be of considerable interest to examine which functional effects mutations homologous to those described here can cause in other classes of G protein-coupled receptors.
