Identification of an Agonist-induced Conformational Change Occurring Adjacent to the Ligand-binding Pocket of the M₃ Muscarinic Acetylcholine Receptor*

To study the conformational changes that convert G protein–coupled receptors (GPCRs) from their resting to their active state, we used the M₃ muscarinic acetylcholine receptor, a prototypical class A GPCR, as a model system. Specifically, we employed a recently developed in situ disulfide cross-linking strategy that allows the formation of disulfide bonds in Cys-substituted mutant M₃ muscarinic receptors present in their native membrane environment. At present, little is known about the conformational changes that GPCR ligands induce in the immediate vicinity of the ligand-binding pocket. To address this issue, we generated 11 Cys-substituted mutant M₃ muscarinic receptors and characterized these receptors in transfected COS-7 cells. All analyzed mutant receptors contained an endogenous Cys residue (Cys-532) located within the exofacial segment of transmembrane domain (TM) VII, close to the agonist-binding site. In addition, all mutant receptors harbored a second Cys residue that was introduced into the exofacial segment of TM III, within the sequence Leu-142-Asn-152. Disulfide cross-linking studies showed that muscarinic agonists, but not antagonists, promoted the formation of a disulfide bond between Ser-151 and Cys-532. A three-dimensional model of the inactive state of the M₃ muscarinic receptor indicated that Cys-532 and Ser-151 face each other in the center of the TM receptor core. Our cross-linking data therefore support the concept that agonist activation pulls the exofacial segments of TMs VII and III closer to each other. This structural change may represent one of the early conformational events triggering the more pronounced structural reorganization of the intracellular receptor surface. To the best of our knowledge, this is the first direct demonstration of a conformational change occurring in the immediate vicinity of the binding site of a GPCR activated by a diffusible ligand.

G protein-coupled receptors (GPCRs) form one of the largest gene families found in nature. Various lines of evidence suggest that the human genome contains nearly 1000 genes coding for distinct GPCRs (1–3). GPCRs are cell-surface receptors that are activated by extraordinarily diverse group of extracellular ligands (4–7). Structurally, all GPCRs are composed of a bundle of seven transmembrane helices (TMs I–VII) that are connected by alternating intracellular and extracellular loops (see Fig. 1) (4–7).

GPCR ligands recognize their target receptors by interacting with specific amino acids located on the extracellular receptor surface (4–11). For example, the classic biogenic amine neurotransmitters bind to their target GPCRs within a cavity formed by the ring-like arrangement of the seven TMs (8–11). In this case, the key residues contributing to ligand recognition are located primarily within the exofacial segments of TMs III and V–VII (8–11).

Currently, bovine rhodopsin in its inactive state is the only GPCR for which high resolution structural information is available (12, 13). Most GPCRs share a considerable degree of structural homology with rhodopsin and are therefore also referred to as rhodopsin-like or family A GPCRs. However, rhodopsin is unique among GPCRs in that its endogenous ligand, 11-cis-retinal, is covalently bound to the receptor protein and keeps rhodopsin in an inactive state by acting as an inverse agonist (14, 15). It therefore remains unclear to what extent the activation mechanism of rhodopsin differs from that of GPCRs that are activated by diffusible ligands.

Following ligand binding, GPCRs undergo conformational changes that must be propagated from the ligand-binding domain to the intracellular receptor surface (5, 14–17). The current view is that GPCR activation opens a cleft on the intracellular side of the receptor that enables the receptor to productively interact with heterotrimeric G proteins (5, 14–17). Biophysical and biochemical studies carried out with bovine rhodopsin (14, 15, 17, 18) and the β₂-adrenergic receptor (16, 19–24) have revealed several activity-dependent conformational changes involving the intracellular receptor surface. The light-induced activation of rhodopsin is predicted to involve a reorientation of the cytoplasmic end of TM VI and changes in the relative disposition of TMs VI and III, along with smaller movements involving several other TMs (for recent reviews, see Refs. 14 and 15). Several studies suggest that similar conformational changes occur in GPCRs activated by diffusible ligands such as the β₂-adrenergic receptor (16, 19–24).

Although much attention has been focused on identifying the conformational changes occurring on the intracellular receptor surface, little is known about the structural changes that GPCR ligands induce in the immediate vicinity of the ligand-binding domain (25). However, such information is essential for understanding how ligand binding to the extracellular receptor surface can lead to major structural changes on the cytoplasmic side of the receptor.

During the past few years, we have used the rat M₃ muscarinic receptor, a prototypical class A GPCR (9), as a model system to study activity-dependent changes in GPCRs activated by diffusible ligands. Specifi-
cally, we developed a novel in situ disulfide cross-linking strategy that allows the detection of disulfide bonds that can form between two Cys residues that are located adjacent to each other in the three-dimensional structure of the M₃ receptor protein (26). One major advantage of this approach is that Cys-substituted mutant M₃ muscarinic receptors can be characterized in their native membrane environment. In contrast, most studies examining activity-dependent changes in rhodopsin have been carried out with mutant proteins present in the solution state. However, as discussed by Hubbell et al. (15), the structural and dynamic properties of the solution state of rhodopsin may not be identical to those found in native disk membranes.

Recent in situ disulfide cross-linking studies showed that M₃ receptor activation is accompanied by pronounced conformational changes on the intracellular receptor surface (26, 27). For example, we demonstrated that muscarinic agonists induce structural changes that increase the proximity of the cytoplasmic ends of TMs V and VI (26) and TMs I and VII (27). The observed cross-linking patterns also suggest that these conformational changes are accompanied by pronounced rotational movements of the cytoplasmic ends of TMs VI and VII (26, 27).

The goal of this study was to identify agonist-induced conformational changes in the M₃ muscarinic receptor that occur in the immediate vicinity of the ligand-binding pocket. Classic muscarinic agonists are known to interact with their target receptors within a cleft enclosed by the ring-like arrangement of TMs I–VII, ∼10–15 Å from the membrane surface (9, 11). The key amino acids involved in the binding of acetylcholine (ACh) and other classic muscarinic agonists are predicted to be located on the exofacial segments of TMs III and V–VII, including a TM III Asp residue that is conserved among all biogenic amine GPCRs (Asp-1473.32; the superscript indicates the amino acid position according to the Ballesteros-Weinstein numbering system (28)) (see Fig. 1) and several muscarinic receptor-specific tyrosine residues (Tyr-1483.33, Tyr-5066.51, Tyr-5297.39, and Tyr-5337.43) (see Fig. 1) (9, 11).

In this study, we tested the hypothesis that muscarinic agonists, by simultaneously contacting residues present within TMs III and VII, can increase the proximity of the exofacial segments of TMs III and VII. To address this question, we took advantage of the fact that the modified version of the M₃ muscarinic receptor (referred to as M₃’(3C)-Xa) that we developed as a template for Cys substitution mutagenesis contains only one single free Cys residue (Cys-5327.42) within the TM receptor core (see Fig. 1). In agreement with a recently developed three-dimensional model of the M₃ muscarinic receptor (27), we demonstrated previously that Cys-532 is located adjacent to the ligand-binding pocket projecting into the center of the TM receptor core (29). Specifically, we examined whether Cys-532 is able to form activity-dependent cross-links with Cys residues introduced into the exofacial segment of TM III (Leu-1423.27–Asn-1523.37) (see Fig. 1).

We found that muscarinic agonists, but not antagonists, promoted the formation of a disulfide bond between Ser-151 and Cys-532 in the S1513.30C mutant receptor. Based on the predicted locations of Ser-151 and Cys-532 in the three-dimensional structure of the M₃ receptor protein, this finding strongly suggests that agonist activation of the M₃ muscarinic receptor increases the proximity of the exofacial segments of TMs III and VII. Because all class A GPCRs share a considerable degree of structural homology, a similar conformational change may occur in other members of this GPCR superfamily.

**Experimental Procedures**

**Materials**—N-Ethylmaleimide, carbamylcholine chloride (carbachol), ACh bromide, atropine sulfate, and mammalian protease inhibitor mixture were purchased from Sigma. Acetyltriethylcholine (N-(Et3)-ACh) was generously provided by Dr. Edward Hulme (Medical Research Council, Mill Hill, London). N-[3H]Methylscopolamine ([3H]NMS; 79–83 Ci/mmol) and myo-[3H]inositol (20 Ci/mmol) were from Perkin-Elmer Life Sciences; factor Xa protease and digoxigenin were from Roche Applied Science; and precast Novex Tris/glycine-polyacrylamide gels and SeeBlue Plus 2 pre-stained molecular mass standards were from Invitrogen. HybondTM ECLTM nitrocellulose membranes, horseradish peroxidase-conjugated anti-rabbit IgG antibody, ECLTM detection reagents, and HyperfilmTM ECLTM chemiluminescence film were obtained from Amersham Biosciences. All other reagents used were of the highest grade commercially available.

**Site-directed Mutagenesis**—A PCRam-based expression plasmid coding for a modified version of the rat M₃ muscarinic receptor, referred to as M₃’(3C)-Xa (see Fig. 1), was used as a template for Cys substitution mutagenesis. As described previously (30), the M₃’(3C)-Xa receptor contains an N-terminal hemagglutinin epitope tag and lacks all five potential N-terminal N-glycosylation sites and most endogenous Cys residues, except for Cys-140, Cys-220, and Cys-532 (see Fig. 1). Moreover, the central portion of the i3 loop (the third intracellular loop of GPCRs; Ala-274–Lys-469) is replaced with two factor Xa cleavage sites (30). Cys residues were reintroduced into the M₃’(3C)-Xa construct, one at a time, at Leu-1423.27–Asn-1523.37 (see Fig. 1) using the QuikChangeTM site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The identity of all mutant constructs was verified by DNA sequencing.

**Transient Expression of Cys-substituted Mutant M₃ Muscarinic Receptors in Mammalian Cells**—All receptor constructs were transiently expressed in COS-7 cells. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ incubator. Approximately 1 × 10⁶ cells were seeded into 100-mm dishes ∼24 h prior to transfections. Cells were transfected with 4 μg of receptor plasmid DNA/dish using the Lipofectamine Plus kit (Invitrogen) according to the manufacturer’s instructions. To increase receptor expression levels, 1 μM atropine was routinely added to the incubation medium for the last 24 h of culture as described previously by us (26, 27, 29).

**Preparation of Membranes from Transfected COS-7 Cells**—Cells were harvested ∼48 h after transfections. To ensure complete removal of atropine that was included in the incubation medium during the last 24 h of culture, cells were washed twice (10 min each wash) with 10 ml of ice-cold phosphate-buffered saline (pH 7.4). After this washing step, 2 ml of ice-cold buffer A (25 mM sodium phosphate and 5 mM MgCl₂ (pH 7.4)) was added to each 100-mm dish, followed by a 15-min incubation at 4 °C. Cells were then scraped off the plates and homogenized using a Polytron tissue homogenizer (setting 5, 20 s), followed by a 15-min centrifugation at 20,000 × g at 4 °C. The membrane pellets were then resuspended in buffer A (1 ml/100-mm dish), re-homogenized, frozen on dry ice, and stored at −70 °C until used. Protein concentrations were measured using the Micro BCA protein assay reagent kit (Pierce) with bovine serum albumin as a standard.

**Radioligand Binding Studies**—Radioligand binding assays were carried out as described previously (26). In brief, membrane homogenates prepared from transfected COS-7 cells (∼10–20 μg of membrane protein/tube) were incubated in 1 ml of buffer A for 2 h at room temperature (22 °C) in the presence of the muscarinic antagonist [3H]NMS. In saturation binding assays, six different concentrations of [3H]NMS (ranging from 20 to 3000 pM) were used. In competition binding assays, a fixed concentration of [3H]NMS (500 pM) was employed in the presence of 10 different concentrations of the unlabeled competitor. Reac-
tions were terminated by rapid filtration over Brandel GF/C filters, followed by three washes with ice-cold distilled water (−4 ml/wash). Nonspecific binding was assessed as binding remaining in the presence of 1 μM atropine. The amount of bound radioactivity was determined by liquid scintillation spectrometry. Binding data were analyzed using the nonlinear curve-fitting program Prism 3.0 (GraphPad Software, Inc.).

Phosphatidylinositol (PI) Assays—To examine the ability of the different mutant receptors to productively interact with G proteins, we studied carbachol-induced increases in intracellular inositol monophosphate (IP$_1$) levels using transiently transfected COS-7 cells grown in 6-well plates (30). Cells were first labeled for 20−24 h with myo-[3H]inositol (3 μCi/ml) and then incubated in the presence of 10 mM LiCl for 1 h at 37 °C with increasing concentrations of carbachol. The inositol monophosphate fraction was isolated and quantitated as described (30). Carbachol concentration-response curves were analyzed using the nonlinear curve-fitting program Prism 3.0.

Luciferase Assay—COS-7 cells were seeded in 24-well plates (~50,000 cells/well) and cultured as described above. Approximately 24 h later, cells were cotransfected with 0.2 μg of receptor DNA and 0.2 μg of the luciferase reporter plasmid pAP-1-Luc (Stratagene) using the Lipofectamine Plus kit according to the manufacturer’s instructions. The pAP-1-Luc plasmid contains the luciferase reporter gene driven by a basic promoter element (TATA box) joined to an activator protein-1 motif. About 48 h after transfections, cells were grown for 5 h in the presence of increasing concentrations of carbachol or N-(Et$_3$)-ACh. Cells were then washed twice with phosphate-buffered saline and lysed with 0.5 ml of lysis buffer (25 mM glycyglycine, 15 mM MgSO$_4$, 4 mM EGTA, 1 mM dithiothreitol, 1% Triton X-100, 15 mM KH$_2$PO$_4$, and 2 mM ATP (pH 7.8)). Cell lysates (100-μl aliquots) were then transferred to 24-well flat bottom plates (Costar 3912) and mixed automatically with 125 μl of reaction buffer (lysis buffer without Triton X-100) and 25 μl of 0.8 μM luciferin in reaction buffer. Luminescence was then measured for 10 s using an Applied Biosystems TR717 microplate luminometer.

Cross-linking Studies—Receptor-containing membranes prepared from transfected COS-7 cells (see above) were thawed at room temperature and homogenized as described under "Preparation of Membranes from Transfected COS-7 Cells." To induce the formation of disulfide bonds, membranes prepared from one 100-mm dish (−1 mg of protein present in a 1-ml volume) were incubated in microcentrifuge tubes with end-over-end rotation (30 rpm) for 10 min at room temperature with molecular iodine (20 μM) in either the absence or presence of different concentrations of the muscarinic agonist carbachol or other muscarinic ligands. Reactions were terminated by the addition of EDTA and N-ethylmaleimide (10 mM each), followed by a 10-min incubation on ice.

To obtain membrane lysates (26), samples were then centrifuged at 8000 × g for 10 min at 4 °C, and the resulting membrane pellets were incubated with 250 μl of 0.2% digitonin in phosphate-buffered saline (pH 7.4) for 20 min on ice to remove peripheral membrane proteins. Following another centrifugation step (8000 × g for 10 min at 4 °C), membrane pellets were incubated with 1.2% digitonin in 50 mM Tris-HCl (pH 8), 100 mM NaCl, and 1 mM CaCl$_2$, for 90 −120 min at 4 °C with end-over-end rotation (30 rpm). After another centrifugation step (same conditions as described above), the supernatants (membrane lysates containing solubilized mutant M$_3$ muscarinic receptors) were transferred to fresh microcentrifuge tubes. Membrane lysates (~15 μg of protein) were then incubated with factor Xa protease (final concentration of 0.1 μg/μl) at room temperature for 16−20 h (final volume of 50 μl). The reactions were terminated by incubation for 30 min at room temperature with a mammalian protease inhibitor mixture (1.25 dilution). Samples were then used directly for SDS-PAGE or stored at −70 °C until used.

Western Blot Analysis—SDS-PAGE was performed essentially as described by Ward et al. (26). Samples were incubated for 30 min at 37 °C with Laemmli loading buffer under nonreducing conditions and then loaded onto 10−20% Tris/glycine-polyacrylamide gels, which were run at 125 V in the presence of 0.1% SDS. Western blot studies were carried out using the anti-M$_3$ antibody directed against the C-terminal 18 amino acids of the M$_3$ receptor protein (26, 31). Receptor proteins were visualized using ECL detection reagents by autoradiography. The intensities of immunoreactive bands were quantitated by scanning densitometry using the program ImageQuant TL (Amersham Biosciences).

Molecular Modeling—We recently described a three-dimensional model of the TM core of the rat M$_3$ muscarinic receptor (27) that was built using homology modeling of the high resolution x-ray structure of the inactive state of bovine rhodopsin (12). All calculations were performed on a Silicon Graphics Octane workstation (300-MHz MIPS R12000 (IP30) processor) using the SYBYL 7.0 program (Tripos, Inc.). Overall, the M$_3$ muscarinic receptor model showed high structural similarity to that of the rhodopsin template (27).

ACh was constructed using the Sketch Molecule function of SYBYL 7.0 and subjected to a grid search, rotating all rotatable bonds by 60° increments. Merck Molecular Force Field (32) and charge were applied using distance-dependent dielectric constants and the conjugate gradient method until the gradient reached 0.05 kcal/mol/Å. After clustering the low energy conformers resulting from the conformational search, the relative stabilities of various representative conformers from all groups were checked by semi-empirical molecular orbital calculations using the PM3 method in the MOPAC 6.0 package (33).

The lowest energy conformer of ACh was docked into the binding crevice of the rat M$_3$ muscarinic receptor using the previously established three-dimensional model of this receptor (27). Flexible docking was facilitated through the FlexiDock utility in the Biopolymer module of SYBYL 7.0. Taking into consideration the results of previous mutagenesis studies (9, 11), ACh was prepositioned in the putative receptor binding site in a fashion similar to that described for the M$_1$ muscarinic receptor subtype (11). Flexible docking allowed for flexibility of all rotatable bonds in ACh and the side chains of the surrounding amino acids. To increase binding interactions, the torsion angles of the side chains that were located within 5 Å of the ligand (according to the results of FlexiDock) were adjusted manually. The structure of the ACh-receptor complex was minimized using an AMBER force field with a fixed dielectric constant (4.0) until the conjugate gradient reached 0.05 kcal/mol/Å.

RESULTS

This study was designed to monitor agonist-induced conformational changes in the M$_3$ muscarinic receptor that occur close to the ligand-binding pocket. Toward this goal, we generated 11 Cys-substituted mutant M$_3$ muscarinic receptors and characterized these receptors in radioligand binding, functional, and disulfide cross-linking studies.

Generation of Cys-substituted Mutant M$_3$ Muscarinic Receptors—Single Cys substitutions were introduced into a modified version of the rat M$_3$ muscarinic receptor (M3*(3C)-Xa) (30) that lacks most native Cys residues and contains two factor Xa cleavage sites within the i3 loop (Fig. 1). Specifically, 11 consecutive residues in TM III (Leu-142−Asn-152) (Fig. 1) were replaced, one at a time, with Cys residues. As a result, all generated mutant receptors contained two Cys residues within the TM receptor core: an endogenous Cys residue (Cys-5327.42) located within
Characterization of Mutant M3 Muscarinic Receptors in Radioligand Binding and Functional Studies—All Cys-substituted mutant M3 muscarinic receptor constructs as well as the M3(3C)-Xa construct contain only three remaining Cys residues, Cys-140, C-220, and Cys-532 (black boxes). All other native Cys residues are replaced with serine or alanine residues (white boxes). In addition, the central portion of the I3 loop (Ala-274–Lys-469) is replaced with two factor Xa cleavage sites ((IGR)2) underlined. To detect the different mutant M3 muscarinic receptors via Western blotting, we used the rabbit anti-M3 polyclonal antibody directed against the indicated C-terminal receptor sequence (31). Numbers refer to amino acid (aa) positions in the rat M3 muscarinic receptor (39).

To determine receptor densities, we carried out saturation binding studies with the muscarinic antagonist [3H]NMS using membranes prepared from transiently transfected COS-7 cells. This analysis showed that the M3(3C)-Xa receptor was expressed at a density of 2.85 pmol/mg protein (Bmax). The expression levels of most Cys-substituted mutant receptors differed from this value by <2.5-fold (TABLE ONE). However, the L144C, S145C, and Y148C receptors showed a ~3–8-fold reduction in Bmax values (TABLE ONE).

Like the M3(3C)-Xa construct, most analyzed mutant receptors were able to bind [3H]NMS with high affinity (K_D = 235–684 pM) (TABLE ONE). However, three mutant receptors (W143C, D147C, and Y148C) showed a significant reduction (~4–6-fold) in [3H]NMS binding affinities (TABLE ONE).

To determine the affinity of the different mutant M3 muscarinic receptors for the muscarinic agonist carbachol, we next carried out a series of [3H]NMS/ carbachol inhibition binding experiments. These studies showed that seven of the Cys-substituted mutant receptors displayed carbachol binding affinities (Ki) that differed from the carbachol affinity of the M3(3C)-Xa construct (K_i = 35.0 ± 15.8 μM) by <2.2-fold (TABLE ONE). However, four mutant receptors (D147C, Y148C, A150C, and N152C) showed an ~3–9-fold reduction in carbachol binding affinities (TABLE ONE).

To examine whether the Cys-substituted mutant receptors were still able to couple to G proteins, we next studied their ability to mediate carbachol-induced increases in inositol monophosphate production (PI hydrolysis). Seven of the analyzed mutant receptors retained the ability to stimulate PI hydrolysis with high efficacy (E_m, expressed as fold increase in inositol monophosphate production above basal levels) (TABLE TWO). The V149C construct showed a pronounced reduction in G protein coupling efficacy (TABLE TWO). Strikingly, the S145C, D147C, and Y148C mutant receptors were completely devoid of functional activity (TABLE TWO).

In the PI assays, five of the analyzed mutant receptors (W143C, L144C, I146C, A150C, and N152C) showed clearly reduced (>9-fold) carbachol potencies (increased EC_50 values) compared with the M3(3C)-Xa construct (EC_50 = 20 ± 11 nM) (TABLE TWO). The L144C construct showed by far the most pronounced decrease in carbachol potency, displaying an ~500-fold reduction in carbachol potency compared with the M3(3C)-Xa receptor (TABLE TWO).

---

**TABLE ONE**

| Receptor    | [3H]NMS binding  | Carbachol binding Ki |
|-------------|------------------|----------------------|
|             | K_D              | B_max                | Ki     |
| M3(3C)-Xa   | 240 ± 5          | 2.85 ± 0.14          | 35.0 ± 15.8 |
| L142C       | 235 ± 39         | 1.35 ± 0.24          | 20.7 ± 2.7  |
| W143C       | 1147 ± 299       | 0.20 ± 0.97          | 19.1 ± 4.7  |
| L144C       | 684 ± 15         | 0.82 ± 0.23          | 41.5 ± 15.2 |
| S145C       | 658 ± 27         | 0.37 ± 0.04          | 15.2 ± 10.1 |
| I146C       | 581 ± 226        | 4.87 ± 2.86          | 75.4 ± 22.0 |
| D147C       | 1178 ± 381       | 1.42 ± 0.46          | 119.4 ± 65.4 |
| Y148C       | 1493 ± 421       | 0.63 ± 0.08          | 261.1 ± 124.1 |
| V149C       | 324 ± 52         | 1.90 ± 0.32          | 64.6 ± 1.2  |
| A150C       | 276 ± 21         | 3.92 ± 0.43          | 213.3 ± 61.9 |
| S151C       | 651 ± 137        | 1.15 ± 0.28          | 39.1 ± 5.2  |
| N152C       | 669 ± 60         | 2.92 ± 0.13          | 311.4 ± 29.4 |
TABLE TWO

| Receptor     | EC₅₀ (carbachol) | Basal activity | Eₘₐₓ |
|--------------|-----------------|----------------|------|
| M₃(3C)-Xa   | 20 ± 11 dpm/well | 100            | 9.0 ± 1.5 |
| L142C       | 59 ± 22 dpm/well | 54 ± 1         | 13.1 ± 1.2 |
| W143C       | 386 ± 122 dpm   | 118 ± 51       | 7.3 ± 1.5 |
| L144C       | 10,553 ± 4257 dpm | 41 ± 13      | 9.2 ± 1.7 |
| S145C       | —               | 17 ± 6         | —    |
| I146C       | 184 ± 25 dpm    | 66 ± 16        | 15.5 ± 7.2 |
| D147C       | —               | 21 ± 8         | —    |
| Y148C       | —               | 34 ± 13        | —    |
| V149C       | 15 ± 9          | 113 ± 28       | 2.6 ± 0.2 |
| A150C       | 244 ± 30 dpm    | 37 ± 11        | 4.8 ± 2.6 |
| S151C       | 59 ± 29         | 195 ± 46       | 9.0 ± 1.0 |
| N152C       | 555 ± 192 dpm   | 58 ± 7         | 16.8 ± 6.1 |

*— no significant increase in IP₁, levels above basal levels.

Dissulfide Cross-linking Studies—As described above, transient expression of all analyzed Cys-substituted mutant M₃ muscarinic receptors resulted in the appearance of a significant number of [³H]NMS-binding sites. Consistent with this observation, all mutant receptors could be easily detected in Western blot studies using membrane lysates prepared from transfected COS-7 cells and the anti-M₃ antibody polyclonal antibody directed against the C-terminal portion of the rat M₃ muscarinic receptor (data not shown).

To examine the potential spatial proximity of the Cys pairs present within the TM core of the 11 Cys-substituted mutant receptors, we studied their ability to form intramolecular disulfide bonds. All disulfide cross-linking studies were performed with mutant receptors present in their natural membrane environment (in situ) using membrane preparations obtained from transfected COS-7 cells (26, 27, 29). To promote the formation of disulfide bonds, receptor-containing membrane preparations were exposed to molecular iodine (20 μl) at 10 min at room temperature in either the absence or presence of the muscarinic agonist carbachol (IC₅₀, 1 mM). Receptors were then solubilized and digested to completion with factor Xa as described under “Experimental Procedures.” Samples containing equal amounts of protein (5 μg) were then run under nonreducing conditions on 10–20% Tris/glycine-polyacrylamide gels, followed by Western blot analysis using the anti-M₃ antibody. Note that only one of the investigated Cys mutant receptors (S151C) displayed agonist-dependent cross-linking, as indicated by the appearance of a strong ~38-kDa full-length receptor band. Another mutant receptor (A150C) gave a cross-linking signal that was similar in intensity in the absence or presence of carbachol. The data shown are representative of three independent experiments.

Carbachol treatment had no significant effect on the intensity of the 38-kDa band in the case of the A150C construct. In contrast, carbachol simulation led to a pronounced increase in the intensity of the cross-linking signal in the case of the S151C mutant receptor (Fig. 2). When Western blot studies were carried out under reducing conditions, the ~38-kDa bands were no longer observed (data not shown), indicating that these bands were not caused by incomplete digestion by factor Xa. The observed disulfide cross-linking signals required the exposure of receptor-containing membrane preparations to iodine (data not shown).

Agonist Dependence of the Dissulfide Cross-linking Signal Observed with the S151C Receptor—To study the agonist dependence of the dissulfide cross-linking signal displayed by the S151C mutant receptor in more detail, membranes prepared from cells expressing this mutant construct were oxidized in the presence of different carbachol concentrations (0.01–10 μM). As shown in Fig. 3A, carbachol treatment led to a concentration-dependent increase in the intensity of the 38-kDa cross-linking signal under nonreducing conditions. Fig. 3B summarizes the results of three independent carbachol cross-linking experiments carried out with the S151C mutant receptor based on the quantification of the intensity of the 38-kDa receptor species. The M₃(3C)-Xa construct was included in all experiments for control purposes.

The S151C Receptor Does Not Form Intramolecular Disulfide Bonds—The M₃ muscarinic receptor (34), like many other GPCRs (35, 36), is known to form dimers or oligomers. We therefore wanted to exclude the possibility that agonist-promoted disulfide cross-linking between Cys-151 and Cys-532 occurred intermolecularly (between two or more receptor molecules) rather than intramolecularly. To address this issue, membranes prepared from S151C-expressing COS-7 cells were treated with iodine (20 μl) in either the absence or presence of carbachol (1 μM). Samples were then processed for SDS-PAGE and Western blotting in the same fashion as described above under nonreducing conditions, except that the factor Xa cleavage step was omitted. Under these conditions, the formation of disulfide cross-links between receptor monomers is predicted to lead to the appearance of immunoreactive species of >75 kDa in size. However, Western blot studies showed that, like the M₃(3C)-Xa construct, the S151C mutant receptor did not yield significant amounts of high molecular mass receptor species in either the absence or presence of carbachol (Fig. 4). The Western blot studies...
revealed several immunoreactive bands: a major band corresponding to
the receptor monomer and two considerably less intense bands migrating
at around 40 and 50 kDa, respectively. Although the molecular identity
of these additional bands remains unclear at present, they may be due to
cross-linking of the S151C mutant and M3’(3C)-Xa receptors to other
membrane proteins. In any case, our data indicate that the formation of
disulfide bond between Cys-151 and Cys-532 in the S151C construct
does not involve interactions between different receptor monomers.

Effect of Additional Muscarinic Ligands on Disulfide Bond Formation
in the S151C Mutant Receptor—To confirm that the carbachol-induced
disulfide cross-link between Cys-151 and Cys-532 in the S151C receptor
reflected a conformational change associated with receptor activation,
we carried out additional cross-linking studies using several other
muscarinic ligands (Fig. 5). Like the ACh derivative carbachol, ACh (1 mM)
alone led to a pronounced increase in the intensity of the cross-linking
signal when membranes prepared from S151C-expressing cells
were subjected to oxidizing conditions (Fig. 6). No such effect was seen
with the classic muscarinic antagonist atropine (1 mM) (Fig. 6).

A structural hallmark of all highly efficacious muscarinic agonists,
including ACh and carbachol, is the presence of a compact ammonium
head group (Fig. 5) (37, 38). Replacement of the three N-methyl groups of
ACh with larger alkyl substituents leads to partial muscarinic agonists
or antagonists (37, 38). In this study, we therefore also examined an ACh
analog, N-(Et3)-ACh, in which all three methyl groups were replaced
with ethyl moieties (Fig. 5). Radioligand binding studies showed that
N-(Et3)-ACh retained the ability to bind to the S151C and M3’(3C)-Xa
receptors, although with 2–5-fold lower affinity than carbachol (TABLE THREE). To examine the functional properties of N-(Et3)-
ACh, we used a luciferase reporter assay that is based on the ability of
gonist-stimulated Gα coupled receptors to activate protein kinase C,
which in turn triggers the activation of a basic promoter element
(TATA box) containing an activator protein-1 motif, resulting in the
expression of the luciferase reporter gene (39, 40). We used this luciferase
reporter assay rather than the PI assay to determine the functional
activity of Et3-ACh because only a very small amount of N-(Et3)-ACh
was available to us. (The luciferase assay could be carried out in a very
small volume in a 24-well format.) As indicated in TABLE THREE, even
the highest concentration of N-(Et3)-ACh (1 mM) failed to stimulate
luciferase activity in COS-7 cells cotransfected with the S151C mutant or
M3’(3C)-Xa receptor and the activator protein-1/luciferase reporter
construct. However, under the same experimental conditions, carbachol
stimulation of the S151C mutant or M3’(3C)-Xa receptor led to a
pronounced increase in luciferase activity (TABLE THREE). These
responses were characterized by carbachol EC50 values that were in a
similar range as the corresponding EC50 values determined in PI assays
(TABLE TWO). These data indicate that N-(Et3)-ACh can bind to the
S151C and M3’(3C)-Xa receptors, but is unable to activate these recep-
tors, characteristic of the behavior of a muscarinic antagonist. Consist-

**FIGURE 3.** Agonist dependence of disulfide cross-linking studied with the S151C mutant receptor. A, membranes prepared from COS-7 cells expressing the S151C mutant or M3’(3C)-Xa (control) receptor were incubated for 10 min at room temperature with iodine (20 μl) in either the absence or presence of increasing concentrations of the muscarinic agonist carbachol. Receptors were then solubilized and digested with factor Xa, followed by SDS-PAGE and Western blotting under nonreducing conditions using the anti-M3 antibody (for details, see “Experimental Procedures”). Equal amounts of protein were loaded onto each lane. The 38-kDa receptor bands that are indicative of disulfide bond formation are shown. B, shown is a summary of cross-linking experiments. The intensities of the 38-kDa receptor species were determined by scanning densitometry using the program ImageQuant TL. The data are expressed as the percent agonist-dependent increase in the intensity of the 38-kDa band above basal levels (no carbachol). The signal observed in the absence of carbachol (Fig. 3A) was set equal to 100%. The EC50 for the agonist-stimulated Gq-coupled receptors to activate protein kinase C,

**FIGURE 4.** Oxidative conditions have no effect on the electrophoretic mobility of the S151C mutant receptor. Membrane extracts prepared from COS-7 cells transfected with the M3’(3C)-Xa or S151C mutant receptor were treated with the oxidizing agent iodine (20 μl) for 10 min at room temperature in either the absence or presence of carbachol (0.1 mM). Samples were subjected to SDS-PAGE and Western blotting using the anti-M3 antibody under nonreducing conditions. Note that incubation of the S151C mutant receptor (or M3’(3C)-Xa) with the oxidizing agent did not result in the formation of a significant amount of dimeric or oligomeric receptor species (see “Results” for more details). Protein molecular mass standards (in kilodaltons) are indicated to the right.

**FIGURE 5.** Chemical structures of the muscarinic ligands used in this study. Whereas ACh and carbachol are classic muscarinic agonists, N-(Et3)-ACh represents a muscarinic antagonist, similar to atropine (see “Results” for details).
M₃ Muscarinic Receptor Activation

The ligand-dependent activation of GPCRs has been shown to lead to pronounced structural changes on the cytoplasmic surface of the receptor proteins (14–17, 25). These conformational changes are thought to be essential for productive receptor/G protein coupling. At present, little is known about the molecular mechanisms by which ligand binding to the extracellular receptor surface triggers the functionally critical conformational changes on the cytoplasmic side of the receptor. Thus, this study was undertaken to investigate whether diffusible ligands can induce conformational changes in the immediate vicinity of the ligand-binding pocket of a class A GPCR. To address this question, we employed a previously developed in situ disulfide cross-linking strategy (26, 27, 29) using the M₃ muscarinic receptor as a model system.

ACh and other classic muscarinic agonists are predicted to bind to the M₃ muscarinic receptor and other members of the muscarinic receptor family (M₁–M₅) within a central binding crevice formed by the ring-like arrangement of the seven TMs (9, 11). The amino acids that play a key role in ACh binding have been shown to be located within the exofacial segments of TMs III and V–VII (9, 11). In general, other biogenic amine neurotransmitters are predicted to bind to their target GPCRs in a similar fashion (8, 10).

The x-ray structure of bovine rhodopsin (12, 13) and our newly generated M₃ muscarinic receptor model (27) suggest that the exofacial segments of TMs III and VII face each other in the three-dimensional structure of class A GPCRs. These receptor segments contain several residues that have been shown to be critically involved in ACh binding, including Asp-1473.32, Tyr-1483.33, Tyr-5297.39, and Tyr-5337.43 (Figs. 1 and 8) (42, 43). In this study, we therefore tested the hypothesis that agonist binding triggers changes in the relative orientation of the exofacial segments of TMs III and VII. Specifically, we generated 11 mutant M₃ muscarinic receptors, all of which contained a single endogenous Cys residue (Cys-5327.42) within TM VII and a second Cys residue within the exofacial segment of TM III (Leu-1423.27–Asn-1523.37). Biochemical (29) and molecular modeling (27) studies suggest that Cys-5327.42 projects into the interior of the TM receptor core. We therefore considered Cys-5327.42 a good "reporter" to detect agonist-induced changes occurring in the vicinity of the ligand-binding site.

Consistent with the known role of the exofacial segment of TM III in the binding of muscarinic ligands (9, 11), several of the analyzed mutant receptors, including D147C and Y148C, exhibited reduced ligand binding affinities (TABLE ONE). Moreover, PI assays showed that three of the examined mutant receptors (S145C, D147C, and Y148C) lost the ability to interact with G proteins (TABLE TWO), indicating that these TM III residues play important roles in agonist-induced M₃ receptor activation. Similar results were obtained when the M₃ muscarinic receptor was subjected to alanine substitution mutagenesis (44).

To induce the formation of disulfide cross-links, the Cys-substituted mutant M₃ muscarinic receptors were exposed to oxidizing conditions in either the absence or presence of muscarinic agonists. In a previous study, we showed that molecular iodine, because of its relatively small size, is more efficient than the frequently used, more bulky oxidizing agent Cu(II)-phenanthroline in facilitating the formation of disulfide bonds between Cys residues present in the TM receptor core (29). In this study, we therefore used molecular iodine to promote the formation of disulfide bonds.

Strikingly, disulfide cross-linking experiments led to the identification of a single mutant receptor (S151C) that exhibited agonist-dependent disulfide bond formation. The S151C mutant receptor showed little cross-linking activity in the absence of ligands or in the presence of the muscarinic antagonist atropine (Figs. 2 and 6). In contrast, this construct displayed a pronounced, concentration-dependent cross-linking signal in the presence of the agonists carbamol and ACh (Figs. 2, 3, and 6). The S151C mutant receptor was able to bind muscarinic ligands with high affinity (TABLE ONE) and retained the ability to couple to G proteins with high efficiency (TABLE TWO). These observations clearly indicate that the cross-linking pattern observed with the S151C variant with this concept, disulfide cross-linking studies showed that, like the classic muscarinic antagonist atropine, N-(Et₃)-ACh failed to promote disulfide bond formation in the S151C mutant construct (Fig. 6).

Predicted Locations of Ala-150, Ser-151, and Cys-532 in the Three-dimensional Structure of the M₃ Muscarinic Receptor—To facilitate the interpretation of disulfide cross-linking data, we recently built a three-dimensional model of the TM core of the rat M₃ muscarinic receptor (27) using homology modeling based on the high resolution x-ray structure of the inactive state of bovine rhodopsin (12). As described in detail previously (27), the calculated model of the inactive state of the M₃ muscarinic receptor displayed high structural similarity to the rhodopsin template.

Fig. 7 shows that Ala-1503.35, Ser-1513.36, and Cys-5327.42 are located at about the same level within the TM receptor core (also see Fig. 1). Moreover, Fig. 7 illustrates that Ser-151 and Cys-532 directly face each other at the TM III/VII interface. Ala-150 is also located in the vicinity of Cys-532, but does not project toward Cys-532 directly.

We also used a molecular modeling strategy to dock ACh into the ligand-binding crevice of the rat M₃ muscarinic receptor (for details, see “Experimental Procedures”). Based on the results of previous mutagenesis studies (9, 11), ACh was prepositioned in the binding site in a fashion similar to that described for the M₁ muscarinic receptor subtype (11). In agreement with the results of a recent NMR study examining the binding of two ACh analogs to the M₃ muscarinic receptor (41), ACh bound to the M₃ muscarinic receptor in the energetically preferred gauche conformation, displaying an O–C–2–C–1–N dihedral angle of about +60°. Fig. 8 shows the location of Asp-1473.32 and several Tyr residues (Tyr-1483.33, Tyr-5066.51, Tyr-5297.39, and Tyr-5337.43) known to play key roles in ACh binding to the M₁ (9) and M₃ (11) muscarinic receptors. It also illustrates that Ala-150, Ser-151, and Cys-532 are located slightly below the plane defined by the bound ACh ligand. These observations, together with the results of the disulfide cross-linking studies, strongly suggest that agonist activation of the M₃ muscarinic receptor is associated with a conformational change that moves the exofacial portions of TMs III and VII closer to each other.

DISCUSSION

The ligand-dependent activation of GPCRs has been shown to lead to pronounced structural changes on the cytoplasmic surface of the receptor proteins (14–17, 25). These conformational changes are thought to be essential for productive receptor/G protein coupling. At present, little is known about the molecular mechanisms by which ligand binding

FIGURE 6. Effect of various muscarinic ligands on disulfide bond formation in the S151C mutant receptor. Membranes prepared from COS-7 cells expressing the S151C mutant or M₃(3C)-Xa (control) receptor were incubated for 10 min at room temperature with iodine (20 μM) either in the presence of the indicated ligands (1 μM each) or in the absence of any ligand (Control). Receptors were then solubilized and digested with factor Xa, followed by SDS-PAGE and Western blotting under nonreducing conditions using the anti-M₃ antibody (for details, see “Experimental Procedures”). Equal amounts of protein (5 μg) were loaded onto each lane. The 38-kDa receptor bands that are indicative of disulfide bond formation are shown. The data shown are representative of three independent experiments.
are shown. Note that Ser-151 and Cys-532 are adjacent to Cys-532 directly. For functional assays, COS-7 cells were cotransfected with receptor DNA and an activator protein-1-luciferase reporter construct. Approximately 48 h after transfections, luciferase assays were carried out as described under "Experimental Procedures." Binding and functional data were analyzed using the nonlinear curve-fitting program Prism 3.0. Data are given as means ± S.E. of three independent experiments, each performed in duplicate.

### TABLE THREE

| Binding affinity ($K_i$) | Functional activity$^a$ | M3(3C)-Xa | S151C |
|--------------------------|-------------------------|-----------|-------|
|                          | $E_{max}$ | EC$_{50}$ | $F_{max}$ | EC$_{50}$ | $F_{max}$ |
| N-(Et$_3$)-ACh           | 150 ± 22  | 78.1 ± 15.0 | —       | —       | —       |
| Carbachol                | 29.7 ± 15.9 | 34.4 ± 4.1  | 62.6 ± 11.2 | 4.6 ± 0.2 | 22.3 ± 8.6 | 4.2 ± 0.3 |

$^a$ $E_{max}$ values are expressed as fold stimulation of luciferase activity (relative light units) above basal levels measured in the absence of ligand. Basal levels were as follows (relative light units/well): M3(3C)-Xa, 5498 ± 631; and S151C, 5357 ± 608.

$^a$ = no increase in luciferase activity (relative light units) at concentrations of up to 1 mM N-(Et$_3$)-ACh.

**FIGURE 7.** Predicted locations of Ala-150$^{3.35}$, Ser-151$^{3.36}$, and Cys-532$^{7.42}$ in the three-dimensional structure of the M$_3$ muscarinic receptor. A, extracellular view of the M$_3$ muscarinic receptor parallel to the path of the exofacial segment of TM III. B, extracellular view of the M$_3$ muscarinic receptor parallel to the path of the exofacial segment of TM VII. A three-dimensional model of the inactive state of the rat M$_3$ muscarinic receptor was built via homology modeling using the high resolution x-ray structure of bovine rhodopsin as a template (12, 27). For the sake of clarity, only the exofacial segments of TMs II, III, VI, and VII are shown. Note that Ser-151$^{3.36}$ lies adjacent to Cys-532$^{7.42}$ at the TM III/VII interface. Ala-150$^{3.35}$ is also located in the proximity of Cys-532, but does not project toward Cys-532 directly.

**FIGURE 8.** Model of ACh binding to the M$_3$ muscarinic receptor. ACh (shown in orange) was docked into the ligand-binding crevice of the rat M$_3$ muscarinic receptor (41), ACh bound to the M$_3$ muscarinic receptor in the energetically preferred gauche conformation, displaying an O–C–2–C–1–N dihedral angle of about ± 60°. The highlighted aromatic amino acids (Tyr-148$^{3.31}$, Tyr-506$^{6.53}$, Tyr-529$^{7.39}$, and Tyr-537$^{7.43}$) and Asp-147$^{3.32}$ are known to play key roles in ACh binding to the M$_3$ and M$_1$ muscarinic receptors. Note that Ala-150$^{3.35}$, Ser-151$^{3.36}$, and Cys-532$^{7.42}$ are located slightly below the ACh ligand.

**FIGURE 9.** Model of the S151C mutant receptor. protein is not an artifact caused by improper folding of the receptor protein. To exclude the possibility that agonist-promoted disulfide cross-linking between Cys-151 and Cys-532 involves the cross-linking of different receptor monomers, membranes prepared from S151C-expressing COS-7 cells were treated with iodine (20 μM) in either the absence or

presence of carbachol (1 mM) and then processed for SDS-PAGE and Western blotting under nonreducing conditions without cleaving the receptor with factor Xa. Under these conditions, like the M3(3C)-Xa construct, the S151C mutant receptor did not yield significant amounts of high molecular mass receptor species in either the absence or presence of carbachol (Fig. 4), suggesting that the formation of a disulfide bond between Cys-151 and Cys-532 in the S151C construct most likely does not involve interactions between different receptor monomers.

Our three-dimensional model of the inactive form of the M$_3$ muscarinic receptor suggests that S151$^{3.36}$ and Cys-532$^{7.42}$ face each other in the interior of the TM receptor bundle (Fig. 7). This model also predicts that the distance between the C-α atoms of Ser-151 and Cys-532 is ~8.2 Å. The distance between the C-α atoms of two Cys residues engaged in a disulfide bond usually ranges from ~3.8 to 6.8 Å (17, 45), providing a
possible explanation as to why S151C and Cys-532 do not easily form a disulfide bond in the inactive state of the M3 muscarinic receptor. The observed cross-linking pattern is therefore consistent with a model in which agonist binding to the M3 muscarinic receptor increases the proximity of the exofacial segments of TMs III and VII, thus allowing the formation of a disulfide bridge between S151C and Cys-532.

The cross-linking signal displayed by the S151C mutant receptor in the absence of ligands may be due to thermal motions of the polypeptide backbone of the transmembrane helical bundle (46). Consistent with this concept, disulfide cross-linking studies with Cys-substituted versions of a bacterial chemoreceptor of known structure indicate that disulfide bonds can form between two C-α atoms that are up to ~12 Å apart (46). Atropine treatment had no significant effect on the intensity of the basal cross-linking signal displayed by the S151C mutant receptor, suggesting that this signal is not due to constitutively active S151C mutant receptors.

In contrast to the S151C construct, the A150<sup>33</sup> mutant receptor gave a weak cross-linking signal that remained essentially unchanged after the addition of the agonist carbachol (Fig. 2). Like Ser-151<sup>3.36</sup>, Ala-150<sup>3.35</sup> is also located in the vicinity of Cys-532<sup>7.42</sup> (estimated distance between the C-α atoms of Ala-150 and Cys-532 of 10.8 Å) (Fig. 7). However, in contrast to Ser-151, Ala-150 does not face Cys-532 directly, but projects toward TM II (Fig. 7), providing a possible explanation for the observed cross-linking pattern.

Fig. 8 depicts a three-dimensional model of ACh docked onto the M3 muscarinic receptor protein. This model illustrates that Ala-150<sup>3.35</sup>, Ser-151<sup>3.36</sup>, and Cys-532<sup>7.42</sup> are located slightly below the ACh ligand, explaining why ACh binding does not sterically block disulfide cross-links between S151C (or A150C) and Cys-532.

As reviewed previously (9, 11), the positively charged ammonium head group of ACh is predicted to lie adjacent to several aromatic amino acids located in TMs III, VI, and VII, including Tyr-148<sup>3.33</sup>, Tyr-506<sup>6.51</sup>, Tyr-529<sup>7.39</sup>, and Tyr-535<sup>7.42</sup>, respectively. In addition, the negatively charged side chain of Asp<sup>147<sup>3.33</sup></sup> and Asp<sup>147<sup>3.33</sup></sup> (Figs. 1 and 8) also contributes the relative orientation of TMs III and VII. In two related studies, metal ion-binding sites (i.e., His or Cys residues) were introduced into the exofacial segments of TMs III and VII at positions 3.32 and 7.39, respectively, of the β<sub>2</sub>-adrenergic and tachykinin NK<sub>1</sub> receptors (56, 57). Interestingly, Zn<sup>2+</sup> or Cu<sup>2+</sup> ions acted as partial agonists on the resulting mutant receptors, most likely by directly contacting the introduced Cys/His residues. Consistent with this concept, positions 3.32 and 7.39 face each other within the TM receptor core in the three-dimensional structure of the inactive state of rhodopsin (12).

In agreement with our disulfide cross-linking data, site-directed mutagenesis studies with rhodopsin (54) and the C5a receptor (55) also suggest that activation of class A GPCRs is associated with changes in the relative orientation of TMs III and VII. In contrast, the agonist-induced conformational change observed in this study is propagated to the intracellular receptor surface remains unknown. However, because TM VII is engaged in multiple contacts with TM VI (12, 13), it is possible that a movement of the exofacial segment of TM VII, as identified in this study, triggers conformational changes that are propagated to the endofacial segments of both TMs VI (26) and VII (27).

In a previous study, Struthers et al. (58) used cysteine scanning mutagenesis and disulfide cross-linking in a split rhodopsin construct to investigate the proximity of Cys residues introduced at the o2 loop (the second extracellular loop of GPCRs)/TM V junction and the extracellular end of TM VI. Under mild oxidizing conditions, Cys residues introduced at positions 198<sup>6.33</sup>, 204<sup>6.53</sup>, and 204<sup>6.59</sup> (o2 loop/TM V junction) were able to form disulfide bonds with a Cys residue introduced at position 276<sup>6.59</sup> (top of TM VI) in the inactive state of rhodopsin. However, none of the three disulfide bonds interfered with 11-cis-retinal binding or light-induced rhodopsin activation (58). These data support the concept that the relative orientation of the extracellular ends of TMs V and VI is similar in the dark state and the light-activated state of M<sub>3</sub> Muscarinic Receptor Activation
M3 Muscarinic Receptor Activation

rhodopsin (58). In contrast to the results of this study examining changes in the relative orientation of the exofacial segments of TMs III and VII, rhodopsin activation therefore does not seem to involve significant movements of the extracellular ends of TMs V and VI relative to each other.

To the best of our knowledge, this is the first study providing direct evidence for a conformational change occurring in the immediate vicinity of the binding pocket of a GPCR activated by a diffusible ligand. The extension of the approach described here to other TMs and residues that are buried more deeply in the TM receptor core should eventually provide a detailed view of the early conformational events triggering GPCR activation. Because all class A GPCRs share a high degree of structural homology, it is likely that the results that we obtained with the M3 muscarinic receptor are also relevant for other members of this very large protein family.

Acknowledgments—We thank Dr. Susanne Neumann for help with the luciferase reporter assay and Dr. Jianhua Li for carrying out several disulfide cross-linking experiments.

REFERENCES

1. Fredriksson, R. Lagerström, M.C., Lundin, L. G., and Schioth, H. B. (2003) Mol. Pharmacol. 63, 1256–1272

2. Foord, S. M. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 437–467

3. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. F., and Guenet, C. (1999) J. Mol. Biol. 299, 7309–7315

4. Narita-Matsuishi, H., Goto, S., and Schioth, H. B. (2003) Eur. J. Pharmacol. 463, 319–333

5. Angers, S., Saladpour, A., and Bourne, M. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 499–435

6. Kobilka, B. K. (2003) J. Biol. Chem. 278, 17033–17041

7. Nissenson, R. A., and Bourne, H. R. (1999) J. Biol. Chem. 274, 17033–17041

8. Jensen, A. D., Guarnieri, F., Rasmussen, S. G., Asmar, F., Ballesteros, J. A., and Gether, U. (2001) J. Biol. Chem. 276, 9279–9290

9. Ghanouni, P., Steenhuis, J. J., Farrens, D. L., and Kobilka, B. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5997–6002

10. Bissantz, C. (2003) J. Recept. Signal Transduct. Res. 23, 123–153

11. Gether, U., and Kobilka, B. K. (1998) J. Biol. Chem. 273, 2247–2257

12. Han, S.-J., Hamdan, F., Kim, S.-K., Jaakola, K. A., Brinca, L., Bloodworth, L. M., Li, J. H., and Weiss, J. (2005) J. Biol. Chem. 280, 24870–24879

13. Ballesteros, J. A., and Weinstein, H. (1995) Methods Neurosci. 25, 366–428

14. Ward, S. D., Ward, S. D., Siddiqui, N. A., Bloodworth, L. M., and Weiss, J. (2002) Biochemistry 41, 7647–7658

15. Zeng, F. Y., Oldner, A., Schöneberg, T., and Weiss, J. (1999) J. Neurochem. 72, 2404–2414

16. Hamdan, F. D., Ward, S. D., Siddiqui, N. A., Bloodworth, L. M., and Weiss, J. (2002) Biochemistry 41, 7647–7658

17. Vatini-Pagliuca, A., and Gershengorn, M. C. (1997) Endocrinology 138, 1471–1475

18. Holsten, B., Elling, C. E., and Schwartz, T. W. (2000) J. Biol. Chem. 275, 17597–17604

19. Hubbell, W. L., Altenbach, C., Hubbell, C. M., and Khorana, H. G. (2003) Science 299, 7309–7315

20. Ballesteros, J. A., and Weinstein, H. (1995) Trends Pharmacol. Sci. 16, 587–593

21. Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., and Oprian, D. D. (1992) J. Biol. Chem. 267, 12299–12302

22. Zeng, F. Y., Oldner, A., Schöneberg, T., and Weiss, J. (1999) J. Neurochem. 72, 2404–2414
Identification of an Agonist-induced Conformational Change Occurring Adjacent to the Ligand-binding Pocket of the M₃ Muscarinic Acetylcholine Receptor

Sung-Jun Han, Fadi F. Hamdan, Soo-Kyung Kim, Kenneth A. Jacobson, Lanh M. Bloodworth, Bo Li and Jürgen Wess

J. Biol. Chem. 2005, 280:34849-34858. doi: 10.1074/jbc.M506711200 originally published online August 10, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M506711200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 26 of which can be accessed free at http://www.jbc.org/content/280/41/34849.full.html#ref-list-1