

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

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Jie Liu, Nathalie Blin, Bruce R. Conklin<sup>‡</sup>, and Jürgen Wess<sup>§</sup>

From the Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland 20892 and the <sup>‡</sup>Departments of Medicine and Pharmacology, Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, California 94141-9100

We have recently shown that a four-amino acid epitope (VTIL) on the m2 muscarinic receptor (corresponding to Val<sup>385</sup>, Thr<sup>386</sup>, Ile<sup>389</sup>, and Leu<sup>390</sup>) 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 Leu<sup>390</sup>. Based on the geometry of an  $\alpha$ -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 Leu<sup>390</sup> 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  $\alpha$ -helically arranged transmembrane do-

main (TM I-VII)<sup>1</sup> connected by three extracellular and three intracellular loops (i1-i3) (Watson and Arkininstall, 1994). 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 $\beta$ ) (Peralta *et al.*, 1988; Parker *et al.*, 1991; Berstein *et al.*, 1992; Offermanns *et al.*, 1994).

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  $\alpha_q$  (G $\alpha_q$ ) 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 $\alpha$  subunits of the G<sub>i/o</sub> family. Consistent with this notion, only those mutant receptors that contained the VTIL motif were able to activate mutant G $\alpha_q$  subunits in which the last five amino acids of G $\alpha_q$  were replaced with the corresponding  $\alpha_{i2}$  (qi5) or  $\alpha_o$  (qo5) 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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§ To whom correspondence should be addressed: NIDDK, National Institutes of Health, Laboratory of Bioorganic Chemistry, Bldg. 8A, Rm. B1A-09, Bethesda, MD 20892. Tel.: 301-402-4745; Fax: 301-402-4182.

<sup>1</sup> 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<sup>385</sup>, Thr<sup>386</sup>, Ile<sup>389</sup>, and Leu<sup>390</sup> 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 $\alpha_{i/o}$  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<sup>390</sup> (see Fig. 1). Consequently, a series of mutant m2 receptors containing one or more additional Ala residues C-terminal of Leu<sup>390</sup> were created and studied for their ability to mediate inhibition of adenylyl cyclase and to functionally interact with C-terminally modified mutant G $\alpha_q$  subunits such as qo5 or qi5. For comparison, the functional effects of inserting one or two additional Ala residues immediately N-terminal of Val<sup>385</sup> 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 $\alpha_{q/11}$ -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<sup>390</sup> 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 $\alpha_{i/o}$ - and G $\alpha_{q/11}$ -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 *NheI* site was introduced into Hm2pcD (at codons Leu<sup>393</sup>-Ala<sup>395</sup>) by oligonucleotide-directed mutagenesis without changing the amino acid sequence (Wess *et al.*, 1989). The 38-base pair *SmaI*-*NheI* fragment was cut out from the resulting plasmid and replaced in a three-piece ligation with synthetic DNA fragments containing the desired mutations. To insert additional alanine codons into the m3 muscarinic receptor gene (after the Lys<sup>487</sup> or Ser<sup>493</sup> codon, respectively), a 699-base pair *BstXI*-*NheI* restriction fragment was removed from Rm3pcD and replaced with fragments containing the altered sequences. The mutated fragments were generated by using standard polymerase chain reaction mutagenesis techniques (Higuchi, 1989). The identity of all mutant constructs and the correctness of all polymerase chain reaction-derived sequences were verified by dideoxy sequencing of the mutant plasmids (Sanger *et al.*, 1977).

The construction of pcDNA1-based expression plasmids coding for wild type murine G $\alpha_q$ , wild type murine G $\alpha_{i2}$  (Sullivan *et al.*, 1986), and the various mutant G $\alpha_q$  subunits has been described previously (Conklin *et al.*, 1993).

**Transient Expression of Mutant Receptors**—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO<sub>2</sub> incubator. For transfections, 2 × 10<sup>6</sup> cells were seeded into 100-mm dishes. About 24 h later, cells were transfected with the various muscarinic receptor constructs (4 μg of plasmid DNA/dish) by a DEAE/dextran method (Cullen, 1987). In coexpression experiments involving wild type and mutant G $\alpha_q$  subunits, COS-7 cells were cotransfected with 4 μg of individual receptor constructs and 1 μg of G $\alpha$  subunits.

**Radioligand Binding Assays**—Transfected COS-7 cells were harvested 72 h after transfections. Binding assays were carried out with membrane homogenates prepared as described previously (Dörje *et al.*, 1991). Incubations were carried out for 3 h at 22 °C, in a 25 mM sodium phosphate buffer (pH 7.4) containing 5 mM MgCl<sub>2</sub>. In [<sup>3</sup>H]N-methylsco-

polamine ([<sup>3</sup>H]NMS, 81.4 Ci/mmol; DuPont NEN) saturation binding experiments, six different radioligand concentrations (12.5–400 pM) were used. In carbachol competition binding experiments, ten different carbachol concentrations (50 nM to 1 mM) were tested in the presence of 200 pM [<sup>3</sup>H]NMS. Nonspecific binding was measured in the presence of 1 μM atropine. Protein concentrations were determined by the method of Bradford (1976). Binding data were analyzed by nonlinear least squares curve-fitting procedures, using the computer programs LIGAND (saturation binding data; Munson and Rodbard, 1980) or KALEIDAGRAPH (competition binding data; Synergy Software).

**PI Hydrolysis Assays**—About 24 h after transfections, cells were split into 6-well dishes (ca. 0.75 × 10<sup>6</sup> cells/well) in culture medium supplemented with 3 μCi/ml [<sup>3</sup>H]myo-inositol (20 Ci/mmol; American Radio-labeled Chemicals Inc.). After a 24-h labeling period, cells were preincubated for 30 min at 37 °C in 2 ml of 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 100-mm dishes with 4 μg of muscarinic receptor DNA, 1 μg of V2pcD-PS (an expression plasmid coding for the human V2 vasopressin receptor; Liu *et al.*, 1995b), and 1 μg of wild type G $\alpha_{i2}$ . Approximately 24 h after transfections, cells were transferred into 6-well plates (~0.75 × 10<sup>6</sup> cells/well), and 2 μCi/ml of [<sup>3</sup>H]adenine (15 Ci/mmol, American Radio-labeled 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 1 mM 3-isobutyl-1-methyl-xanthine. Cells were then stimulated for 30 min at 37 °C with 0.5 nM of [Arg<sup>8</sup>]vasopressin (AVP) in the absence or the presence of carbachol (0.1 mM). Maximum cAMP production (set at 100%) induced by 0.5 nM 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. [<sup>3</sup>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 DVPDYA 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 μg of crude membrane protein prepared from transfected COS-7 cells were resolved by SDS-polyacrylamide gel electrophoresis (12%), electroblotted onto nitrocellulose, and probed with the monoclonal antibody 12CA5 (Boehringer Mannheim). Immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Corp.) and visualized using an enhanced chemiluminescence system (Amersham Corp.).

**Drugs**—All drugs, with the exception of pertussis toxin, which was purchased from List, were obtained through Sigma.

#### 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* + *y*A) (or m3(*x* + *y*A)) 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<sup>385</sup>, Thr<sup>386</sup>, Ile<sup>389</sup>, and Leu<sup>390</sup>; 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 $\alpha_{i/o}$  family. Initially, we created a mutant m2 receptor, m2(390 + 1A), that contained one extra Ala residue immediately C-terminal of Leu<sup>390</sup>. We speculated that this mutation might mimic (at least partially) the conformational changes in the receptor protein

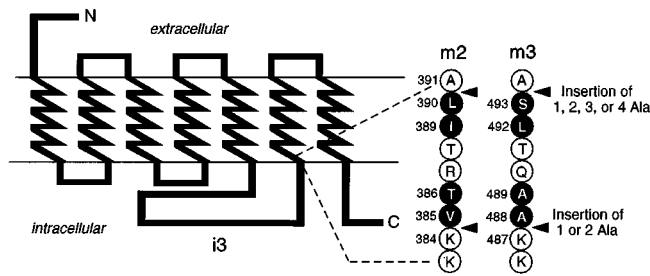


FIG. 1. Amino acid sequences of the m2 and m3 muscarinic receptors at the junction between the i3 loop and TM VI. Numbers refer to amino acid positions in the human m2 and the rat m3 muscarinic receptors, respectively (Bonner *et al.*, 1987). Mutant muscarinic receptors were created by inserting one 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, Gln; 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).

physiologically induced by agonist binding.

We first examined the ability of the m2(390 + 1A) mutant receptor to functionally interact with  $G\alpha_q$  subunits in which the last five amino acids were replaced with the corresponding  $\alpha_{i2}$  (qi5) or  $\alpha_o$  (qo5) sequences. Consistent with published results (Liu *et al.*, 1995a), the wild type m2 receptor, when coexpressed with qo5 or qi5 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  $G\alpha_q$  (q(wt)) or a mutant  $G\alpha_q$  subunit (qs5) in which the last five amino acids of q(wt) were replaced with the corresponding  $\alpha_s$  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 q(wt) and qs5 but efficient coupling to qo5 and qi5). However, in contrast to the wild type receptor, m2(390 + 1A) was able to activate qo5 and qi5 even in the absence of agonist (3–4-fold stimulation in phospholipase C activity above basal; Fig. 2). The addition of carbachol led to only a minor increase (10–20%) in the magnitude of this response (Fig. 2). The agonist-independent increase in inositol phosphate production seen with cells coexpressing m2(390 + 1A) and qo5 or qi5 could be completely prevented by the addition of the muscarinic antagonist, atropine (5  $\mu$ M; Fig. 2).

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  $G\alpha_{i2}$ ) 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).

**Functional Properties of Mutant m2 Receptors Containing Multiple Extra Ala Residues C-terminal of the VTIL Motif**—Based on the results obtained with the m2(390 + 1A) mutant receptor, three additional m2 mutant receptors were created that contained two, three, or four extra Ala residues C-terminal of Leu<sup>390</sup>. As shown in Figs. 3 and 4, the resulting mutant receptors (m2(390 + 2A), m2(390 + 3A), and m2(390 + 4A), respectively) displayed G protein coupling properties very similar to those found with m2(390 + 1A). Whereas all three mutant receptors were unable to functionally interact with q(wt), they were capable of stimulating the production of ino-

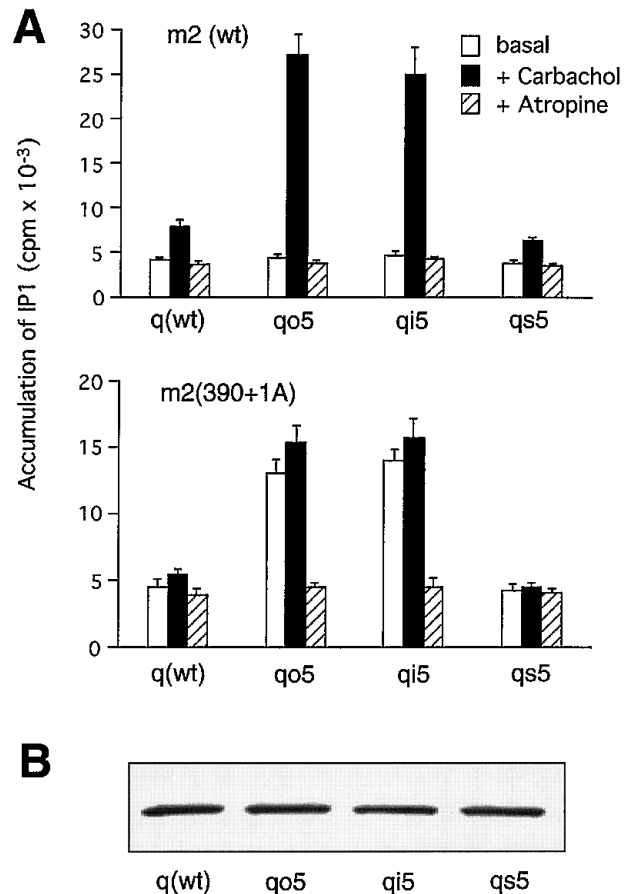


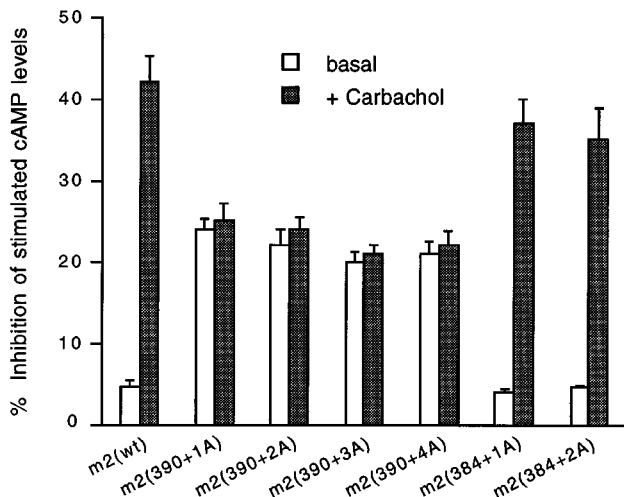
FIG. 2. Stimulation of PI hydrolysis mediated by the wild type m2 and the m2(390 + 1A) mutant muscarinic receptor after coexpression with mutant  $G\alpha_q$  subunits. *A*, COS-7 cells were cotransfected with expression plasmids coding for the wild type m2 (*top*) or the m2(390 + 1A) mutant receptor (*bottom*) and the indicated  $G\alpha_q$  subunits. In qo5, qi5, and qs5, the last five amino acids of wild type  $G\alpha_q$  (q(wt); *EYNLV*) were replaced with the corresponding sequences derived from  $\alpha_o$  (*GCGLY*),  $\alpha_{i2}$  (*DCGLF*), or  $\alpha_s$  (*QYELL*), respectively. About 48 h after transfections, cells were incubated for 1 h (at 37 °C) in the presence of the agonist carbachol (1 mM) or the antagonist atropine (5  $\mu$ M) 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  $\pm$  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  $G\alpha_q$  subunits in COS-7 cells cotransfected with the wild type m2 receptor.  $G\alpha$  subunits were detected by Western blotting using the 12CA5 antibody as described under "Experimental Procedures" (Wedegaertner *et al.*, 1993).

sitol phosphates (3–4-fold above basal) via interaction with qo5 even in the absence of agonist (Fig. 4). The addition of the agonist carbachol (1 mM) had no significant effect on the magnitude of these responses. Consistent with these results, cAMP assays showed that the m2(390 + 2A), m2(390 + 3A), and m2(390 + 4A) mutant receptors gained the ability to activate wild type  $G_i$  in an agonist-independent fashion (20–25% inhibition of AVP-stimulated cAMP production; Fig. 3).

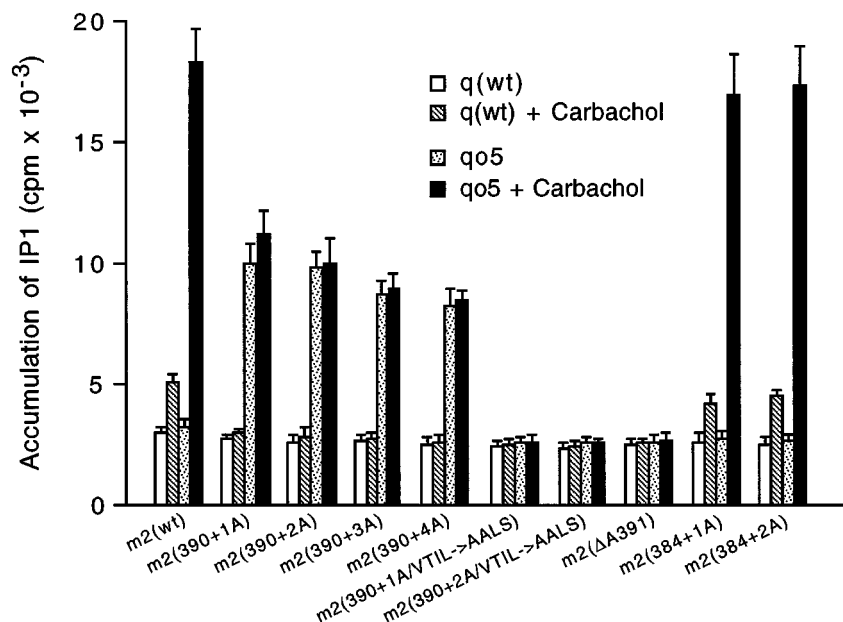
To examine whether the presence of the VTIL motif was required for agonist-independent signaling, Val<sup>385</sup>, Thr<sup>386</sup>, Ile<sup>389</sup>, and Leu<sup>390</sup> were replaced with the corresponding m3 receptor residues (AALS; corresponding to Ala<sup>488</sup>, Ala<sup>489</sup>, Leu<sup>492</sup>, and Ser<sup>493</sup>; Fig. 1) in the m2(390 + 1A) and m2(390 + 2A) mutant receptors (resulting in m2(390 + 1A/VTIL->AALS) and m2(390 + 2A/VTIL->AALS), respectively). As illustrated in Fig. 4, this modification completely abolished the ability of m2(390 + 1A) and m2(390 + 2A) to functionally interact with

the mutant  $G\alpha_q$  subunit, q05 (in the absence or the presence of agonist). Similar results were obtained when Ala<sup>391</sup> was deleted from the wild type m2 receptor (yielding m2( $\Delta$ A391); Fig. 4).

**Functional Activity of Mutant m2 Receptors Containing Extra Ala Residues N-terminal of the VTIL Motif**—In another set



**FIG. 3. Inhibition of adenylyl 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  $G\alpha_{i2}$  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:  $1780 \pm 220$  cpm/well). The data are expressed as the percentage of inhibition of maximum cAMP production induced by 0.5 nM AVP (100%;  $18 \pm 3$ -fold above basal levels), determined in the presence of pertussis toxin (500 ng/ml). The data are given as means  $\pm$  S.E. of triplicate determinations in a single experiment; a separate experiment gave similar results.

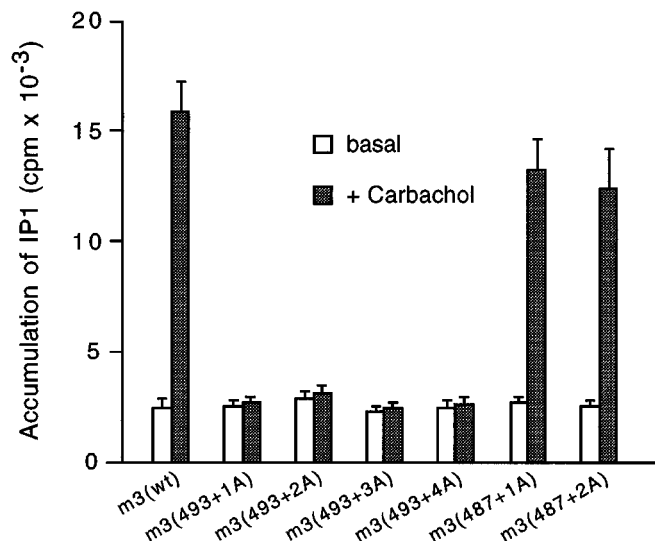


**FIG. 4. Stimulation of PI hydrolysis mediated by wild type and mutant m2 muscarinic receptors coexpressed with wild type  $G\alpha_q$  (q(wt)) or the mutant  $G\alpha_q$  subunit (q05).** The structures of the various m2 insertion mutants are given in Fig. 1. In the m2(390 + 1A/VTIL->AALS) and m2(390 + 2A/VTIL->AALS) mutant receptors, Val<sup>385</sup>, Thr<sup>386</sup>, Ile<sup>389</sup>, and Leu<sup>390</sup> (m2 receptor sequence) were replaced with the corresponding m3 receptor residues (Ala<sup>488</sup>, Ala<sup>489</sup>, Leu<sup>492</sup>, and Ser<sup>493</sup>; Fig. 1). In m2( $\Delta$ A391), Ala<sup>391</sup> was deleted from the wild type m2 receptor. q05 is a mutant  $G\alpha$  subunit in which the C-terminal five amino acids of q(wt) were replaced with the corresponding  $\alpha_0$  sequence. COS-7 cells coexpressing individual mutant receptors and q(wt) or q05 were incubated for 1 h (at 37 °C) in the absence or the presence of the agonist carbachol (1 mM). The resulting increases in intracellular inositol monophosphate levels were determined as described under "Experimental Procedures." The data are expressed as means  $\pm$  S.E. and are representative of a single experiment carried out in triplicate; three to five additional experiments gave similar results.

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<sup>384</sup>; Fig. 1). As shown in Figs. 3 and 4, the two resulting mutant receptors, m2(384 + 1A) and m2(384 + 2A), displayed functional properties similar to those of the wild type receptor. None of the two mutant receptors displayed significant functional activity in the absence of agonist. However, in the presence of agonist (carbachol), both mutant receptors retained the ability to efficiently interact with the mutant  $G\alpha_q$  subunit, q05 (5–7-fold increase in phospholipase C activity above basal; Fig. 4) and wild type  $G_i$  (35–40% inhibition of AVP-stimulated cAMP accumulation; Fig. 3).

**Functional Activity of Mutant m3 Receptors Containing Ala Insertions**—The m3 muscarinic receptor was structurally modified in a fashion analogous to that described above for the wild type m2 receptor, in order to examine whether receptor-mediated activation of  $G_{q/11}$  proteins is controlled by molecular mechanisms similar to those found with the  $G_{i/o}$ -coupled m2 receptor. Initially, one to four extra Ala residues were inserted C-terminal of Ser<sup>493</sup> (Ser<sup>493</sup> corresponds to Leu<sup>390</sup> in the m2 receptor; Fig. 1). In contrast to the homologous m2 receptor mutants, the resulting m3 receptor mutants (m3(493 + 1A), m3(493 + 2A), m3(390 + 3A), and m3(390 + 4A), respectively) virtually completely lost the ability to productively couple to G proteins (Fig. 5). Whereas the wild type m3 receptor was able to stimulate inositol phosphate production by 6–8-fold (in the presence of 1 mM carbachol), none of the four mutant receptors containing extra Ala residues after Ser<sup>493</sup> displayed detectable functional activity (Fig. 5). Similar results were obtained when the four mutant receptors were coexpressed with q(wt) (data not shown).

In addition, two m3 mutant receptors were created (m3(487 + 1A) and m3(487 + 2A)) that contained one or two extra Ala residues N-terminal of Ala<sup>488</sup> (Ala<sup>488</sup> corresponds to Val<sup>385</sup> in the m2 receptor; Fig. 1). Consistent with the results obtained



**FIG. 5. Stimulation of PI hydrolysis mediated by wild type and mutant m3 muscarinic receptors.** The structures of the various m3 receptor insertion mutants are given in Fig. 1. COS-7 cells transiently expressing the indicated wild type and mutant receptor constructs were incubated for 1 h (at 37 °C) either in the absence (*basal*) or the presence of the agonist carbachol (1 mM). The resulting increases in intracellular inositol monophosphate levels were determined as described under "Experimental Procedures." The data are given as means  $\pm$  S.E. and are representative of four independent experiments, each carried out in triplicate.

with the structurally homologous m2 receptor mutants (Figs. 3 and 4), m3(487 + 1A) and m3(487 + 2A) retained the ability to mediate a robust agonist-dependent PI response (5–6-fold increase in phospholipase C activity; Fig. 5).

**Comparison of Ligand Binding Properties of Wild Type and Mutant Receptors**—All wild type and mutant muscarinic receptors were assayed for their ability to bind the antagonist, [<sup>3</sup>H]NMS, and the agonist, carbachol. The results of the radioligand binding studies are summarized in Table I. [<sup>3</sup>H]NMS saturation binding studies showed that all m2 and m3 mutant receptors (except m2(390 + 3A) and m2(390 + 4A), which were expressed at 2–3-fold lower levels) were expressed at densities ( $B_{max}$ ) comparable with those found with the two wild type receptors. Similarly, the [<sup>3</sup>H]NMS  $K_D$  values determined for the different mutant receptors very closely resembled the respective wild type values. Moreover, all mutant m2 and m3 receptors displayed agonist binding properties ( $IC_{50}$ ,  $n_H$ ) that were very similar to those determined with the wild type m2 and m3 receptor, respectively.

#### DISCUSSION

Based on the results of a previous study (Liu *et al.*, 1995a), we speculated that the insertion of one or more extra Ala residues into the endofacial segment of TM VI of the m2 muscarinic receptor immediately C-terminal of Leu<sup>390</sup> might be able to mimic (at least partially) the agonist-induced conformational changes in the receptor protein. Radioligand binding studies showed that the resulting m2 mutant receptors displayed agonist and antagonist binding affinities that closely resembled those found with the wild type m2 receptor. Because the binding of muscarinic ligands is known to involve specific residues on TM VI and several other TM domains (Hulme *et al.*, 1990; Wess, 1993), this observation indicates that the insertion of extra Ala residues C-terminal of Leu<sup>390</sup> did not interfere with the proper assembly of the TM helical bundle. The presence of extra residues at the N terminus of TM VI is therefore predicted to "push" the adjacent VTIL motif further away from the lipid bilayer into the cytoplasm.

Consistent with our working hypothesis, we found that all mutant m2 receptors that contained additional (one to four) Ala residues after Leu<sup>390</sup> were constitutively active. Even in the absence of agonist, all four mutant receptors were able to mediate inhibition of adenylyl cyclase (via activation of  $G_i$ ) and to efficiently stimulate phospholipase C activity when coexpressed with a mutant  $G\alpha_q$  subunit containing  $\alpha_o$  (or  $\alpha_i$ ) sequence at its C terminus. The addition of agonist had no or little effect on the magnitude of these responses ( $E_{max}$  ~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 Leu<sup>390</sup> rendered the resulting m2 mutant receptors constitutively active, deletion of Ala<sup>391</sup> from the wild type m2 receptor resulted in a mutant receptor (m2( $\Delta$ 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  $G\alpha_{i/o}$  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° 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  $G\alpha_{i/o}$  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  $G\alpha$  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 Leu<sup>390</sup> and Ala<sup>391</sup> (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  $\alpha$ -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<sup>373</sup> (initially erroneously referred to as Thr<sup>348</sup>) in the  $\alpha_{2A}$ -adrenergic receptor (correspond-

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

Radioligand binding studies were carried out with membrane homogenates prepared from transfected COS-7 cells (4  $\mu\text{g}$  of receptor DNA/100-mm dish), as described under "Experimental Procedures." The [ $^3\text{H}$ ]NMS concentration used in the carbachol competition binding studies was 200 pM. The data are presented as means  $\pm$  S.E. of three independent experiments, each performed in duplicate.

Receptor	$[^3\text{H}]$ NMS binding		Carbachol binding	
	$K_D$ $\text{pM}$	$B_{\text{max}}$ $\text{fmol/mg}$	$\text{IC}_{50}$ $\mu\text{M}$	$n_H^a$
Wild type and mutant m2 receptors				
m2(wt)	72 $\pm$ 8	562 $\pm$ 68	16.0 $\pm$ 4.1	0.69 $\pm$ 0.06
m2(390 + 1A)	84 $\pm$ 6	508 $\pm$ 79	13.2 $\pm$ 1.4	0.72 $\pm$ 0.05
m2(390 + 2A)	80 $\pm$ 10	489 $\pm$ 48	17.8 $\pm$ 1.7	0.67 $\pm$ 0.04
m2(390 + 3A)	73 $\pm$ 11	273 $\pm$ 52	12.4 $\pm$ 3.2	0.70 $\pm$ 0.07
m2(390 + 4A)	87 $\pm$ 13	214 $\pm$ 43	14.9 $\pm$ 1.5	0.59 $\pm$ 0.08
m2(390 + 1A/VTIL $\rightarrow$ AALS)	67 $\pm$ 4	519 $\pm$ 59	18.8 $\pm$ 2.1	0.65 $\pm$ 0.05
m2(390 + 2A/VTIL $\rightarrow$ AALS)	92 $\pm$ 13	455 $\pm$ 64	15.2 $\pm$ 1.9	0.58 $\pm$ 0.06
m2( $\Delta$ A391)	97 $\pm$ 8	345 $\pm$ 60	11.5 $\pm$ 1.8	0.62 $\pm$ 0.05
m2(384 + 1A)	65 $\pm$ 7	521 $\pm$ 28	19.2 $\pm$ 3.5	0.52 $\pm$ 0.10
m2(384 + 2A)	57 $\pm$ 8	547 $\pm$ 49	23.6 $\pm$ 2.5	0.65 $\pm$ 0.06
Wild type and mutant m3 receptors				
m3(wt)	32 $\pm$ 5	684 $\pm$ 72	89 $\pm$ 8	0.70 $\pm$ 0.06
m3(493 + 1A)	28 $\pm$ 3	614 $\pm$ 84	162 $\pm$ 10	0.58 $\pm$ 0.04
m3(493 + 2A)	29 $\pm$ 6	546 $\pm$ 67	182 $\pm$ 7	0.63 $\pm$ 0.03
m3(493 + 3A)	39 $\pm$ 8	482 $\pm$ 71	155 $\pm$ 10	0.52 $\pm$ 0.06
m3(493 + 4A)	48 $\pm$ 4	348 $\pm$ 51	194 $\pm$ 10	0.57 $\pm$ 0.03
m3(487 + 1A)	42 $\pm$ 3	658 $\pm$ 44	81 $\pm$ 10	0.68 $\pm$ 0.07
m3(487 + 2A)	33 $\pm$ 5	609 $\pm$ 69	97 $\pm$ 9	0.74 $\pm$ 0.06

<sup>a</sup> Hill coefficient.

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  $\alpha_{1B}$ -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).<sup>2</sup> 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  $G_{i/o}$ -coupled m4 muscarinic receptor can activate  $G_{i/o}$  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  $G_{i/o}$ -coupled  $\alpha_{2A}$ -adrenergic receptor could be chemically cross-linked to  $G_{\alpha_o}$  and  $\beta$  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  $G_{i/o}$ -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>360</sup>  $\rightarrow$  Ala) mutant muscarinic receptor) (Högger *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\*) and that agonists, by preferentially binding to the R\* form, shift this equilibrium to the active receptor conformation. According to this model, a similar shift in the equilibrium toward R\* 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\* 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

<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<sup>493</sup> into the G<sub>q/11</sub>-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 G<sub>αq</sub>; 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<sub>10</sub>- and G<sub>q/11</sub>-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<sup>488</sup>, Ala<sup>489</sup>, Leu<sup>492</sup>, and Ser<sup>493</sup>) corresponding to the VTIL motif in the m2 receptor are not essential for G<sub>q/11</sub> 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<sub>q/11</sub> 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<sub>q/11</sub> (Blin *et al.*, 1995). However, consistent with previous mutagenesis studies (Kunkel and Peralta, 1993; Högger *et al.*, 1995), the complete lack of agonist-dependent signaling observed with the mutant m3 receptors containing additional Ala residues after Ser<sup>493</sup> 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 Arkininstall, 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; Högger *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 consid-

erable interest to examine which functional effects mutations homologous to those described here can cause in other classes of G protein-coupled receptors.

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