Pronounced Conformational Changes following Agonist Activation of the M₃ Muscarinic Acetylcholine Receptor*§

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The conformational changes that convert G protein-coupled receptors (GPCRs) activated by diffusible ligands from their resting into their active states are not well understood at present. To address this issue, we used the M₃ muscarinic acetylcholine receptor, a prototypical class A GPCR, as a model system, employing a recently developed disulfide cross-linking strategy that allows the formation of disulfide bonds using Cys-substituted mutant M₃ muscarinic receptors present in their native membrane environment. In the present study, we generated and analyzed 30 double Cys mutant M₃ receptors, all of which contained one Cys substitution within the C-terminal portion of transmembrane domain (TM) VII (Val-541 to Ser-546) and another one within the C-terminal segment of TM I (Val-88 to Phe-92). Following their transient expression in COS-7 cells, all mutant receptors were initially characterized in radioligand binding and second messenger assays (carbachol-induced stimulation of phosphatidylinositol hydrolysis). This analysis showed that all 30 double Cys mutant M₃ receptors were able to bind muscarinic ligands with high affinity and retained the ability to stimulate G proteins with high efficacy. In situ disulfide cross-linking experiments revealed that the muscarinic agonist, carbachol, promoted the formation of cross-links between specific Cys pairs. The observed pattern of disulfide cross-links, together with receptor modeling studies, strongly suggested that M₃ receptor activation induces a major rotational movement of the C-terminal portion of TM VII and increases the proximity of the cytoplasmic ends of TM I and VII. These findings should be of relevance for other family A GPCRs.

The superfamily of G protein-coupled receptors (GPCRs)¹ represents the largest group of cell surface receptors found in nature (1–3). A structural hallmark of all GPCRs is the presence of a bundle of seven transmembrane helices (TM I–VII) that are connected by alternating intracellular and extracellular loops (4–6) (Fig. 1). The structural elements determining ligand binding and G protein recognition have been studied in considerable detail, at least for some members of the GPCR superfamily (5–8). In contrast, the conformational changes that activating ligands induce in their target receptors are still not well understood at present. The currently available evidence suggests that GPCR activation opens a cleft on the intracellular side of the receptor that promotes the recognition and activation of specific G protein heterotrimers (4, 6, 9, 10, 34).

At present, bovine rhodopsin (in its inactive state) is the only GPCR for which high resolution structural information is currently available (11). Most GPCRs share a considerable degree of structural homology with bovine rhodopsin (12) and are therefore referred to as rhodopsin-like or family A GPCRs. However, whereas the endogenous ligand of rhodopsin, 11-cis-retinal, is covalently bound to the receptor protein, all other family A GPCRs known to date are activated by diffusible ligands. The possibility therefore exists that the precise structural mechanisms involved in receptor activation may not be identical between rhodopsin and other class A GPCRs.

During the past decade, considerable progress has been made in elucidating the light-induced conformational changes in bovine rhodopsin (9, 10, 13). Biophysical and biochemical studies suggest that rhodopsin activation triggers a reorientation of the cytoplasmic end of TM VI and changes in the relative disposition of TM VI and III, along with smaller movements involving several other TM helices (10, 14–16). Considerable evidence indicates that a similar movement (reorientation of the cytoplasmic end of TM VI versus that of TM III) occurs in other GPCRs, including the β₂-adrenergic receptor (17–21). More specifically, site-directed spin labeling studies (15) suggested that rhodopsin activation involves a rigid body movement of the cytoplasmic end of TM VI (away from the C terminus of TM III) that is accompanied by a rotational movement of ~30° (clockwise as viewed from the cytoplasm).

The pioneering biophysical and biochemical studies carried out with bovine rhodopsin have led to important new insights into the structural mechanisms involved in rhodopsin activation. However, the vast majority of these studies were carried out with mutant versions of rhodopsin in the solution state (receptor proteins were solubilized in dodecyl maltoside micelles), and some data suggest that the structural and dynamic properties of rhodopsin present in solution may not be identical of G protein-coupled receptors; IP₃, inositol monophosphate; TM I–VII, the seven transmembrane domains of G protein-coupled receptors.
with those found in native disk membranes (10). Thus, the development of techniques that would allow the monitoring of agonist-induced conformational changes in GPCRs present in their native membrane environment would be highly desirable. To address this issue, we recently described a novel in situ disulfide cross-linking strategy that allows the formation of disulfide bonds using Cys-substituted mutant M₃ muscarinic acetylcholine receptors present in their native membrane environment (22, 23). The M₃ muscarinic receptor is a prototypical class A GPCR that preferentially interacts with G proteins of the Gₛ family (24).

Agonist binding to the M₃ muscarinic receptor and most other class A GPCRs involves, among other sites of contact, several key residues present within the exofacial portion of TM VII (24, 25). Moreover, the endofacial segment of TM VII contains the highly conserved NPXXY motif (corresponding to Asn-539 to Tyr-543 in the rat M₂ receptor sequence) (Fig. 1), which may provide a point of flexibility for agonist-induced structural changes. We therefore tested the hypothesis that diffusible ligands may induce conformational changes within the cytoplasmic segment of TM VII (the region located C-terminal of the NPXXY motif), using our previously developed in situ disulfide cross-linking strategy (22, 23).

Since the C-terminal segment of TM VII is predicted to be located in the vicinity of the C-terminal portion of TM I (11), we generated 30 double Cys mutant M₃ muscarinic receptors, all of which contained one Cys substitution within the C-terminal portion of TM VII (Val-541–Val-546) and another Cys substitution within the C-terminal segment of TM I (Val-88–Val-92) (the superscripts indicate amino acid positions according to the nomenclature proposed by Ballesteros and Weinstein (36)). All Cys mutations were introduced into a modiﬁed version of the rat M₃ muscarinic receptor (M3'/3C)-Xa (22, 26) that lacked most native Cys residues and contained a factor Xa cleavage site within the third intracellular loop (i3 loop) (Fig. 1).

Disulfide cross-linking experiments, carried out in the absence or the presence of a muscarinic agonist (carbachol), led to the identiﬁcation of three double Cys mutant M₃ muscarinic receptors (V88C’/3C)-Y543C’/3C (V formerly (26). After labeling of cells for 20–24 h with [3H]Methylscopolamine (behavior, was replaced by two factor Xa cleavage sites. Cys residues were reintroduced into the M3'/3C-Xa construct at positions Val-541-Phe-92 and Val-541-Ser-546 by using the QuikChange™ site-directed mutagenesis kit (Stratagene), according to the manufacturer’s instructions. Double Cys mutant receptors were obtained by subcloning a 1.7-kb BglII-NdeI fragment derived from the mutant M3'/3C-Xa constructs containing single Cys substitutions at positions Val-541 to Val-546 into the M3'/3C-Xa cleavage sites. Single Cys substitutions at positions Val-88 to Phe-92. The identity of all mutant constructs was veriﬁed by DNA sequencing.

Expression of Receptor Constructs in Mammalian Cells—All mutant M₃ muscarinic receptors were transiently expressed in COS-7 cells. Cells were cultured in Dulbecco’s modiﬁed Eagle’s medium supple-mented with 10% fetal bovine serum, 2 mm l-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ incubator. Approximately 24 h prior to transfections, 1 × 10⁶ cells were seeded into 100-mm dishes. Cells were transfected with 4 μg of receptor plasmid DNA/dish using the Lipofectamine™ Plus kit (Invitrogen), according to the manufacturer’s recommendations. In order to increase muscarinic receptor expression levels, 1 μM atropine was routinely added to the incubation medium for the last 24 h.

Preparations of Membranes from Transfected COS-7 Cells—Transfected cells were harvested ~48 h after transfections. To ensure complete removal of atropine that was present 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). Subsequently, 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 of the plate and homогenized 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), homogenized, frozen on dry ice, and stored at −70 °C until needed. Protein concentrations were measured using the Micro BCA protein assay reagent kit (Pierce) as a standard.

Radioligand Binding Studies—Radioligand binding assays were carried out using membrane homogenates prepared from transfected COS-7 cells essentially as described previously (22). In brief, all incubations were carried out in 1 ml of buffer A (~10–20 μg of membrane protein/tube) for 2 h at room temperature (22 °C). In saturation binding assays, six different concentrations (ranging from 20 to 3,000 pM) of [3H]NMS were used. In competition binding assays, a fixed concentra- tion of [3H]NMS (500 pM) was employed in the presence of 10 different concentrations (13 pM to 10 μM) of the cold competitor, carbachol, a muscarinic agonist. Reactions were terminated by rapid ﬁltration over GF/C Brandel ﬁlters followed by three washes (~4 ml each) with ice-cold distilled water. In all assays, nonspeciﬁc binding was deﬁned as the 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).

Agonist-induced Stimulation of Phosphatidylinositol Hydrolysis—The ability of the muscarinic agonist, carbachol, to stimulate increases in intracellular inositol monophosphate (IP₁) levels was determined using transfected COS-7 cells grown in 6-well plates, as described previously (26). After labeling of cells for 20–24 h with myo-[3H]inositol (3 μCi/ml), cells were incubated in the presence of 10 mM LiCl for 1 h at 37 °C with increasing concentrations of carbachol. The IP₁ fraction was isolated and quantitated as described (26). Carbachol concentration-response curves were analyzed using the nonlinear curve-ﬁtting program Prism 3.0 (GraphPad).

Oxidation, Solubilization, and Factor Xa Treatment of Mutant M₃ Muscarinic Receptors—Membrane preparations obtained from transfected COS-7 cells were thawed at room temperature and rehomogenized as described under “Preparations of Membranes from Transfected COS-7 Cells.” Membranes from one 100-mm dish (~1 mg of protein present in a 1-ml volume) were incubated in microcentrifuge tubes for 30 min at room temperature (30 rpm; 10 min at room temperature) with the oxidizing agent, Cu(II)-phenanthroline (2.5 μM), either in the absence or the presence of different concentrations of the muscarinic agonist, carbachol, or the antagonist, atropine. 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 (22), samples were then centrifuged at 8,000 × g for 10 min at 4 °C. 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 (8,000 × g for 10 min at 4 °C), membrane pellets were incubated with 1.2% digitonin in buffer B (50 mM Tris-HCl, pH 8, 100 mM NaCl, and 1 mM CaCl₂) for 90–120 min at 4 °C with end-over-end rotation (30 rpm). After another centrifugation step (same conditions as above), the supernatants (membrane lysates containing solubilized mutant M₃ muscarinic receptors) were transferred to fresh microcentrifuge tubes. Membrane lysates (~15 μg of protein) were then incubated with factor Xa protease (final concentration, 0.1 μg/μl) at room temperature for 16–20 h (final volume, 50 μl). The reactions were then terminated by incubation for 30 min at room temperature with a mammalian protease inhibitor mixture (1:25 dilution; Sigma). Samples were then used directly for SDS-PAGE or stored at −70 °C until use.

**Western Blot Analysis**—SDS-PAGE was performed essentially as described (22). Samples were incubated for 30 min at 37 °C with Læmmli loading buffer (nonreducing conditions) and then loaded onto 10–20% Tris-glycine polyacrylamide gels, which were run at 125 V in mmli loading buffer (nonreducing conditions) and then loaded onto 10–20% Tris-glycine polyacrylamide gels, which were run at 125 V in

**Results**

**Generation of 30 Double Cys Mutant M₃ Muscarinic Receptors**—This study was designed to monitor agonist-induced conformational changes in the M₃ muscarinic receptor with the receptor being present in its native membrane environment. Our major goal was to detect potential activity-dependent structural changes occurring at the cytoplasmic end of TM VII. Toward this aim, we used an in situ disulfide cross-linking strategy to monitor the positions of six consecutive amino acids located at the C terminus of TM VII, relative to a string of residues located at the C terminus of TM I. Altogether, we generated 30 double Cys mutant receptors, all of which contained one Cys substitution within the C-terminal segment of TM I (Val-88 to Phe-92) and another Cys substitution within the sequence Val-541 to Ser-546 (cytoplasmic end of TM VII). To detect the various mutant receptors via Western blotting, we used a rabbit polyclonal antibody (anti-M3) directed against the last 18 amino acids of the receptor protein (27). The numbers refer to amino acid positions in the rat M₃ muscarinic receptor sequence (28).

**Transient Expression of Mutant M₃ Muscarinic Receptors and Radioligand Binding Studies**—All 30 double Cys mutant M₃ receptor constructs, along with the M₃(3C)-Xa “backbone” receptor, were transiently expressed in COS-7 cells and initially examined for their ability to bind the muscarinic radioligand, [³H]NMS. To increase receptor expression levels, transfected cells were incubated with atropine (1 μM) for the last 24 h of culture. We previously demonstrated that this strategy leads to a pronounced increase in the density of the M₃(3C)-Xa receptor and all Cys-substituted mutant receptors derived from this construct (22, 23).

**Saturation binding studies with the muscarinic antagonist, [³H]NMS, showed that the M₃(3C)-Xa receptor was expressed at a density of 3.54 ± 0.01 pmol/mg protein (B₅₀).** The expression levels of the majority of the 30 double Cys mutant recepto
M₃ Muscarinic Receptor Activation

The indicated mutant M₃ muscarinic receptors were transiently expressed in COS-7 cells. All double Cys mutant receptors were derived from the M3(3C)-Xa construct. Bₘ₅ₐₓ and Kᵢ values for [³H]NMS were determined from saturation binding experiments using membranes homogenates prepared from transfected COS-7 cells. Carbachol binding affinities (Kᵢ) were determined in [³H]NMS competition binding assays (nᵢ = Hill coefficient). Carbachol binding data were corrected for the Cheng-Prusoff shift. Binding data were analyzed using the nonlinear curve-fitting program Prism 3.0 (GraphPad). Data are given as means ± S.E. from 2–5 independent experiments, each performed in duplicate.

Table I

| Receptor          | [³H]NMS binding | Carbachol binding |
|-------------------|-----------------|-------------------|
|                   | Kᵢ (μM)         | Bₘ₅ₐₓ (pmol/mg)  | Kᵢ μM      | nᵢ |
| M³(3C)-Xa         |                 |                   |            |    |
| 240 ± 45          | 3.54 ± 0.01     | 15.2 ± 1.7        | 0.55 ± 0.02 |
| 254 ± 110         | 2.15 ± 0.49     | 12.1 ± 0.3        | 0.54 ± 0.01 |
| 300 ± 73          | 5.30 ± 0.37     | 38.1 ± 7.7        | 0.61 ± 0.05 |
| 364 ± 120         | 1.92 ± 0.52     | 9.4 ± 1.3         | 0.49 ± 0.03 |
| 343 ± 43          | 3.86 ± 0.01     | 22.6 ± 4.8        | 0.55 ± 0.01 |
| 288 ± 71          | 3.33 ± 0.63     | 12.1 ± 0.3        | 0.53 ± 0.02 |
| 267 ± 1           | 2.53 ± 1.05     | 28.3 ± 10.3       | 0.51 ± 0.06 |
| 277 ± 83          | 2.04 ± 0.30     | 14.5 ± 6.0        | 0.43 ± 0.01 |
| 241 ± 66          | 4.55 ± 0.67     | 39.5 ± 4.2        | 0.54 ± 0.04 |
| 341 ± 185         | 1.48 ± 0.54     | 5.4 ± 1.8         | 0.41 ± 0.08 |
| 260 ± 68          | 2.47 ± 0.53     | 22.2 ± 0.2        | 0.44 ± 0.01 |
| 280 ± 68          | 3.01 ± 1.62     | 15.2 ± 1.6        | 0.51 ± 0.03 |
| 405 ± 169         | 4.23 ± 0.39     | 102.8 ± 49.0      | 0.78 ± 0.12 |
| 334 ± 85          | 19.06 ± 0.53    | 43.6 ± 11.5       | 0.62 ± 0.06 |
| 334 ± 108         | 8.84 ± 0.40     | 77.2 ± 9.3        | 0.64 ± 0.02 |
| 365 ± 71          | 4.00 ± 0.60     | 12.3 ± 2.4        | 0.48 ± 0.02 |
| 280 ± 47          | 4.80 ± 0.65     | 96.0 ± 23.1       | 0.61 ± 0.07 |
| 238 ± 114         | 5.61 ± 1.32     | 27.4 ± 4.3        | 0.63 ± 0.04 |
| 223 ± 20          | 3.76 ± 0.61     | 11.4 ± 1.6        | 0.72 ± 0.07 |
| 282 ± 121         | 5.01 ± 1.43     | 6.7 ± 1.9         | 0.50 ± 0.02 |
| 280 ± 57          | 5.99 ± 0.76     | 21.1 ± 4.7        | 0.63 ± 0.00 |
| 324 ± 79          | 4.37 ± 0.39     | 5.6 ± 2.3         | 0.53 ± 0.10 |
| 297 ± 51          | 5.20 ± 1.10     | 17.1 ± 1.8        | 0.57 ± 0.01 |
| 221 ± 69          | 4.85 ± 0.63     | 7.9 ± 1.3         | 0.59 ± 0.01 |
| 297 ± 33          | 4.07 ± 0.64     | 27.3 ± 2.5        | 0.56 ± 0.07 |
| 277 ± 128         | 3.96 ± 1.36     | 2.3 ± 0.7         | 0.57 ± 0.03 |
| 293 ± 52          | 7.81 ± 1.69     | 4.5 ± 1.0         | 0.50 ± 0.04 |
| 427 ± 88          | 11.69 ± 6.93    | 2.8 ± 1.1         | 0.62 ± 0.06 |
| 272 ± 47          | 10.98 ± 4.86    | 1.6 ± 0.1         | 0.59 ± 0.03 |
| 288 ± 52          | 16.42 ± 0.26    | 2.0 ± 0.3         | 0.67 ± 0.06 |
| 183 ± 3           | 3.01 ± 0.94     | 30.4 ± 17.5       | 0.64 ± 0.05 |

tors differed from this value by less than 2-fold (Table I). Interestingly, several mutant receptors yielded Bₘ₅ₐₓ values that were significantly higher than that observed with the M³(3C)-Xa construct (Table I). The mutant receptors displaying the highest receptor densities were V90C/S546C and F92C/V541C, which exhibited Bₘ₅ₐₓ values of 19.06 ± 0.53 and 16.42 ± 0.26 pmol/mg, respectively. All 30 double Cys mutant receptors were able to bind [³H]NMS with high affinity (Table I). The [³H]NMS Kᵢ values displayed by these receptors differed from the Kᵢ value determined for the M³(3C)-Xa “base mutant” (Kᵢ = 240 ± 45 pmol/mg) by less than 2-fold.

Competition binding studies with the muscarinic agonist, carbachol, showed that most of the 30 double Cys mutant receptors displayed carbachol binding affinities that were similar to or even higher than that found for the M³(3C)-Xa construct (Kᵢ = 15.2 ± 1.7 μM; Table I). Interestingly, most mutant receptors containing the F92C point mutation exhibited carbachol affinities that were increased by ∼3–10-fold, as compared with the M³(3C)-Xa “base mutant” (Table I). In contrast, only two of the analyzed double Cys mutant receptors, I89C/S546C and V90C/S546C, showed a clear reduction (by ∼3–10-fold) in carbachol binding affinities (Table I).

G Protein-coupling Properties of Double Cys Mutant M₃ Muscarinic Receptors—To examine whether the 30 double Cys mutant receptors were still able to couple to G proteins, we next studied their ability to mediate carbachol-induced increases in inositol monophosphate (IP₇) production (phosphatidylinositol hydrolysis). As shown in Table II, all analyzed double Cys mutant receptors retained the ability to stimulate phosphatidylinositol hydrolysis with high efficacy (Eₘ₅ₐₓ, expressed as -fold increase in IP₇ production above basal levels). In general, the apparent increase in Eₘ₅ₐₓ values displayed by several of the double Cys mutant receptors, as compared with the M³(3C)-Xa construct, correlated well with reduced basal IP₇ levels displayed by these mutant receptors (Table II). This observation suggests that the observed increases in Eₘ₅ₐₓ values observed for several of the double Cys mutant receptors probably do not reflect a “true” gain in G protein coupling efficacy.

Most of the analyzed double Cys mutant receptors showed reduced carbachol potencies (increased EC₅₀ values), as compared with the M³(3C)-Xa construct (EC₅₀ = 0.025 ± 0.004 μM; Table II), indicative of impaired receptor/G protein coupling efficiency. This observation is consistent with the concept that residues located within the C-terminal segment of TM VII play an important role in receptor/G protein coupling (28, 29).

Taken together, the ligand binding studies indicated that all 30 double Cys mutant receptors were able to bind muscarinic ligands with high affinity, indicating that all mutant receptors were properly folded. Moreover, all double Cys mutant receptors retained the ability to activate G proteins, although the efficiency of receptor/G protein coupling was reduced in most cases.

Disulfide Cross-linking Studies—The results of the [³H]NMS binding studies indicated that all 30 double Cys mutant receptors, similar to the M³(3C)-Xa construct, were properly expressed, at moderate to high levels, in transiently transfected COS-7 cells. Consistent with these results, Western blotting studies using a polyclonal antibody directed against the C-terminal portion of the rat M₃ muscarinic receptor (referred to as “anti-M₃ antibody”) showed that all double Cys mutant receptors.
receptors could be easily detected in membrane lysates prepared from transfected COS-7 cells (data not shown) in a fashion similar to the M₃/(3C)-Xa receptor (22, 23).

To probe the potential proximity of the Cys pairs present in the 30 double Cys mutant receptors, we examined the ability of the 30 Cys pairs to form intramolecular disulfide bonds. Disulfide cross-linking studies were performed with mutant receptors being present in their natural membrane environment (in situ), using membrane preparations obtained from transfected COS-7 cells (22, 23). To facilitate the formation of disulfide bonds, all reactions were carried out in the presence of a low concentration (2.5 μM; 10-min incubation at room temperature) of the mild oxidizing agent, Cu(II)-phenanthroline. Subsequently, receptors were solubilized, digested to completion with factor Xa, and subjected to SDS-PAGE and Western blotting (reducing and nonreducing conditions). In this system, the appearance of a ~38-kDa receptor band under nonreducing conditions (2.5 μM Cu(II)-phen) was evident that corresponded to properly folded cell surface receptors.

The results of the cross-linking studies obtained with the 30 double Cys mutant M₃ receptors are summarized in Fig. 2. Under basal conditions (no carbachol added to the incubation medium), only the V88C/Y543C receptor yielded a robust 38-kDa signal (under nonreducing conditions) (Fig. 2), indicat-

### Table II

**Functional properties of double Cys mutant M₃ muscarinic receptors**

The indicated mutant M₃ muscarinic receptors were transiently expressed in COS-7 cells. All double Cys mutant receptors were derived from the M₃/(3C)-Xa construct. After labeling of cells for 20–24 h with myo-[3H]inositol (3 μCi/ml), cells were incubated in 6-well plates for 1 h at 37 °C with increasing concentrations of carbachol. Carbachol-induced increase in IP₁ levels were determined as described under “Experimental Procedures.” Carbachol EC₅₀ and E₉₀ values were analyzed using the nonlinear curve-fitting program Prism 3.0 (GraphPad). Data are given as means ± S.E. from 2–5 independent experiments, each performed in duplicate.

| Receptor | EC₅₀ (carbachol) | Basal activity | E₉₀ |
|----------|-----------------|----------------|-----|
| M₃/(3C)-Xa | 0.025 ± 0.004 | 11,942 ± 1,490 | 6 ± 1 |
| V88C/Y543C | 0.27 ± 0.07 | 6,868 ± 1,670 | 17 ± 1 |
| V88C/Y543C | 1.11 ± 0.11 | 4,766 ± 567 | 12 ± 1 |
| V88C/Y543C | 2.56 ± 1.35 | 4,169 ± 504 | 17 ± 1 |
| V88C/Y543C | 0.17 ± 0.01 | 4,686 ± 1,602 | 11 ± 3 |
| V88C/Y543C | 0.25 ± 0.01 | 5,788 ± 972 | 15 ± 1 |
| V88C/Y543C | 0.92 ± 0.26 | 4,673 ± 540 | 28 ± 4 |
| I91C/A544C | 0.15 ± 0.05 | 5,779 ± 1,996 | 6 ± 1 |
| I91C/A544C | 0.068 ± 0.006 | 9,516 ± 1,424 | 12 ± 1 |
| I91C/A544C | 0.58 ± 0.11 | 3,871 ± 2,044 | 7 ± 1 |
| I91C/A544C | 0.53 ± 0.15 | 2,957 ± 549 | 12 ± 1 |
| I91C/A544C | 0.21 ± 0.08 | 9,225 ± 2,066 | 11 ± 2 |
| I91C/S546C | 0.021 ± 0.005 | 6,200 ± 1,738 | 9 ± 3 |
| V90C/Y543C | 0.059 ± 0.009 | 7,728 ± 1,748 | 9 ± 1 |
| V90C/A544C | 0.094 ± 0.038 | 6,783 ± 484 | 11 ± 1 |
| V90C/A544C | 1.08 ± 0.41 | 2,622 ± 461 | 16 ± 3 |
| V90C/A544C | 8.19 ± 4.28 | 4,392 ± 499 | 22 ± 1 |
| V90C/A544C | 0.72 ± 0.48 | 6,412 ± 970 | 8 ± 1 |
| V90C/A544C | 0.016 ± 0.001 | 16,199 ± 561 | 7 ± 3 |
| A91C/A544C | 0.016 ± 0.006 | 16,355 ± 5,016 | 5 ± 1 |
| A91C/A544C | 0.89 ± 0.27 | 16,445 ± 2,021 | 6 ± 1 |
| A91C/A544C | 0.19 ± 0.03 | 7,942 ± 4,575 | 11 ± 2 |
| A91C/A544C | 0.15 ± 0.12 | 5,809 ± 729 | 10 ± 1 |
| A91C/A544C | 0.11 ± 0.09 | 7,749 ± 732 | 9 ± 2 |
| A91C/A544C | 0.015 ± 0.01 | 25,771 ± 7,269 | 4 ± 1 |
| F92C/A544C | 0.014 ± 0.004 | 14,242 ± 3,020 | 9 ± 1 |
| F92C/A544C | 0.24 ± 0.21 | 13,093 ± 3,043 | 6 ± 1 |
| F92C/A544C | 2.88 ± 2.03 | 6,296 ± 2,882 | 13 ± 4 |
| F92C/A544C | 0.34 ± 0.17 | 10,804 ± 2,684 | 8 ± 1 |
| F92C/A544C | 1.41 ± 1.03 | 9,629 ± 4,454 | 7 ± 3 |
| F92C/A544C | 1.15 ± 0.02 | 14,988 ± 1,286 | 9 ± 2 |

**Fig. 2.** Agonist-induced disulfide cross-linking in mutant M₃ muscarinic receptors studied by Western blot analysis. Thirty M₃/(3C)-Xa-based double Cys mutant receptors (see Fig. 1 for the structure of the mutant receptors) were incubated with the oxidizing agent, Cu(II)-phen (2.5 μM), for 10 min at room temperature, either in the absence or the presence of the muscarinic agonist, carbachol (1 μM). Receptors were then solubilized and digested to completion with factor Xa, as indicated 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 blotting analysis using the anti-M₃ antibody. Note that three of the 30 investigated double Cys mutant receptors (V88C/Y543C, A91C/L545C, and A91C/S546C) displayed agonist-dependent cross-linking, as indicated by the appearance of a clearly visible ~38-kDa full-length receptor band. Data shown here are representative of three independent experiments.
DISULFIDE BONDS FORM INTRAMOLECULARLY RATHER THAN INTERMOLECULARLY—Like many other GPCRs (30, 31), M₃ muscarinic receptors have been shown to form dimers or oligomers (32). To exclude the possibility that the agonist-dependent formation of disulfide bonds observed with the V88C/Y543C, A91C/L545C, and A91C/S546C mutant receptors are due to the formation of intermolecular cross-links (involving Cys residues located on different receptor molecules), we carried out an additional set of experiments. We first generated five M₃(3C)-Xa-based mutant receptors containing the V88C, A91C, Y543C, L545C, and S546C single Cys substitutions. Radioligand binding studies showed that these mutant receptors were properly expressed in transiently transfected COS-7 cells (data not shown). We then cotransfected COS-7 cells with the following pairs of single Cys mutant receptor constructs: V88C-Y543C, A91C + L545C, and A91C + S546C. In the absence of Cu(II)-phenanthroline and factor Xa treatment, Western blotting studies using membrane lysates prepared from these cotransfected COS-7 cells collected at appearance of prominent 38-kDa receptor species were incubated with Cu(II)-Phen (2.5 μM; 10 min) in the absence of carbachol and in the presence of the muscarinic agonist, atropine (0.1 and 1 μM). Receptors were then solubilized and digested with factor Xa, followed by SDS-PAGE and Western blotting (nonreducing conditions), using the anti-M3 antibody (for details, see “Experimental Procedures”). Equal amounts of protein (~5 μg) were loaded in each lane. The ~38-kDa full-length receptor bands are shown. Data are representative of three independent experiments.

Under the same experimental conditions, the muscarinic agonist, atropine (0.1 and 1 μM), had no significant effect on the weak cross-linking signals observed in the absence of carbachol (Fig. 4). The results of three independent carbachol cross-linking experiments carried out with the V88C/Y543C, A91C/L545C, and A91C/S546C mutant receptors are summarized in Fig. 3B, based on the quantification of the intensity of the 38-kDa receptor species. To obtain a quantitative measure of agonist-induced disulfide cross-linking, ratios were formed between band intensities determined in the presence versus the absence of carbachol. As shown in Fig. 3B, the relative increases in agonist-induced disulfide cross-linking were most pronounced for the A91C/L545C and A91C/S546C receptors.

Agonist Dependence of Disulfide Cross-linking Signals—To examine the agonist dependence of the disulfide cross-linking signals in more detail, we carried out additional cross-linking experiments in which the V88C/Y543C, A91C/L545C, and A91C/S546C receptors were oxidized (in situ) in the presence of different carbachol concentrations (0.01–10 mM). In all three mutant receptors, carbachol induced concentration-dependent increases in the intensity of the 38-kDa cross-linking signal (nonreducing conditions) (Fig. 3A). As expected, no cross-linking was observed with the M₃(3C)-Xa “background” receptor, which served as a negative control.
pronounced cross-linking signals (Fig. 5), as expected (Figs. 2 and 3). Taken together, these data strongly support the concept that carbachol promotes the formation of intramolecular rather than intermolecular disulfide bonds in the V88C/Y543C, A91C/L545C, and A91C/S546C mutant receptors.

A Three-dimensional Model of the Rat M₃ Muscarinic Receptor—To facilitate the interpretation of the disulfide cross-linking data, we built a three-dimensional model of the TM core of the rat M₃ muscarinic receptor (including the various loop regions and helix 8) using homology modeling based on the high resolution x-ray structure of bovine rhodopsin as a template (11) (see “Experimental Procedures” and Supplemental Data for details). A, side view of the cytoplasmic portion of the TM helical bundle and helix 8 (purple) of the M₃ muscarinic receptor. B, cytoplasmic view of a selected region of the intracellular surface of the M₃ muscarinic receptor. The side chains of the 11 amino acids that were subjected to Cys mutagenesis are shown. Cys substitution of the residues highlighted in yellow resulted in double Cys mutant receptors that showed agonist-dependent disulfide cross-linking (88, 91, 543, 545, and 546) whereas, Val-88 (TM I) and Tyr-543 (TM VI) directly face each other in the inactive state of the M₃ muscarinic receptor. Leu-545 and Cys-546 (TM VII) point toward the lipid face and TM VI, respectively, away from Ala-91 on TM I. Amino acid position numbers according to the Ballesteros/Weinstein nomenclature (36) are: Val-541, Cys-542, Tyr-543, Ala-544, Leu-545, Cys-546, Val-88, Ile-89, Val-90, Ala-91, and Phe-92.

core of the rat M₃ receptor (with the primary focus on TM I and VII), indicates that residues 88 and 543 directly face each other at the TM I/TM VII interface. In striking contrast, residues 545 and 546 face away from TM I, including residue 91. This observation, together with the relatively large distances between the Cα atoms in the 91/545, and 91/546 residue pairs, strongly suggests that the C-terminal portion of TM VII undergoes a major conformational change following M₃ receptor activation. The potential structural nature of these changes are discussed in detail below.

DISCUSSION

Many biochemical and biophysical studies of bovine rhodopsin have revealed the structural changes involved in light-induced activation of rhodopsin in considerable detail (10, 13). In contrast, little is known about the agonist-induced conformational changes that occur in GPCRs activated by diffusible ligands (6, 34). In addition, almost all rhodopsin studies were carried out with mutant versions of rhodopsin in the solution state (10). Thus, studies that allow the monitoring of agonist-induced conformational changes in GPCRs present in their native membrane environment should be highly informative.

To address these issues, we recently developed a novel disulfide cross-linking strategy that allows the formation of disulfide...
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Biochemical and biophysical analysis of bovine rhodopsin suggests that the C-terminal segment of TM VII undergoes significant light-induced conformational changes. Site-directed spin labeling studies showed that Tyr-306 and Phe-307 underwent a light-induced mobility change, which was interpreted as an increase in the polarity of the environment of Tyr-306 (37). Abdulaev and Ridge (38) demonstrated that a monoclonal antibody against amino acids 304-311 of bovine rhodopsin did not recognize the inactive state of rhodopsin but efficiently bound to light-activated rhodopsin, suggesting that rhodopsin activation is associated with pronounced conformational changes at the cytoplasmic end of TM VII. However, the precise molecular nature of these activity-dependent structural changes remains unknown.

Thus, one major goal of the present study was to investigate whether activation of GPCRs by diffusible ligands also leads to conformational changes involving the C-terminal portion of TM VII. To address this question, we employed a previously developed disulfide cross-linking strategy (22, 23). Moreover, we speculated that this analysis would yield more specific information about the molecular nature of the agonist-induced conformational changes occurring in this region.

Specifically, we generated 30 double Cys mutant M3 muscarinic receptors, all of which contained one Cys substitution within the C-terminal portion of TM VII (Val-541-Ser-546) and another Cys substitution with the C-terminal segment of TM I (Val-881-Phe-921). The x-ray structure of bovine rhodopsin and our newly generated M3 muscarinic receptor model suggest that these receptor segments lie adjacent to each other in the three-dimensional structure of class A GPCRs.

In the inactive state of the M3 receptor (in the absence of carbachol), only the V88C/Y543C double Cys mutant receptor showed a robust disulfide cross-linking signal (Fig. 2). This observation suggests that Val-88 and Tyr-543 directly face each other at the TM I/TM VII interface, consistent with the newly developed three-dimensional model of the M3 muscarinic receptor (Fig. 6B). This model suggests that the distance between the Cα atoms of Val-88 and Tyr-543 is about 9.6 Å. Although the distance between the Cα carbons of two Cys residues engaged in a disulfide bond usually ranges from about 3.8 to 6.8 Å (9, 39), disulfide cross-linking studies carried out with Cys-substituted versions of a bacterial chemoreceptor of known structure indicate that disulfide bonds can readily form when the two Cα carbons are less than ~12 Å apart (40).

Interestingly, carbachol activation of the V88C/Y543C double Cys receptor led to a concentration-dependent increase in the intensity of the disulfide cross-linking signal (increase in intensity of the 38-kDa band following digestion with factor Xa; Fig. 3), suggesting that Val-88 and Tyr-543 move closer to each other following M3 receptor activation. Mutational analysis of many different class A GPCRs suggests that the conserved Tyr7 residue plays a key role in receptor activation (28, 29, 41-43). In the inactive state of the receptor, Tyr7 is predicted to interact with a highly conserved Phe residue (position 7.60) located in helix 8 (11, 41, 42). A recent study provided evidence that this interaction is disrupted following light-induced activation of rhodopsin (42). Our disulfide cross-linking data therefore suggest that the loss of this interaction may be due to the activity-dependent change in the position of Tyr7 (Tyr-543 in the M3 muscarinic receptor).

Strikingly, following carbachol treatment, two additional double Cys mutant M3 receptors, A91C/L545C and A91C/S546C, also showed pronounced disulfide cross-linking (Fig. 2). In both cases, as seen with the V88C/Y543C receptor, the intensity of the cross-linking signals increased with increasing agonist concentrations (Fig. 3), indicating that the number of agonist-occupied receptors correlates well with the extent of disulfide cross-linking.

In contrast to carbachol, the muscarinic antagonist, atropine, did not stimulate disulfide cross-link formation in the case of the V88C/Y543C, A91C/L545C, and A91C/S546C mutant receptors (Fig. 4), consistent with the concept that the observed conformational changes are agonist-specific. Atropine, like all other classical muscarinic antagonists that have been tested so far, is able to act as an inverse agonist, being able to suppress constitutive signaling by wild-type or mutant muscarinic receptors (43-45). At present, it remains unclear why atropine failed to reduce the weak cross-linking signals observed with the V88C/Y543C, A91C/L545C, and A91C/S546C mutant receptors in the absence of carbachol.

In the inactive state of the M3 muscarinic receptor, the Cα atoms of Ala-91 and Leu-545 are predicted to be about 15.1 Å apart, as suggested by the M3 muscarinic receptor model. A similar distance (15.5 Å) lies between the Cα atoms of Ala-91 and Ser-546 (46). Fig. 6B also shows that Leu-545 and Ser-546, located at the C terminus of TM VII, face the lipid bilayer and TM VI, respectively, pointing away from Ala-91 (TM I). The ability of the muscarinic agonist, carbachol, to promote the formation of disulfide cross-links in the A91C/L545C and A91C/S546C mutant receptors suggests that activation of M3 receptors in their native membrane environment triggers major conformational changes at the cytoplasmic end of TM VII. A side view of the M3 muscarinic receptor (Fig. 6A) shows that Leu-545 and Ser-546 are located at approximately the same level within the TM receptor core as Ala-91. The most straightforward explanation for the disulfide cross-linking patterns observed with the A91C/L545C and A91C/S546C mutant receptors therefore is that carbachol activation leads to a rotational movement of the C-terminal end of TM VII (clockwise as viewed from the cytoplasm) that brings residues 545 and 546 within the cross-linking distance of position 91. One might argue that such a rotational movement would move Tyr-543 further away from Val-88, thus reducing the likelihood of disulfide cross-link formation between positions V88C and Y543C. One possible explanation for the observation that carbachol promoted rather than reduced disulfide cross-linking between these two residues is that the proposed rotational movement of the cytoplasmic end of TM VII follows an initial lateral movement of TM VII that moves Y543C closer to V88C.

TM VII, in contrast to TM I, contains several amino acids (e.g., Tyr-529 and Tyr-537 in the M3 muscarinic receptor) that are involved in the binding of muscarinic agonists (24, 25). Moreover, the endofacial segment of TM VII contains the highly conserved NPXXY motif (corresponding to Asn-539 in the rat M3 receptor; Fig. 1) that is known to be critically involved in receptor activation. The rhodopsin x-ray structure indicates that the Pro residue contained within the conserved NPXXY motif induces a pronounced kink in TM VII (11). A similar kink can be observed in the three-dimensional model of the M3 muscarinic receptor. Since proline kinks in TM helices are considered points of flexibility within otherwise rigid helical rods (12, 46), it is possible that the agonist-induced structural changes observed within the C-terminal segment of TM VII depend on the presence of the conserved TM VII Pro7 residue.
In contrast to the conclusions based on our in situ disulfide cross-linking experiments, a site-directed spin labeling study using double Cys rhodopsin mutants in solution suggested that light-induced activation of rhodopsin increases the distance between position 651.60 (located at the C terminus of TM I) and 3067.53 (located at the C terminus of TM VII) or 3107.57 (located between TM VII and helix 8) (47). Based on this observation, the authors proposed that rhodopsin activation results in a movement of the cytoplasmic portion of TM VII away from TM I (10, 47). There are several possible explanations for the discrepant conclusions drawn by Altenbach et al. (47) and those drawn from the results of the present study, suggesting that the cytoplasmic ends of TM I and VII move closer to each other following M₃ receptor activation. For example, the possibility exists that the discrepant conclusions drawn from the two studies are caused by the different experimental procedures used. Whereas the spectroscopic techniques used for studying conformational changes in rhodopsin measure the average of multiple possible receptor conformations (10), the disulfide cross-linking approach used in the present study allows the detection of agonist-induced receptor conformations that may be relatively short lived by “trapping” these conformations via disulfide bond formation (40, 48). Another possibility is that the structural mechanisms involved in receptor activation are not identical between rhodopsin and class A GPCRs activated by diffusible ligands. Finally, it is also possible that the differences observed between the two studies are caused by the fact that the disulfide cross-linking experiments were carried out with receptors being present in their native membrane environment, whereas the rhodopsin studies were performed with mutant rhodopsin molecules in the solution state. In the present study, the activity-dependent formation of disulfide cross-links was restricted to a rather small number of amino acids (Cys pairs). It is therefore unlikely that the observed disulfide cross-linking pattern was caused by a generalized increase in the mobility of TM VII. In any case, our findings emphasize the need to explore activity-dependent dynamic changes in receptor conformation by different (complementary) experimental techniques, preferably with receptors being present in their native membrane environment.

The high resolution x-ray structure of bovine rhodopsin indicates that TM VII is followed by another helical segment, referred to as helix 8, that lies nearly perpendicular to TM VII (11). Several lines of evidence suggest that GPCR activation leads to a structural rearrangement of helix 8 (37, 41, 49–54). Moreover, studies with different class A GPCRs including rhodopsin reveal that helix 8 contains amino acids that are important for G protein recognition and activation (8, 55–57). One possible scenario therefore is that the activity-dependent structural changes observed at the C terminus of TM VII are propagated to the adjacent helix 8 region, allowing helix 8 to contribute to productive receptor/G protein interactions.

Fig. 7 shows an intracellular view of the cytoplasmic surface of the M₃ muscarinic receptor, highlighting the structural changes that accompany M₃ receptor activation, as predicted by the results of our in situ disulfide cross-linking studies. We previously presented data suggesting that agonist activation moves the cytoplasmic end of TM VI closer to that of TM V (22). In addition, a recent disulfide cross-linking study using double Cys mutant M₃ receptors containing Cys substitutions at the intracellular ends of TM III and VI suggested that the cytoplasmic end of TM VI undergoes an agonist-dependent clockwise rotational movement, consistent with previous biochemical and biological studies carried out with rhodopsin (15) and the β₂-adrenergic receptor (17, 18, 35). The results of the present study support the concept that the cytoplasmic end of TM VII also undergoes an activity-dependent rotational movement and, at the same time, moves closer to the cytoplasmic end of TM I. It remains unclear at present whether all of these conformational changes occur in a concerted fashion or whether a primary structural change (e.g., the reorientation of TM VI) triggers secondary changes in the structure and orientation of other TM helices. In any case, our in situ disulfide cross-linking data are consistent with the concept that agonist activation of the M₃ muscarinic receptor opens a cleft on the intracellular receptor surface that increases the accessibility of various residues located at the cytoplasmic ends of different TM helices including TM III, VI, and VII (Fig. 7). These activity-dependent changes are predicted to promote receptor binding to heterotrimERIC G proteins, ultimately triggering productive receptor/G protein coupling.

In sum, our findings provide novel insights into the activity-dependent conformational changes occurring in a GPCR activated by a diffusible ligand. Our results indicate that the applied in situ disulfide cross-linking strategy, combined with molecular modeling studies, represents a powerful approach for studying activity-dependent changes in GPCRs present in their native membrane environment. Systematic application of this strategy involving the entire TM receptor core should eventually lead to a refined model of the dynamic structural changes that convert a nonrhodopsin GPCR from its resting into its activated state. Given the high structural homology found among class A GPCRs, our results should also be relevant for other members of this receptor superfamily.

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Pronounced Conformational Changes following Agonist Activation of the M₃ Muscarinic Acetylcholine Receptor
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