Distinct Structural Changes in a G Protein-coupled Receptor Caused by Different Classes of Agonist Ligands*

The activity of G protein-coupled receptors can be modulated by different classes of ligands, including agonists that promote receptor signaling and inverse agonists that reduce basal receptor activity. The conformational changes in receptor structure induced by different agonist ligands are not well understood at the molecular level. Given the high degree of structural homology found among most G protein-coupled receptors, our findings should be of broad general relevance.

The superfamily of G protein-coupled receptors (GPCRs) represents the largest group of cell surface receptors found in nature (1–5). GPCR-dependent signaling pathways play critical roles in regulating an extraordinarily large number of important physiological functions. The major structural hallmark shared by all GPCRs is a transmembrane (TM) core formed by a bundle of seven TM helices (TM I–VII) (1–4) that are connected by three intracellular and three extracellular loops. Drugs acting on specific GPCRs are of great therapeutic relevance, representing ~40% of drugs in current clinical use (4).

A better understanding of how GPCRs function at the molecular level is considered essential for the development of novel classes of clinically useful drugs. The structural features of the ligand binding and G protein coupling domains have been studied extensively for a large number of GPCRs (1–4, 6). Likewise, the conformational changes involved in the activation of the photoreceptor rhodopsin have been mapped in considerable detail (7–11). In contrast, relatively little is known about the sequence of molecular events that trigger the activation of GPCRs that bind diffusible ligands such as neurotransmitters and hormones.

According to their pharmacological characteristics, GPCR ligands are currently classified into agonists, neutral antagonists, and inverse agonists (12–14). Inverse agonists are drugs that can reduce GPCR-mediated G protein activation observed in the absence of ligands (basal GPCR activity). In fact, accumulating evidence suggests that most classic GPCR antagonists need to be reclassified as inverse agonists (12–14). At present, little is known about the nature of the structural changes that inverse agonists induce in their target receptors. Biophysical studies carried out with different adrenergic receptor subtypes strongly suggest that adrenergic agonists endowed with different efficacies, including inverse agonists, induce or stabilize different receptor conformations (15–20). However, how these various conformations differ at the molecular level remains unclear at present.

To shed light on this issue, we have used the rat M₃ muscarinic acetylcholine receptor, a prototypic class I GPCR (21, 22), as a model system. To monitor ligand-induced changes in receptor structure, we employed an in situ disulfide cross-linking strategy that allows the detection of disulfide bond formation between Cys residues that are adjacent to each other in the three-dimensional structure of the receptor (23–26). One major advantage of this strategy is that ligand-dependent conformational changes can be detected in receptors present in their native membrane environment, without the need for any receptor purification and reconstitution steps.

In this study, we examined whether different classes of muscarinic ligands (full versus inverse muscarinic agonists) had different effects on the relative orientation of helix 8 relative to the C terminus of TM I (Fig. 1). Helix 8 represents a cytoplasmic α-helical extension of TM VII to which it is connected via a short linker sequence (Fig. 1) (27). Considerable evidence suggests that helix 8 plays an important role in productive receptor/G protein coupling (6, 28–31). High resolution structural data of bovine rhodopsin (27), complimented by biophysical
and biochemical studies (11), indicate that several residues contained within helix 8 are located close to the cytoplasmic end of TM I. We therefore hypothesized that Cys residues substituted into this segment of TM I might serve as useful reporters to detect potential ligand-induced movements of helix 8 in disulfide cross-linking studies.

Specifically, we introduced pairs of Cys residues into a modified version of the M3 muscarinic receptor that lacked most native Cys residues and contained two factor Xa cleavage sites within the third intracellular loop (this receptor is referred to as “M3(3C)-Xa” receptor throughout this study; see “Experimental Procedures” for details; Fig. 1). Previous studies have shown that the M3(3C)-Xa receptor exhibits ligand binding and G protein coupling properties similar to the wild-type M3 muscarinic receptor (35). We generated 20 double Cys mutant M3 receptors, all of which contained one Cys substitution within the cytoplasmic end of TM I (Ala591–Asn595) and a second one within the N-terminal segment of helix 8 (Lys548–Arg551; Fig. 1).

Disulfide cross-linking studies using membranes prepared from transfected COS-7 cells showed that muscarinic agonists and inverse muscarinic agonists had different effects on the efficiency of disulfide bond formation in specific double Cys mutant M3 receptors. In conjunction with a three-dimensional model of the M3 muscarinic receptor, this study provides the first piece of direct structural information as to how the receptor conformations induced (or stabilized) by GPCR agonists and inverse GPCR agonists differ from each other at the molecular level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbamylcholine chloride (carbachol), acetylcholine bromide, atropine sulfate, N-methylscopolamine bromide, cupric sulfate (CuSO4), 1,10-phenanthroline, N-ethylmaleimide, and mammalian protease inhibitor mixture were purchased from Sigma. [3H]NMS (82.0 Ci/mmol) and myo-[3H]inositol (20 Ci/mmol) were obtained from PerkinElmer Life Sciences. Factor Xa protease and digitonin were from Roche Applied Science. Precast Novex Tris-glycine polyacrylamide gels and SeeBlue Plus 2 prestained molecular mass standards were purchased from Invitrogen. Hybond™ ECL™ nitrocellulose membranes, anti-rabbit IgG antibody conjugated to horseradish peroxidase, ECL™ detection reagents, and Hyperfilm™ ECL™ chemiluminescence film were obtained from Amersham Biosciences. The β-actin antibody (human) used was from Cell Signaling. All other reagents used were of the highest grade commercially available. CuSO4 was mixed with 1,10-phenanthroline at a molar ratio of 1:3 (32). The concentrations indicated in the text for the Cu(II)-(1,10-phenanthroline)3 complex refer to molar copper concentrations.

**Construction of Cys-substituted Mutant M3 Muscarinic Receptors**—All Cys substitutions were introduced into a pCD-based expression plasmid coding for a modified version of the rat M3 muscarinic receptor referred to as M3(3C)-Xa (35) (Fig. 1). The M3(3C)-Xa receptor construct contains an N-terminal hemagglutinin epitope tag and lacks all five potential N-terminal N-glycosylation sites and most endogenous Cys residues, except for Cys140, Cys220, and Cys532, as described previously (35). In addition, the central portion of the i3 loop (Ala274–Lys289) was replaced by two factor Xa cleavage sites. Cys residues were substituted into the M3(3C)-Xa construct by using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The identity of all mutant receptors was confirmed by sequencing the entire receptor coding sequences.

**Transient Expression of Cys-substituted Mutant M3 Muscarinic Receptors in COS-7 Cells**—The M3(3C)-Xa receptor construct and all M3(3C)-Xa-derived Cys-substituted mutant receptors 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. About 24 h prior to transfections, ~1 × 106 cells were seeded into 100-mm dishes. Cells were transfected with 4 μg/dish of receptor plasmid DNA using the Lipofectamine Plus kit (Invitrogen), according to the manufacturer’s instructions. To achieve higher receptor expression levels, transfected cells were incubated with 1 μM atropine for the last 24 h of culture, as described previously (23–26).

**Preparation of Membranes from Transfected COS-7 Cells**—To prepare cell membranes, COS-7 cells were harvested ~48 h after transfections. Initially, cells were washed twice (10 min each wash) with 10 ml of ice-cold phosphate-buffered saline, pH 7.4. This washing step was included to ensure the complete removal of atropine that was present in the culture medium during the last 24 h of culture. 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 the plates and homogenized using a Polytron tissue homogenizer (setting 5; 20 s). After a 15-min centrifugation at 20,000 × g (4 °C), membrane pellets were resuspended in buffer A (1 ml/100-mm dish), rehomogenized, frozen on dry ice, and stored at −70 °C until use.

Protein concentrations were determined using the Micro BCA protein assay reagent kit using bovine serum albumin as a standard.

**Radioligand Binding Studies**—The NMS and carbachol binding properties of the receptors analyzed in this study were determined in radioligand binding assays. [3H]NMS saturation and carbachol competition binding studies were carried out essentially as described previously (24). In brief, membrane homogenates prepared from transfected COS-7 cells (~10–20 μg of membrane protein per tube) were incubated with the muscarinic agonist/inverse agonist, [3H]NMS, for 2 h (22 °C) in 1 ml of buffer A. In saturation binding assays, six different [3H]NMS concentrations ranging from 20 to 3,000 pM were employed. In competition binding assays, a fixed concentration of [3H]NMS (500 pM) was present in all tubes, using 10 different concentrations of the cold competitor, carbachol. Nonspecific binding was assessed as binding remaining in the presence of 1 μM atropine. Binding reactions were terminated by rapid filtration over GF/C Brandel filters, followed by three washes (~4 ml per wash) with ice-cold distilled water. The amount of radioactivity that remained bound to the filters was determined by liquid scintillation spectrometry. To analyze the
saturation and carbachol competition binding data, the nonlinear curve-fitting program Prism 4.0 (GraphPad) was used.

Measurement of Receptor-mediated Phosphatidylinositol Hydrolisis—To study whether the different mutant receptors analyzed in this study retained the ability to activate G proteins, we determined carbachol-mediated increases in intracellular inositol monophosphate (IP) levels. Transiently transfected COS-7 cells grown in 6-well plates were labeled with myo-[3H]inositol (3 μCi/ml) for the last 20–24 h of culture. On the day of the assay, the labeled cells were incubated with increasing concentrations of carbachol in the presence of 10 mM LiCl for 1 h at 37 °C. The IP fraction was isolated and quantitated as described previously (24). The nonlinear curve-fitting program Prism 4.0 (GraphPad) was used to derive EC<sub>50</sub> and E<sub>max</sub> values from carbachol concentration-response curves.

Disulfide Cross-linking, Solubilization, and Factor Xa Digestion of Cys-Substituted M<sub>3</sub> Muscarinic Receptors—Disulfide cross-linking studies were carried out as described in detail previously (24). In brief, receptor-containing membranes prepared from one 100-mm dish (~1 mg of protein) were suspended in 1 ml of buffer A containing 25 μM Cu(II)-phenanthroline, either in the presence or in the absence of different muscarinic ligands. Reactions were carried out for 10 min at room temperature (22 °C) and then terminated by the addition of EDTA and N-ethylmaleimide (10 mM each), followed by a 10-min incubation on ice. Membrane proteins were then solubilized by incubating samples with 1.2% digitonin, as described by Han et al. (24). Receptor-containing membrane lysates (~15 μg of protein) were then treated in 30 μl of factor Xa digestion buffer with factor Xa protease (final concentration, 0.1 μg/μl) at room temperature for 16 h. Reactions were terminated by addition of 1 μl of mammalian protease inhibitor mixture (1:25 dilution; see Ref. 24). Samples were then stored at −70 °C or used directly for SDS-PAGE.

Urea Treatment of Receptor-containing Membranes—To inactivate heterotrimeric G proteins, membranes prepared from transfected COS-7 cells were treated with a high concentration of urea (23, 33). Receptor-containing membranes were incubated on ice for 30 min either in the presence or in the absence of 5 μM urea and then used for disulfide cross-linking studies, exactly as described by Ward et al. (23).

Western Blot Analysis—SDS-PAGE was carried out as described previously (23). Factor Xa-treated membrane lysates were incubated with Laemmli loading buffer for 30 min at 37 °C, either under nonreducing or under reducing conditions (in the absence or presence of 50 mM DTT, respectively). Samples were then immediately loaded onto 10–20% Tris-glycine polyacrylamide gels and run at 125 V in the presence of 0.1% SDS. Western blotting studies were performed essentially as described (24), using a rabbit polyclonal anti-M<sub>3</sub> receptor antibody directed against the C-terminal 18 amino acids of the rat M<sub>3</sub> receptor sequence (34). M<sub>3</sub> receptor proteins were visualized by using enhanced chemiluminescence detection reagents and autoradiography. To quantitate the intensities of immunoreactive bands, we used scanning densitometry employing the program NIH Image.
bated in the presence of a low concentration (25 μM) of the oxidizing agent Cu(II)-phenanthroline. Incubations were carried out either in the presence or in the absence of different muscarinic ligands. Following complete digestion with factor Xa, receptor proteins were visualized via Western blotting (nonreducing conditions), using an antibody directed against the C terminus of the M3 receptor (Fig. 1; see “Experimental Procedures” for details). Under these conditions, the appearance of a full-length receptor band (38 kDa) is indicative of successful disulfide cross-linking (23–26).

An initial screening of the 20 double Cys mutant receptors showed that two of the mutant receptors, A91C/T549C and F92C/F550C, gave a pronounced cross-linking signal even in the absence of ligand (Fig. 2A). Strikingly, incubation with the full muscarinic agonist, carbachol (1 mM), led to a significant reduction in the intensity of this signal in both mutant receptors (Fig. 2A). Six additional mutant receptors also displayed some degree of disulfide cross-linking (V94C/T549C, V94C/F550C, V94C/R551C, N95C/T549C, N95C/F550C, and N95C/R551C; Fig. 2A). However, the intensity of these cross-linking signals (~38-kDa bands) remained unaffected by carbachol (1 mM) treatment (Fig. 2A). A three-dimensional model of the M3 muscarinic receptor (24) suggests that the two Cys residues contained in each of these mutant receptors are relatively far apart (>12 Å) and do not face each other in the inactive state of the receptor (also see Fig. 9), suggesting that the relatively faint cross-linking signals observed with these mutant receptors may be due to the presence of partially unfolded (or misfolded) receptor subpopulations.

Fig. 3 shows that all 20 double Cys mutant receptors could be detected readily in Western blotting studies, consistent with the results of the radioligand binding experiments (Table 1). In this case, samples were treated in exactly the same fashion as described above (Fig. 2A), except that the factor Xa digestion step was omitted. Taken together, these data suggest that the absence of disulfide cross-linking signals in Fig. 2A is not caused by the poor expression of specific mutant receptor proteins.

After the initial screen of the 20 double Cys mutant receptors (Fig. 2A), the A91C/T549C and F92C/F550C receptors, which showed carbachol-sensitive disulfide cross-linking, were studied in greater detail. By using scanning densitometry of immunoreactive bands, we estimated that about 20–30% of all A91C/
T549C and F92C/F550C receptors underwent disulfide cross-linking under control conditions in the absence of ligands. Fig. 2B demonstrates that increasing concentrations of carbachol led to a progressive impairment in disulfide bond formation in both receptors (see Table 2 for a quantitative analysis of carbachol-mediated inhibition of disulfide bond formation in the A91C/T549C and F92C/F550C receptors). The full-length receptor bands were no longer detectable when Western blotting studies were carried out under reducing conditions (in the presence of 50 mM DTT; data not shown; note that actual Western blots involving the use of carbachol and the A91C/T549C and F92C/F550C mutant receptors run under both nonreducing and reducing conditions are included in Fig. 5 for control purposes). These observations suggest that the full-length A91C/T549C and F92C/F550C receptor bands that could be observed after factor Xa treatment under nonreducing conditions were because of the formation of disulfide bridges rather than incomplete digestion by factor Xa.

The A91C/T549C and F92C/F550C Receptors Do Not Form Intermolecular Cross-links—Like other GPCRs (38), the M₃ muscarinic receptor has been shown to form dimers or oligomers (39, 40). To exclude the possibility that the disulfide bonds formed by the A91C/T549C and F92C/F550C receptors were because of inter- rather than intra-molecular interactions, we carried out an additional set of cross-linking studies. We coexpressed M₃(3C)-Xa-based mutant receptors containing the A91C and F92C single point mutations with M₃(3C)-Xa-derived constructs containing the T549C and F550C single point mutations, respectively. In this case, no specific disulfide cross-linking signal was observed, either in the absence or in the presence of carbachol (1 mM; Fig. 4, A and B, top rows). The A91C/T549C and F92C/F550C double Cys receptors gave cross-linking signals (full-length receptor bands) that were significantly reduced in intensity after treatment of receptor-expressing membranes with carbachol (1 mM; Fig. 4, A and B, top rows), in agreement with the results shown in Fig. 2. All Cys mutant receptors used for these studies could be detected readily in Western blotting studies when the factor Xa incubation step was omitted (Fig. 4, A and B, lower rows). Taken together, these observations support the concept that the Cys residues contained in the A91C/T549C and F92C/F550C constructs formed intra-molecular rather than inter-molecular disulfide bonds.

**Mucarinic Agonists and Inverse Mucarinic Agonists Exert Opposite Effects on Disulfide Bond Formation in the A91C/T549C and F92C/F550C Receptors**—We next examined the effects of several other muscarinic ligands on the disulfide cross-linking signals (~38-kDa bands) observed with the A91C/T549C and F92C/F550C receptors. Like carbachol, the full muscarinic agonist, oxotremorine-M (1 mM), inhibited the formation of disulfide cross-links in both receptors (Fig. 5). Strikingly, treatment of receptor-containing membrane preparations with atropine and NMS, two inverse muscarinic agonists, led to opposite effects on disulfide bond formation in the A91C/T549C and F92C/F550C receptors (Fig. 5). Traditionally, atropine and NMS have been considered classic muscarinic antagonists. However, recent studies have shown that these agents can suppress signaling by constitutively active mutant muscarinic receptors (36, 37), indicating that these ligands need to be reclassified as inverse muscarinic agonists. As shown in Fig. 5, atropine or NMS treatment (100 nM each) led to significantly enhanced disulfide cross-linking in the A91C/T549C and F92C/F550C receptors. When Western blotting studies were carried out under reducing conditions (in the presence of 50 mM DTT), the full-length receptor bands observed under nonreducing conditions were no longer detectable (Fig. 5), confirming the involvement of disulfide bridges. Additional studies indicated that NMS facilitated the formation of disulfide cross-links in these two receptors in a concentration-

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**TABLE 1**

Ligand binding and functional properties of double Cys mutant M₃ muscarinic receptors analyzed in this study

The indicated double Cys mutant M₃ muscarinic receptors were transiently expressed in COS-7 cells. All mutant receptors were derived from the M₃(3C)-Xa construct (see "Experimental Procedures"). Radioligand binding studies and functional assays (carbachol-mediated IP production) were carried out as detailed under "Experimental Procedures." Data are given as means ± S.E. from two to five independent experiments, each performed in duplicate.

| Receptor          | [³H]NMS binding | Carbachol binding, Kᵢ | Carbachol-induced IP production |
|-------------------|-----------------|------------------------|---------------------------------|
|                   | Kᵢ(pM) | B_max (pmol/mg protein) | Kᵢ(pM) | B_max (pmol/mg protein) | EC₅₀ (carbachol) | E_max fold increase above basal |
| M₃(3C)-Xa         | 329 ± 2 | 6.1 ± 1.8               | 23.1 ± 2.0 | 63 ± 3 | 10.8 ± 0.3 |
| A91C/K548C        | 449 ± 22 | 1.2 ± 0.1              | 4.95 ± 0.43 | 38 ± 1 | 7.5 ± 0.2 |
| A91C/T549C        | 667 ± 29 | 2.2 ± 0.3              | 5.94 ± 1.47 | 21 ± 1 | 5.6 ± 0.1 |
| A91C/F550C        | 500 ± 14 | 1.4 ± 0.3              | 4.47 ± 0.62 | 102 ± 1 | 15.5 ± 0.2 |
| A91C/R551C        | 581 ± 61 | 2.1 ± 0.4              | 6.57 ± 3.32 | 30 ± 1 | 9.3 ± 0.1 |
| F92C/K548C        | 561 ± 75 | 2.0 ± 0.2              | 1.26 ± 0.05 | 40 ± 2 | 2.6 ± 0.1 |
| F92C/T549C        | 496 ± 99 | 4.6 ± 2.6              | 1.04 ± 0.20 | 35 ± 4 | 2.9 ± 0.1 |
| F92C/F550C        | 430 ± 28 | 4.2 ± 0.2              | 0.70 ± 0.10 | 43 ± 0.4 | 7.1 ± 1.7 |
| F92C/R551C        | 303 ± 29 | 6.3 ± 0.6              | 0.99 ± 0.19 | 90 ± 1 | 4.4 ± 0.1 |
| K93C/K548C        | 373 ± 72 | 10 ± 0.1               | 9.14 ± 0.49 | 191 ± 80 | 2.6 ± 0.2 |
| K93C/T549C        | 345 ± 6 | 9.1 ± 0.1              | 5.41 ± 1.45 | 197 ± 33 | 3.2 ± 0.3 |
| K93C/F550C        | 337 ± 11 | 4.2 ± 0.2              | 3.22 ± 0.37 | 66 ± 15 | 7.8 ± 2.3 |
| K93C/R551C        | 347 ± 18 | 4.6 ± 0.4              | 6.25 ± 1.97 | 134 ± 1 | 16.5 ± 0.4 |
| V94C/K548C        | 318 ± 8 | 7.3 ± 0.2              | 13.8 ± 0.8 | 67 ± 14 | 22.0 ± 4.4 |
| V94C/T549C        | 418 ± 55 | 3.8 ± 0.3              | 7.69 ± 1.54 | 40 ± 7 | 6.8 ± 1.7 |
| V94C/F550C        | 396 ± 1 | 3.1 ± 0.5              | 6.82 ± 2.91 | 300 ± 120 | 23.5 ± 0.1 |
| V94C/R551C        | 301 ± 8 | 4.4 ± 0.4              | 4.68 ± 0.28 | 130 ± 15 | 10.0 ± 2.1 |
| N95C/K548C        | 270 ± 71 | 1.0 ± 0.2              | 5.52 ± 1.68 | 148 ± 5 | 13.8 ± 3.1 |
| N95C/T549C        | 286 ± 5 | 2.8 ± 0.7              | 4.60 ± 1.51 | 423 ± 163 | 25.9 ± 1.5 |
| N95C/F550C        | 241 ± 7 | 1.6 ± 0.3              | 3.59 ± 0.77 | 120 ± 1 | 20.5 ± 1.9 |
| N95C/R551C        | 300 ± 46 | 1.9 ± 0.2              | 3.41 ± 0.13 | 12,100 ± 810 | 9.8 ± 0.8 |

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**FIGURE 2.** Carbachol inhibits the formation of disulfide bonds in the A91C/T549C and F92C/F550C double Cys mutant M₃ muscarinic receptors. A, membranes prepared from COS-7 cells expressing the indicated double Cys mutant M₃ muscarinic receptors were processed for disulfide cross-linking studies as described under “Experimental Procedures.” Receptors were oxidized with Cu(II)-phenanthroline (25 μM) in the absence or the presence of the muscarinic agonist, CCh (1 mM), digested with factor Xa, and subjected to Western blotting analysis (nonreducing conditions), using the anti-M₃ antibody. All bands shown correspond to the 38-kDa full-length receptor species, which is indicative of successful disulfide cross-linking (23–26). Note that two of the investigated receptors, A91C/T549C and F92C/F550C, gave pronounced cross-linking signals, the intensity of which was significantly reduced in the presence of CCh. B, CCh reduces disulfide cross-link formation in the A91C/T549C and F92C/F550C receptors in a concentration-dependent fashion. Except for the use of different CCh concentrations, all other experimental conditions were the same as in A. In the experiment shown here, the cross-linking signal displayed by the F92C/F550C receptor was reduced by 36% at the highest CCh concentration used (10 mM), as compared with the control sample (no CCh treatment). The bands shown correspond to the 38-kDa full-length receptor species that can be observed after successful disulfide cross-linking (23–26). All Western blots shown are representative of three independent experiments. In each individual experiment, identical amounts of proteins were loaded in each lane (−5 μg/lane).

**TABLE 2** Quantification of the effects of carbachol and NMS on disulfide bond formation in the A91C/T549C and F92C/F550C mutant receptors

Membranes were prepared from COS-7 cells expressing the A91C/T549C and F92C/F550C mutant receptors and processed for Western blotting studies (nonreducing conditions), as described under “Experimental Procedures.” Disulfide cross-linking studies were carried out either in the absence of ligands or in the presence of increasing concentrations of the full muscarinic agonist, carbachol, or the inverse muscarinic agonist, NMS. The intensities of immunoreactive bands corresponding to cross-linked receptors were determined by scanning densitometry (NIH ImageJ). Data were analyzed by using the nonlinear curve-fitting program Prism 4.0 (GraphPad). In each individual experiment, the extent of disulfide cross-linking observed in the absence of ligands was set equal to 100%. Data are given as means ± S.E. of three independent experiments.

| Receptor                  | Carbachol | NMS |
|---------------------------|-----------|-----|
|                           | Eₘₐₓ[μM]| IC₅₀[μM]| Eₘₐₓ[μM]| EC₅₀[μM]|
|                           | % control (100%) | % control (100%) | pM | pM |
| A91C/T549C                | 48 ± 2  | 818 ± 302 | 244 ± 2 | 50 ± 16 |
| F92C/F550C                | 57 ± 1  | 562 ± 241 | 192 ± 2 | 195 ± 90 |

a Maximum degree of disulfide cross-linking is compared with cross-linking in the absence of ligands (control; 100%).

b Carbachol concentration at which the inhibition of disulfide cross-linking corresponds to half of the Eₘₐₓ is shown.

c NMS concentration at which the stimulation of disulfide cross-linking corresponds to half of the Eₘₐₓ is shown.

dependent fashion (Fig. 6; see Table 2 for a quantitative analysis of NMS-mediated stimulation of disulfide bond formation in the A91C/T549C and F92C/F550C receptors).

**Ligand-dependent Changes in Disulfide Cross-linking Patterns Remain Unaffected by Inactivation of HeterotrimERIC G Proteins—**To exclude the possibility that the ligand-induced inhibitory or stimulatory effects on disulfide bond formation observed with the A91C/T549C and F92C/F550C mutant receptors were affected by precoupling of the receptors to heterotrimERIC G proteins, we carried out additional cross-linking experiments. Specifically, we incubated receptor-containing membranes with a high concentration (5 μM) of the chao-
tropic agent urea. Studies with the M₃’(3C)-Xa receptor construct and other GPCRs have shown that this treatment leads to the almost complete inactivation or removal of heterotrimERIC G proteins, without affecting the function of the uncoupled receptors (23, 33, 41). After incubation of membranes expressing the A91C/T549C and F92C/F550C receptors with urea (5 μM, 30 min on ice), samples were treated with Cu(II)-phenanthroline, solubilized, digested with factor Xa, and subjected to Western blotting analysis (nonreducing conditions). We found that urea treatment had no significant effect on the ability of the agonist, carbachol (1 mM), to inhibit disulfide cross-linking in the A91C/T549C and...
F92C/F550C receptors (Fig. 7). Similarly, the ability of the inverse agonist, NMS (100 nM), to promote disulfide cross-linking in these two receptors remained unaffected after incubation with urea (Fig. 7). These findings suggest that the observed ligand-specific effects on disulfide cross-linking were caused by conformational changes intrinsic to the A91C/T549C and F92C/F550C receptor proteins, rather than by disruption of precoupled receptor-G protein complexes.

Atropine and NMS Act as Inverse Agonists in a Functional Assay—To examine whether atropine and NMS acted as inverse agonists at the A91C/T549C and F92C/F550C receptors, we carried out a series of functional studies (IP assays). Incubation of cells expressing either of the two receptors (A91C/T549C or F92C/F550C) with atropine or NMS (10 μM each) led to a significant reduction in basal IP accumulation (Fig. 8), consistent with the concept that atropine and NMS act as inverse muscarinic agonists at these receptors. Moreover, atropine and NMS also suppressed basal IP accumulation in cells transfected with the M3′(3C)-Xa receptor from which the two double Cys mutant receptors were derived (Fig. 8). However, the A91C/T549C and F92C/F550C mutant receptors exhibited greater basal activity than the M3′(3C)-Xa receptor (Fig. 8), suggesting that the introduced Cys substitutions...
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allowed a somewhat larger proportion of unliganded receptors to adopt the active state.

DISCUSSION

During the past few years, we have applied an in situ disulfide cross-linking strategy to gain insight into agonist-induced conformational changes in the M₃ muscarinic receptor, a prototypical class I GPCR. In a recent study (25), we demonstrated that the binding of full muscarinic agonists leads to a structural change adjacent to the acetylcholine-binding site that increases the proximity of the extracellular segments of TM III and VII. Most likely, this agonist-induced structural change represents one of the early conformational events leading to the more pronounced structural changes predicted to occur on the intracellular receptor surface (for recent reviews, see Refs. 11 and 42), ultimately triggering productive receptor/G protein coupling.

In this study, we demonstrated that muscarinic agonists inhibited disulfide bond formation in Cys-substituted mutant M₃ receptors. In fact, this is the first time that we observed agonist-mediated inhibition of disulfide bond formation in Cys-substituted mutant M₃ receptors. The A91C/T549C and F92C/F550C receptors retained the ability to bind muscarinic ligands with high affinity and to couple to G proteins with high efficiency, indicating that the different Cys substitutions did not interfere with proper receptor folding.

Fig. 9 shows a three-dimensional model of the cytoplasmic surface of the M₃ muscarinic receptor, established via homology modeling using the high resolution x-ray structure of bovine rhodopsin as a template (24, 27). Highlighted in yellow in Fig. 9 are the key positions involved in ligand-modulated disulfide cross-linking, Ala₉₁ and Phe₉₂ at the cytoplasmic end of TM I, and Thr₅₄⁹ and Phe₅₅₀ at the N terminus of helix 8,
respectively. Fig. 9 indicates that residues 91/92 lie adjacent to residues 549/550 in the inactive state of the M₃ receptor (estimated distance between Cα atoms, ~9–11 Å). Our data therefore strongly support a model in which full muscarinic agonists trigger a separation of the N-terminal segment of helix 8 from the cytoplasmic end of TM I, thus preventing the formation of disulfide cross-links between Cys residues introduced at positions 91/549 and 92/550.

Our findings are in agreement with the outcome of a site-directed spin labeling study using Cys-substituted purified mutant rhodopsin proteins (46). In this study, Yang et al. (46) demonstrated that illumination increased the distance between spin labels attached to a Cys residue introduced at the cytoplasmic end of TM I (H65C) and a naturally occurring Cys residue (Cys316) present in the middle of helix 8. It is therefore likely that the observed separation between helix 8 and the cytoplasmic end of TM I is a feature associated with the activation of other class I GPCRs.

Helix 8 is connected to the C terminus of TM VII via a short linker sequence (27) (Fig. 1). Studies with the M₃ muscarinic receptor (24) and bovine rhodopsin (11, 47, 48) have demonstrated that GPCR activation is associated with conformational changes within the cytoplasmic end of TM VII. We recently presented disulfide cross-linking data indicating that agonist-induced M₃ receptor activation leads to a conformational change that increases the proximity between the cytoplasmic ends of TM I and VII (24). In addition, the observed disulfide cross-linking pattern suggested that M₃ receptor activation is associated with a rotational movement of the cytoplasmic end of TM VII (24). A likely scenario therefore is that the agonist-induced conformational changes in TM VII are propagated to helix 8 via the short linker sequence connecting these two receptor regions (Fig. 1).

The concept that TM VII and helix 8 are structurally and functionally interconnected is also supported by biophysical and biochemical studies carried out with bovine rhodopsin. The crystal structure of bovine rhodopsin (27) indicates that Tyr306 in TM VII (corresponds to Tyr543 in the M₃ receptor) forms a hydrophobic interaction with Phe313 in helix 8 (corresponds to Phe550 in the M₃ receptor). These two aromatic residues are highly conserved among class I GPCRs. A mutant version of rhodopsin in which these two sites were linked via a disulfide bond was unable to activate G proteins, suggesting that rhodopsin activation requires a separation between these two residues (48).

Studies with different GPCRs have shown that helix 8 contains several residues that play a critical role in the efficiency of receptor/G protein interactions (6, 28–31). It is therefore likely that the agonist-induced reorientation of helix 8 enables specific helix 8 residues to productively interact with heterotrimeric G proteins. It is also possible that the agonist-induced separation between the N-terminal segment of helix 8 and the cytoplasmic end of TM I removes structural restraints, allowing G proteins to productively interact with other functionally critical receptor domains.

Currently, GPCR ligands are subdivided into three major classes, agonists, neutral antagonists, and inverse agonists (12–14). Understanding the structural basis underlying this functional diversity of ligands is considered essential for the development of novel classes of therapeutically useful drugs (12–14). Although agonist-induced structural changes have been mapped in considerable detail in at least some class I GPCRs (see Refs. 11 and 42 for recent reviews), little is known about the structural details of the receptor conformations induced or stabilized by inverse agonists.

Interestingly, Gether et al. (15) showed that agonist treatment of purified β₂-adrenergic receptors carrying a fluorescence tag led to a decrease in fluorescence, whereas inverse agonists induced a small increase in base-line fluorescence. Moreover, using a fluorescence-based approach, Vilardaga et al. (17) recently demonstrated that inverse adrenergic agonists induce structural changes in the α₂A-adrenergic receptor that differ in character and kinetics from those caused by agonist ligands. Although these studies provided important novel mechanistic insights, they did not reveal any structural details regarding the molecular nature of the underlying conformational differences. In contrast, we here provide the first piece of detailed structural information indicating how the conformational changes induced by muscarinic agonists and inverse muscarinic agonists differ from each other at the molecular level.

In this study, we used atropine and NMS as inverse muscarinic agonists. These agents, historically considered prototypic muscarinic antagonists, have been reclassified as inverse muscarinic agonists, because they are able to reduce receptor/G protein coupling that can be observed under certain experimental conditions in the absence of agonist ligands (36, 37) (Fig. 8). In previous studies (23, 24, 26), atropine treatment did not lead to significant changes in the efficiency of disulfide bond formation in any of the investigated double Cys mutant M₃ muscarinic receptors. However, in this study, we demonstrated that atropine and NMS enhanced disulfide bond formation in the A91C/T549C and F92C/F550C mutant receptors. Interestingly, these two receptors showed reduced disulfide cross-linking in the presence of muscarinic agonists (see above). These findings strongly suggest that inverse muscarinic agonists, in contrast to muscarinic agonists, decrease the distance between the cytoplasmic end of TM I and the N-terminal portion of helix 8 (Fig. 9).

In summary, we demonstrated that muscarinic agonists and inverse muscarinic agonists induce distinct conformational changes in the M₃ receptor protein. Our study provides the first piece of direct structural information as to how these conformational changes differ from each other at the molecular level. Class I GPCRs are known to share a considerable degree of structural homology, which is particularly high among receptors activated by biogenic amine ligands, including the muscarinic and adrenergic receptors. It is therefore likely that the findings reported here are also applicable to other class I GPCRs.

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