Use of an in Situ Disulfide Cross-Linking Strategy To Study the Dynamic Properties of the Cytoplasmic End of Transmembrane Domain VI of the M_3 Muscarinic Acetylcholine Receptor†

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Received July 28, 2005; Revised Manuscript Received November 24, 2005

ABSTRACT: The ligand-induced activation of G protein-coupled receptors (GPCRs) is predicted to involve pronounced conformational changes on the intracellular surface or the receptor proteins. A reorientation of the cytoplasmic end of transmembrane domain VI (TM VI) is thought to play a key role in GPCR activation and productive receptor/G protein coupling. Disulfide cross-linking studies with solubilized, Cys-substituted mutant versions of bovine rhodopsin and the M_3 muscarinic acetylcholine receptor suggested that the cytoplasmic end of TM VI is conformationally highly flexible, even in the absence of activating ligands (Farrens, D. L., et al. (1996) *Science 274*, 768−770; Zeng, F. Y., et al. (1999) *J. Biol. Chem. 274*, 16629−16640). To test the hypothesis that the promiscuous disulfide cross-linking pattern observed in these studies was caused by the use of solubilized receptor proteins endowed with increased conformational flexibility, we employed a recently developed in situ disulfide cross-linking strategy that allows the detection of disulfide bonds in Cys-substituted mutant M_3 muscarinic receptors present in their native membrane environment. Specifically, we used membranes prepared from transfected COS-7 cells to analyze a series of double Cys mutant M_3 receptors containing one Cys residue within the sequence $K484^{6.29}$ to $S493^{6.38}$ at the cytoplasmic end of TM VI and a second Cys residue at the cytoplasmic end of TM III ($I169C^{3.54}$). This analysis revealed a disulfide cross-linking pattern that was strikingly more restricted than that observed previously with solubilized receptor proteins, both in the absence and in the presence of the muscarinic agonist, carbachol. Carbachol stimulated the formation of disulfide bonds in only two of the 10 analyzed mutant muscarinic receptors, $I169C^{3.54}/K484C^{6.29}$ and $I169C^{3.54}/A488C^{6.33}$, consistent with an agonist-induced rotation of the cytoplasmic end of TM VI. These findings underline the usefulness of analyzing the structural and dynamic properties of GPCRs in their native lipid environment.

G protein-coupled receptors (GPCRs)[1] play important roles in regulating the function of virtually all physiological processes. The human genome is predicted to contain nearly 1000 genes coding for distinct GPCRs (*1*−*3*). Thus, GPCRs form one of the largest gene families found in nature.

GPCRs are cell surface receptors which are activated by an extraordinarily diverse group of extracellular ligands including neurotransmitters, hormones, and sensory stimuli (*4*−*8*). Structurally, all GPCRs are composed of a bundle of seven transmembrane (TM) helices (TM I−VII) that are connected by alternating intracellular and extracellular loops (*4*−*8*; Figure 1).

Studies with various members of the GPCR superfamily have led to the identification of the structural elements determining the selectivity of ligand binding and G protein recognition (*4*−*8*). On the other hand, much less is known about the conformational changes that activating ligands induce in their target GPCRs. Collectively, biochemical and biophysical studies suggest that GPCR activation is characterized by the opening of a cleft on the intracellular side of the receptor that allows specific receptor residues to productively interact with heterotrimeric G proteins (*6*, *8*−*11*).

Currently, bovine rhodopsin, in its resting state, is the only GPCR for which high-resolution structural information is available (*12*, *13*). Rhodopsin is probably the most thoroughly studied GPCR, primarily due to its abundant expression in the retina and the fact that rhodopsin and mutant versions of rhodopsin can be expressed at high density in cultured cells and can be easily purified in a functional form (*11*). As a result, considerable progress has been made in elucidating the light-induced conformational changes in bovine rhodopsin (*10*, *11*, *14*). As recently reviewed by Hubbell et al. (*11*), light-induced activation of rhodopsin is

† This research was supported by the Intramural Research Program of the NIH, NIDDK.

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[1] Abbreviations: Cu−Phen; Cu(II)−(1,10-phenanthroline)$_3$; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GPCR, G protein-coupled receptor; i3 loop, the third intracellular loops of GPCRs; IP1, inositol monophosphate; [3H]NMS, *N*-[3H]M-methylscopolamine; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-T, PBS (pH 7.4) containing 0.05% Tween-20; PI, phosphatidyl inositol; SDS, sodium dodecyl sulfate; TM, transmembrane domain; TRH, thyrotropin-releasing hormone.

10.1021/bi051503q
Published on Web 12/24/2005

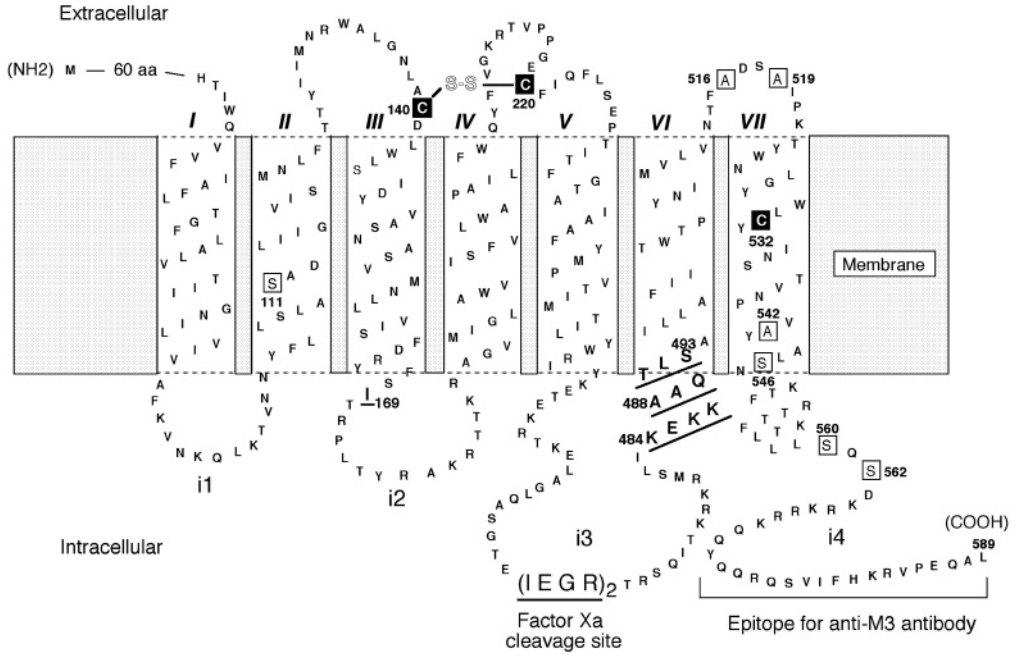

FIGURE 1: Structure of Cys-substituted mutant M_3 muscarinic receptors used in this study. All Cys substitutions were introduced into a modified version of the rat M_3 muscarinic receptor referred to as M3′(3C)-Xa (*26*−*28*). The M3′(3C)-Xa construct contains only three remaining Cys residues, C140, C220, and C532 (filled squares). All other native Cys residues were replaced with serine or alanine residues (open squares). In addition, the central portion of the i3 loop (A274−K469) was replaced with two adjacent factor Xa cleavage sites ($(IEGR)_2$; underlined). Ten consecutive residues located within the cytoplasmic end of TM VI (K484−S493; underlined) were replaced, one at a time, with Cys residues. In addition, all mutant receptor constructs contained the additional I169C substitution at the cytoplasmic end of TM III. A rabbit polyclonal antibody (anti-M3) directed against the indicated C-terminal receptor sequence (*35*) was used to detect the different mutant receptors via Western blotting. Numbers refer to amino acid positions in the rat M_3 muscarinic receptor sequence (*30*).

predicted to involve 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 (*15*−*17*).

Consistent with the structural information gathered from the analysis of bovine rhodopsin, studies with other GPCRs of the rhodopsin family (class I GPCRs), including the β_2-adrenergic receptor (*18*−*22*) and the thyrotropin-releasing hormone (TRH) type I receptor (*46*), suggest that an activity-dependent rotation or reorientation of the cytoplasmic end of TM VI is a structural feature shared by all class I GPCRs. Biochemical studies demonstrated that this conformational change plays a key role in receptor-mediated G protein activation (*16*, *17*, *21*). In the high-resolution X-ray structure of the inactive state of bovine rhodopsin, TM VI extends by two (*12*) or three (*13*) α-helical turns into the cytoplasm. Residues contained within the cytoplasmic end of TM VI are known to play key roles in G protein coupling and activation (*8*).

Besides other experimental strategies, disulfide cross-linking approaches have been successfully used to monitor activity-dependent changes in bovine rhodopsin (*10*, *11*, *23*) and other class I GPCRs including the M_3 muscarinic receptor (*26*, *28*) and the TRH type I receptor (*46*). These studies have led to important new insights into the conformational changes associated with the activation of class I GPCRs.

Early disulfide cross-linking studies yielded somewhat surprising results regarding the structural and dynamic properties of the cytoplasmic end of TM VI. A study with bovine rhodopsin in its inactive state found that Cys residues singly introduced at consecutive positions within the cytoplasmic end of TM VI ($247^{6.30}$−$251^{6.34}$; the superscripts indicate the amino acid positions according to the Ballesteros−Weinstein numbering system; ref *24*) were all able to form a disulfide bond with a Cys residue introduced at the cytoplasmic end of TM III ($I139C^{3.54}$) (*16*). This observation suggested that the cytoplasmic portion of TM VI is conformationally highly flexible, even in the inactive state of the receptor. A high degree of cross-linking promiscuity was also observed when a similar set of residues was subjected to disulfide cross-linking studies in the M_3 muscarinic receptor (*25*). This study (*25*) showed that Cys residues singly introduced at consecutive positions within the segment $484^{6.29}$−$491^{6.36}$ at the cytoplasmic end of TM VI were all able to form disulfide cross-links with a Cys residue introduced at the cytoplasmic end of TM III ($I169C^{3.54}$) in the unliganded state of the M_3 muscarinic receptor.

We note that the disulfide cross-linking study by Farrens et al. (*16*) was carried out with mutant rhodopsin proteins solubilized in dodecyl maltoside micelles. Similarly, Zeng et al. (*25*) analyzed digitonin-solubilized, Cys-substituted mutant M_3 receptors. We therefore speculated that the promiscuous disulfide cross-linking pattern observed in these two studies was caused by the presence of the mutant receptor proteins in detergent micelles rather than in their native membrane environment. To test this possibility, we used a recently developed novel in situ disulfide cross-linking strategy that allows the detection of disulfide bonds in Cys-substituted mutant M_3 muscarinic receptors present in their native membrane environment (*26*−*28*). Specifically, we employed this approach to analyze a series of Cys-substituted mutant M_3 receptors that had been characterized previously in the solution state (*25*). The analyzed double Cys mutant receptors contained one Cys residue within the sequence $K484^{6.29}$−$S493^{6.38}$ at the cytoplasmic end of TM VI and a

second Cys residue at the cytoplasmic end of TM III (I169C$^{3.54}$; Figure 1). All Cys mutations were introduced into a modified version of the rat M_3 muscarinic receptor (M3′-(3C)-Xa; *25*−*28*) that lacked most native Cys residues and contained two adjacent factor Xa cleavage sites within the third intracellular loop (i3 loop; Figure 1).

Analysis of this set of double Cys mutant M_3 receptors present in their native membrane environment revealed a disulfide cross-linking pattern that was strikingly less promiscuous than that observed previously with solubilized receptor proteins (*25*). We found that the muscarinic agonist, carbachol, stimulated the formation of disulfide bonds in only two of the 10 analyzed double Cys mutant M_3 muscarinic receptors, I169C$^{3.54}$/K484C$^{6.29}$ and I169C$^{3.54}$/A488C$^{6.33}$, consistent with an agonist-induced rotation of the cytoplasmic end of TM VI. Our study clearly demonstrates that the outcome of disulfide cross-linking studies using Cys-substituted mutant GPCRs can differ greatly depending on whether the receptor proteins are present in detergent micelles or in their native membrane environment.

EXPERIMENTAL PROCEDURES

Materials. Carbamylcholine chloride (carbachol), atropine sulfate, copper sulfate ($CuSO_4$), 1,10-phenanthroline, and mammalian protease inhibitor cocktail were obtained from Sigma. *N*-[^{3}H]-Methylscopolamine ([^{3}H]-NMS, 79−83 Ci/mmol) and *myo*-[^{3}H]inositol (20 Ci/mmol) were from Perkin-Elmer Life Sciences and American Radiolabeled Chemicals, respectively. The micro BCA protein assay reagent kit was purchased from Pierce. Factor Xa protease and digitonin were from Roche Molecular Biochemicals. Precast Novex 10−20% tris−glycine polyacrylamide gels and SeeBlue Plus 2 prestained molecular mass standards were obtained from Invitrogen. Hybond ECL nitrocellulose membranes, anti-rabbit IgG antibodies conjugated to horseradish peroxidase, ECL detection reagents, and Hyperfilm ECL chemiluminescence film were from Amersham Pharmacia Biotech. Laemmli loading buffer was from Bio-Rad. The QuickChange mutagenesis kit was purchased from Stratagene. All other reagents were of the highest grade commercially available.

Preparation of Cu(II)−(1,10-phenanthroline)$_3$ Solution. Copper sulfate ($CuSO_4$) was mixed with 1,10-phenanthroline at a molar ratio of 1:3 (*29*). Throughout the text, the concentrations indicated for the Cu(II)−(1,10-phenanthroline)$_3$ complex (Cu−Phen) refer to molar copper concentrations.

Site-Directed Mutagenesis. All Cys substitutions were introduced into a modified version of the rat M_3 muscarinic receptor referred to as M3′(3C)-Xa (Figure 1; *25*). Importantly, the M3′(3C)-Xa receptor lacked most endogenous Cys residues, except for C140, C220, and C532. Moreover, the central portion of the i3 loop (A274−K469) was replaced by two adjacent factor Xa cleavage sites (Figure 1). The construction of the 10 double Cys M_3 muscarinic receptors analyzed in this study has been described previously (*25*). Residues are numbered according to their positions in the wild-type rat M_3 muscarinic receptor sequence (*30*) and, where relevant, according to the "generic" numbering system proposed by Ballesteros and Weinstein (*24*).

Transient Expression of Cys-Substituted Mutant M_3 Muscarinic Receptors. All receptor constructs were transiently expressed in COS-7 cells, as described by Ward et al. (*26*). Cells were transfected with 4 μg of receptor plasmid DNA/100 mm dish using the Lipofectamine Plus kit (Invitrogen), according to the manufacturer's instructions. To increase receptor expression levels, 1 μM atropine was added to the incubation medium during the last 24 h of culture (*26*−*28*). Crude membranes were prepared from transfected COS-7 cells as described by Ward et al. (*26*).

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 per tube) were incubated in binding buffer (25 mM sodium phosphate containing 5 mM $MgCl_2$, pH 7.4) for 2 h at room temperature (22 °C) in the presence of the muscarinic antagonist, [^{3}H]-NMS. In saturation binding assays, six different concentrations of [^{3}H]-NMS were used (range of concentrations: 20−3000 pM). In competition binding assays, a fixed concentration of [^{3}H]-NMS (500 pM) was employed in the presence of 10 different concentrations of carbachol. Nonspecific binding was determined in the presence of 1 μM atropine. Bound and free [^{3}H]-NMS were separated by rapid vacuum filtration, and radioactivity was quantified by liquid scintillation spectrometry. Binding data were analyzed using the nonlinear curve-fitting program Prism 3.0 (GraphPad).

Carbachol-Mediated Stimulation of Phosphatidylinositol (PI) Hydrolysis. About 24 h after transfections, COS-7 cells grown in 100 mm dishes were trypsinized and seeded into 6-well plates (∼0.75×10^6 cells/well). Cells were then grown in the presence of 3 μCi/mL of *myo*-[^{3}H]inositol and 1 μM atropine for an additional 24 h. On the assay day, cells were washed twice for 10 min at room temperature with 2 mL of Hank's balanced salt solution (HBSS) to completely remove atropine. After this washing step, cells were preincubated for 20 min at room temperature with 1 mL of HBSS containing 20 mM HEPES (pH 7.4) and 10 mM LiCl. Cells were then incubated with increasing concentrations of carbachol (final concentration: 10^{-9} to 10^{-5} M) for 1 h at 37 °C. Assays were terminated by removal of the assay medium and addition of 0.8 mL of ice-cold 20 mM formic acid, followed by a 30 min incubation at 4 °C. The acidic medium was then neutralized with ∼ 0.35 mL of 60 mM NH_4OH, and the inositol monophosphate (IP_1) fraction was isolated by anion exchange chromatography **(***31***)** and counted on a liquid scintillation counter. Concentration−response curves were analyzed using the nonlinear curve-fitting program Prism 3.0 (Graph Pad).

Cu−Phen Treatment of Receptor-Containing Membrane Preparations. Membranes prepared from COS-7 cells transfected with the different M_3 muscarinic receptor constructs were incubated in microcentrifuge tubes for 10 min at room temperature (22 °C) with end-over-end rotation (30 rpm) with 2.5 μM Cu−Phen, either in the absence of ligands or in the presence of 1 mM carbachol or 1 μM atropine (∼1 mg of membrane protein per tube). Reactions were terminated by addition of EDTA and *N*-ethylmaleimide (10 mM each), followed by a 10 min incubation on ice.

Membrane Solubilization and Factor Xa Digestion. Following treatment with Cu−Phen, membranes prepared from transfected COS-7 cells were solubilized by treatment with 1.2% digitonin for 2 h at 4 °C, exactly as described by Ward et al. (*26*). Subsequently, membrane lysates (∼15 μg of

Table 1: Ligand Binding and Functional Properties of Mutant M_3 Muscarinic Receptors Analyzed in This Study[a]

	[^{3}H]-NMS binding		carbachol binding	carbachol-induced IP_1 production		
	pK_D	B_{max} (pmol/mg protein)	pK_i	pEC_{50}	response (fold above basal)	basal %
M3′(3C)-Xa	9.58 ± 0.32	3.0 ± 0.8	4.45 ± 0.07	7.05 ± 0.12	7.0 ± 1.5	100
I169C/K484C	9.58 ± 0.30	2.4 ± 0.7	5.05 ± 0.20	6.95 ± 0.28	8.1 ± 2.5	64 ± 9
I169C/E485C	9.66 ± 0.20	0.7 ± 0.1	5.03 ± 0.33	6.83 ± 0.12	3.8 ± 0.3	93 ± 18
I169C/K486C	9.58 ± 0.08	1.0 ± 0.3	5.05 ± 0.24	6.63 ± 0.21	5.0 ± 1.2	66 ± 10
I169C/K487C	10.03 ± 0.05	0.9 ± 0.1	5.14 ± 0.07	5.70 ± 0.15	12.6 ± 4.4	42 ± 15
I169C/A488C	9.65 ± 0.17	1.3 ± 0.4	5.38 ± 0.09	7.23 ± 0.19	5.7 ± 0.2	64 ± 12
I169C/A489C	9.65 ± 0.22	1.9 ± 0.4	4.67 ± 0.16	6.77 ± 0.33	5.4 ± 1.2	88 ± 38
I169C/Q490C	9.93 ± 0.12	1.2 ± 0.2	5.14 ± 0.18	6.75 ± 0.13	3.8 ± 0.4	127 ± 6
I169C/T491C	9.82 ± 0.02	0.9 ± 0.2	5.25 ± 0.15	6.14 ± 0.13	6.7 ± 1.3	37 ± 5
I169C/L492C	9.61 ± 0.12	1.8 ± 0.4	4.67 ± 0.05	6.64 ± 0.32	3.0 ± 0.3	116 ± 14
I169C/S493C	9.87 ± 0.04	1.8 ± 0.1	4.81 ± 0.10	6.64 ± 0.17	6.4 ± 1.6	71 ± 22

[a] COS-7 cells were transiently transfected with the indicated M3′(3C)-Xa-derived double Cys mutant M_3 receptor constructs. Radioligand binding and PI assays were performed and analyzed as described in Experimental Procedures. Carbachol binding data were corrected for the Cheng−Prusoff shift. Basal inositol monophosphate (IP_1) levels were 4609 ± 1000 dpm (=100%) for the M3′(3C)-Xa construct. Data are given as means ± SE from at least three independent experiments, each performed in duplicate.

protein) were incubated with factor Xa protease (final concentration ~0.1 μg/μL) at room temperature for 16−20 h. Reactions were terminated by the addition of a mammalian protease inhibitor cocktail (Sigma; 1:25 dilution), followed by a 30 min incubation at room temperature. Samples were then used directly for SDS−PAGE or frozen at −70 °C until needed.

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 (*26*, *32*). Receptor-containing membranes were incubated on ice for 30 min either in the presence or in the absence of 5 M urea and then used for disulfide cross-linking studies, exactly as described by Ward et al. (*26*).

Western Blotting Studies. SDS−PAGE, Western blotting, and ECL were carried out as previously described (*26*). In brief, samples were incubated with Laemmli loading buffer (*26*, *33*) for 30 min at 37 °C (nonreducing conditions) and then loaded onto Tris−glycine polyacrylamide gels run at 125 V in the presence of 0.1% SDS. In a separate set of experiments, 50 mM dithiothreitol was added to the samples prior to the initial incubation step (30 min at 37 °C) to break up existing disulfide bonds (reducing conditions). After blotting of the gels onto nitrocellulose membranes (*26*, *34*), membranes were blocked with 5% fat-free milk in PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T) (16 h at 4 °C). Following a wash with PBS-T, membranes were incubated in PBS-T for 1 h at room temperature with 1 mg/mL of the anti-M3 antibody which is directed against the C-terminus of the rat M_3 receptor protein (*26*, *35*). The blots were then washed three times with PBS-T (10 min each wash) and incubated in PBS-T for 1 h at room temperature with anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:3000 dilution). Following three 15 min washes with PBS-T, receptor proteins were visualized by the use of ECL detection reagents. The intensities of immunoreactive bands were quantitated by scanning densitometry using the program NIH Image (National Institutes of Health).

RESULTS

In this study, we subjected a series of double Cys mutant M_3 muscarinic receptors to a recently developed in situ disulfide cross-linking approach (*26*). All Cys substitutions were present in a modified version of the rat M_3 muscarinic receptor (M3′(3C)-Xa) that lacked most native Cys residues and contained two adjacent factor Xa cleavage sites within the i3 loop (Figure 1; *25*). Previous studies have shown that the M3′(3C)-Xa background receptor retains the ability to bind muscarinic ligands with high affinity and to productively couple to G proteins, in a fashion similar to the wild-type receptor (*25*−*28*). All mutant receptors contained a $I169C^{3.54}$ point mutation at the bottom of TM III and a second Cys substitution within the segment $K484^{6.29}$−$S493^{6.38}$ at the cytoplasmic end of TM VI (Figure 1).

Expression and Ligand Binding Properties of Double Cys Mutant M_3 Muscarinic Receptors. All receptor constructs were transiently expressed in COS-7 cells. Transfected cells were incubated with atropine (1 μM) for the last 24 h of culture. Previous studies (*26*−*28*) have shown that this atropine incubation step significantly increases the expression levels of Cys-substituted mutant M_3 receptors. [^{3}H]-NMS saturation binding studies showed that no significant amounts of atropine remained attached to the cells after two washes with PBS (for details, see Experimental Procedures; data not shown).

Saturation binding studies with the muscarinic antagonist, [^{3}H]-NMS, indicated that all double Cys mutant receptors were expressed at a density (B_{max}) of at least 0.9 pmol/mg of protein (the M3′(3C)-Xa background receptor was expressed at a density of 3.0 ± 0.8 pmol/mg; Table 1). Moreover, all mutant receptors, similar to the M3′(3C)-Xa construct, were able to bind [^{3}H]-NMS with high affinity (range of K_D values: 93−263 pM; Table 1). Likewise, [^{3}H]-NMS competition binding studies showed that the Cys substitutions had no detrimental effect on the binding affinity of the muscarinic agonist, carbachol (range of K_i values: 4−35 μM; Table 1).

Carbachol-Induced PI Hydrolysis. To determine whether the 10 double Cys mutant receptors retained the ability to activate G proteins, we next studied their ability to mediate carbachol-induced stimulation of PI hydrolysis. As shown in Table 1, most mutant receptors displayed maximum functional responses (E_{max}) similar to those observed with the M3′(3C)-Xa construct from which they were derived. The E_{max} values of three of the analyzed double Cys mutant

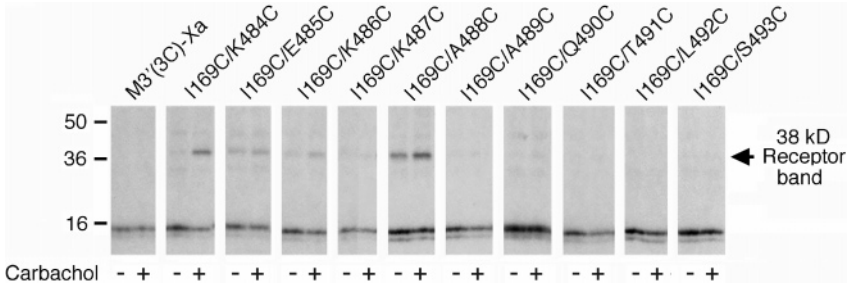

FIGURE 2: Carbachol-induced formation of disulfide bonds in mutant M_3 muscarinic receptors. Membranes prepared from COS-7 cells expressing the indicated mutant M_3 muscarinic receptors were incubated with the oxidizing agent, Cu−Phen (2.5 μM), for 10 min at room temperature, either in the absence or in the presence of the muscarinic agonist, carbachol (1 mM). Receptors were then solubilized and digested to completion with factor Xa (see Experimental Procedures for details). Samples containing equal amounts of protein (5 μg) were then run on 10−20% tris−glycine polyacrylamide gels, followed by Western blotting analysis using the anti-M3 antibody (nonreducing conditions). Two of the 10 investigated double Cys mutant receptors, I169C/K484C and I169C/A488C, displayed an agonist-dependent increase in the intensity of the 38 kDa receptor species, indicative of the formation of disulfide cross-links. Protein molecular mass standards (in kDa) are indicated to the left.

receptors (I169C$^{3.54}$/E485C$^{6.30}$, I169C$^{3.54}$/Q490C$^{6.35}$, and I169C$^{3.54}$/L492C$^{6.37}$) were reduced by about 50%. Cells expressing the I169C$^{3.54}$/T491C$^{6.36}$ construct showed a pronounced reduction in basal activity (IP_1 production in the absence of carbachol; Table 1).

The majority of the analyzed double Cys mutant receptors displayed carbachol EC_{50} values that were similar to the EC_{50} value found with the M3′(3C)-Xa construct (Table 1). Only two of the mutant receptors, I169C$^{3.54}$/K487C$^{6.32}$ and I169C$^{3.54}$/T491C$^{6.36}$, displayed a ∼10−20-fold reduction in carbachol potency (Table 1). Taken together, these results indicated that the majority of the introduced Cys residues did not interfere with the agonist-induced conformational changes required for productive receptor/G protein coupling.

Disulfide Cross-Linking Studies. To investigate the potential proximity between Cys residues introduced into the cytoplasmic end of TM VI and a Cys residue present at the bottom of TM III (I169C), we examined the ability of the 10 double Cys mutant receptors to form intramolecular disulfide bonds. All 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 (*26*−*28*).

To promote the formation of disulfide bonds, receptor-containing membrane preparations were incubated for 10 min at room temperature with a low concentration (2.5 μM) of the oxidizing agent Cu−Phen, either in the absence or in the presence of the muscarinic agonist, carbachol (1 mM). Following Cu−Phen treatment, receptors were solubilized, digested to completion with factor Xa, and subjected to SDS−PAGE and Western blotting under reducing and nonreducing conditions. Under these conditions, the appearance of a ∼38 kDa receptor band under nonreducing conditions is indicative of the formation of an intramolecular disulfide bond (*26*−*28*). Previous studies have shown that this ∼38 kDa immunoreactive species corresponds to properly folded cell surface receptors (*26*, *27*).

The results of a representative cross-linking experiment are shown in Figure 2. In this experiment, samples treated with Cu−Phen and digested to completion with factor Xa were processed for Western blotting studies under nonreducing conditions (no DTT added) to ensure the integrity of disulfide bonds. Like the M3′(3C)-Xa construct, eight of the 10 analyzed Cys-substituted mutant M_3 receptors did not yield a significant 38 kDa band (nonreducing conditions), either in the absence or the presence of carbachol (1 mM). The faint 38 kDa bands displayed by the I169C/E485C and I169C/K486C receptors (Figure 2) were not observed reproducibly. In contrast, the I169C$^{3.54}$/A488C$^{6.33}$ mutant construct consistently yielded a cross-linking signal, both in the absence or in the presence of carbachol (Figure 2). However, the intensity of the 38 kDa band was significantly more pronounced in samples treated with carbachol. Similarly, the I169C$^{3.54}$/K484C$^{6.29}$ construct showed little cross-linking in the absence of carbachol but gave a pronounced 38 kDa band in its presence (Figure 2). When Western blotting studies were carried out under reducing conditions, the 38 kDa bands displayed by the I169C/K484C and I169C/A488C receptors were no longer observed (data not shown), indicating that these bands were not caused by incomplete digestion by factor Xa. Carbachol-mediated facilitation of disulfide cross-link formation observed with the I169C/K484C and I169C/A488C receptors was prevented by co-incubation with atropine (10 μM; data not shown).

As shown in Figure 2, all mutant receptor constructs yielded a ∼15 kDa immunoreactive band. This band corresponds to the C-terminal M_3 receptor fragment generated by factor Xa cleavage (note that the antibody used was directed against the C-terminus of the M_3 receptor protein; Figure 1). To quantitate the degree of agonist-induced cross-linking observed with the I169C/K484C and I169C/A488C receptors, we measured the intensities of the 38 and 15 kDa bands by using scanning densitometry (NIH Image). This analysis revealed that ∼30−40% of the I169C/K484C and I169C/A488C receptors underwent carbachol-induced disulfide cross-linking under the experimental conditions used in this study.

Treatment of membranes expressing the M3′(3C)-Xa background receptor with 2.5 μM Cu−Phen (10 min at room temperature) had no significant effect on [^{3}H]-NMS and carbachol binding affinities ([^{3}H]-NMS K_D values for untreated samples, 180 ± 7 pM, and for Cu−Phen-treated samples, 174 ± 5 pM; carbachol K_i values for untreated samples, 24.8 ± 6.6 μM, and for Cu−Phen-treated samples, 30.9 ± 7.9 μM; n = 2 or 3).

Agonist-Dependence of the Observed Disulfide Cross-Linking Signals. To study the agonist dependence of the disulfide cross-linking signals observed with the I169C/

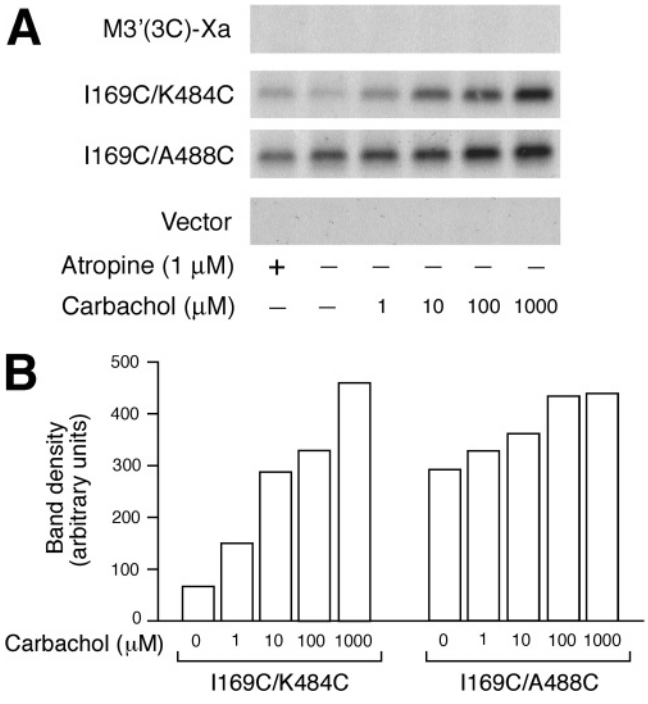

FIGURE 3: Effect of increasing agonist concentrations on the extent of disulfide cross-linking observed with the I169C/K484C and I169C/A488C double Cys mutant M_3 receptors. (A) Membranes prepared from COS-7 cells transfected with the I169C/K484C and I169C/A488C receptor constructs were incubated with the oxidizing agent, Cu−Phen (2.5 μM; 10 min), either in the absence of ligands, in the presence of the antagonist, atropine (1 μM), or in the presence of increasing concentrations of the agonist, carbachol. Cells transfected with the M3′(3C)-Xa background receptor or vector DNA served as controls. Cu−Phen-treated receptors were solubilized and digested with factor Xa, followed by SDS−PAGE and Western blotting (nonreducing conditions), using the anti-M3 antibody. Equal amounts of protein (5 μg) were loaded in each lane. The 38 kDa receptor bands which correspond to functional cell surface receptors (*26*, *27*) are shown. Data from a representative experiment are shown. Two additional experiments gave similar results. (B) Quantitation of the cross-linking data shown in panel A. The intensities of the 38 kDa receptor species were determined by scanning densitometry, as described under Experimental Procedures. Band densities are given in arbitrary units.

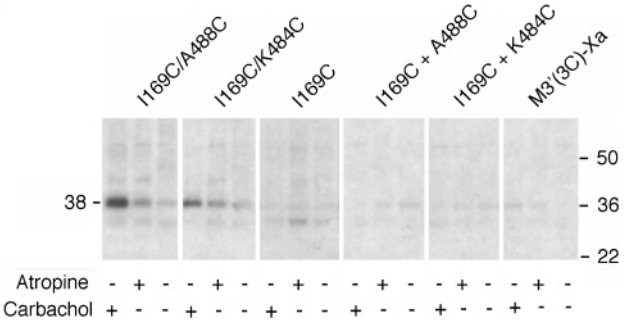

FIGURE 4: Disulfide cross-linking studies with cotransfected single Cys mutant M_3 muscarinic receptors. All Cys mutations were introduced into the M3′(3C)-Xa background receptor. COS-7 cells were cotransfected with a mutant receptor containing the I169C single point mutation and a second mutant receptor construct containing a single Cys substitution at position K484 or A488. For control purposes, cells were also transfected individually with the I169C single Cys mutant receptor and the I169C/K484C and I169C/A488C double Cys mutant constructs. Membranes prepared from transfected cells were incubated with the oxidizing agent, Cu−Phen (2.5 μM; 10 min), either in the presence of the agonist, carbachol (1 mM), or the antagonist, atropine (1 μM), or in the absence of ligands. Receptors were then solubilized and digested to completion with factor Xa, followed by SDS−PAGE and Western blotting (nonreducing conditions) using the anti-M3 antibody. Equal amounts of protein (5 μg) were loaded in each lane. No significant 38 kDa signal was observed in the coexpression experiments, indicating that intermolecular disulfide cross-links are not formed to a significant extent. Protein molecular mass standards (in kDa) are indicated to the right. Data from a representative experiment are shown. Two additional experiments gave similar results.

K484C and I169C/A488C receptors in more detail, membranes prepared from cells expressing these two mutant constructs were oxidized in the presence of increasing carbachol concentrations (1−1000 μM). In both cases, carbachol stimulation led to a concentration-dependent increase in the intensity of the 38 kDa cross-linking signal (nonreducing conditions; Figure 3). In contrast, treatment with the muscarinic antagonist, atropine (1 μM), did not promote the formation of disulfide bonds in either of the two mutant receptors (Figure 3A). The slight reduction in the intensity of the 38 kDa band displayed by the I169C/488C construct in the presence of atropine (Figure 3A) was not observed reproducibly.

The EC_{50} values observed for the carbachol-induced cross-linking signals displayed by the I169C/K484C and I169C/A488C receptors ranged from ~10−70 μM (Figure 3), a range similar to that observed for the corresponding carbachol K_i values determined in radioligand binding studies (Table 1).

Disulfide Cross-Links Are Not Formed among Different Receptor Monomers. Like many other GPCRs (*36*, *37*), the M_3 muscarinic receptor is able to form dimeric or oligomeric arrays (*38*). Thus, to exclude the possibility that the agonist-induced formation of disulfide bonds observed with the I169C/K484C and I169C/A488C mutant receptors occurred intermolecularly rather than intramolecularly, we carried out an additional set of experiments. Specifically, we coexpressed an M3′(3C)-Xa-based mutant receptor containing the I169C single point mutation with M3′(3C)-Xa-derived mutant constructs containing the K484C or A488C single Cys substitutions. The expression levels of these single Cys mutant receptors were similar to those of the I169C/K484C and I169C/A488C double Cys mutant receptors, as determined by [^{3}H]-NMS binding studies (data not shown).

Cu−Phen treatment of membranes prepared from cotransfected COS-7 cells, followed by solublilization of membrane proteins, factor Xa digestion, and Western blotting, did not reveal a significant cross-linking signal (38 kDa receptor band under nonreducing conditions), either in the absence of ligands or in the presence of carbachol (1 mM) or atropine (1 μM) (Figure 4). In contrast, in the presence of carbachol, the I169C/K484C and I169C/A488C double Cys mutant receptors, which were included for control purposes, gave a pronounced agonist-dependent cross-linking signal (Figure 4), consistent with the results shown in Figures 2 and 3. Taken together, these observations strongly suggest that the agonist carbachol promotes the formation of intra- rather than intermolecular disulfide bonds in the I169C/K484C and I169C/A488C mutant receptors.

Agonist-Induced Disulfide Cross-Linking Remains Unaffected by Inactivation of Heterotrimeric G Proteins. We next wanted to exclude the possibility that the agonist-dependent formation of disulfide bonds observed with the I169C/K484C

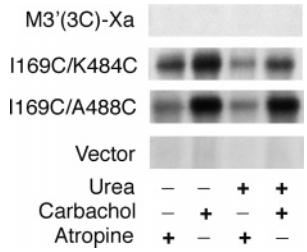

FIGURE 5: Effect of urea treatment on the extent of disulfide cross-linking observed with the I169C/K484C and I169C/A488C double Cys mutant M_3 receptors. Membranes were prepared from COS-7 cells transfected with the I169C/K484C and I169C/A488C receptor constructs. Cells transfected with the M3′(3C)-Xa background receptor or vector DNA served as controls. Cell membranes were either treated with 5 M urea to inactivate heterotrimeric G proteins (*26*, *32*, *39*; for details see Experimental Procedures) or left untreated. Subsequently, membrane preparations were incubated with the oxidizing agent, Cu−Phen (2.5 μM), for 10 min at room temperature. Incubations were carried out either in the presence of the agonist, carbachol (1 mM), or the antagonist, atropine (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. Equal amounts of protein (5 μg) were loaded in each lane. The 38 kDa receptor bands which correspond to functional cell surface receptors (*26*, *27*) are shown.

and I169C/A488C mutant receptors was caused by agonist-induced dissociation of heterotrimeric G proteins precoupled to the inactive state of the receptor. To address this question, receptor-containing membranes were treated with a high concentration (5 M) of the chaotropic agent, urea. Previous 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 (*26*, *32*, *39*). Following incubation of membranes expressing the I169C/K484C or I169C/A488C double Cys mutant receptors with urea (5 M, 30 min on ice), samples were treated with Cu−Phen, solubilized, digested with factor Xa, and subjected to Western blotting analysis (nonreducing conditions). Figure 5 clearly demonstrates that urea treatment did not affect the ability of carbachol (1 mM) to promote disulfide cross-linking in the I169C/K484C and I169C/A488C mutant receptors. These data suggest that the formation of agonist-dependent disulfide cross-links observed with these two mutant receptors was caused by conformational changes intrinsic to the receptor proteins rather than by receptor/G protein precoupling.

DISCUSSION

In the present study, we analyzed a series of double Cys mutant M_3 muscarinic receptors all of which contained a Cys residue at the bottom of TM III ($I169C^{3.54}$) and a second Cys residue introduced into the cytoplasmic end of TM VI ($484^{6.29}-493^{6.38}$; Figure 1). Specifically, we used an in situ disulfide cross-linking strategy (*26*−*28*) that allows the monitoring of disulfide bond formation in receptors present in their native membrane environment. Studies were carried out either in the absence of ligands to obtain cross-links characteristic for the resting state of the receptor or in the presence of the muscarinic agonist, carbachol, to detect conformational changes specific for the activated state of the receptor.

Radioligand binding and functional studies showed that all 10 double Cys mutant receptors were able to bind muscarinic ligands with high affinity and to activate G proteins in an agonist-dependent fashion (Table 1), indicating that the different Cys substitutions did not interfere with proper receptor folding. Although the cytoplasmic segment of TM VI is known to play an important role in the efficiency and selectivity of receptor/G protein coupling (*8*), individual replacement of residues 484−493 with Cys (in the M3′(3C)-Xa background) did not lead to major impairments in receptor/G protein coupling (Table 1). This observation indicates that single Cys substitutions in this region do not severely disrupt overall receptor structure. Cys scanning mutagenesis studies targeting the corresponding region in bovine rhodopsin yielded similar results (*40*). These data are consistent with the concept that receptor/G protein coupling involves many different contact sites on the receptor protein (*8*).

In the absence of carbachol, most of the analyzed mutant receptors did not yield a cross-linking signal (Figure 2). Only two of the mutant receptors, $I169C^{3.54}$/$K484C^{6.29}$ and $I169C^{3.54}$/$A488C^{6.33}$, showed weak but reproducible cross-linking in the absence of carbachol (Figures 2−5). Strikingly, addition of carbachol resulted in a pronounced cross-linking signal in the I169C/K484C and I169C/A488C receptors (Figures 2−5). In both cases, the intensity of the cross-linking signals increased with increasing agonist concentrations (Figure 3), indicating that the number of agonist-occupied receptors correlates well with the extent of disulfide cross-linking. In contrast, the remaining eight mutant receptors failed to form disulfide bonds in the presence of agonist, indicating that the observed pattern of disulfide cross-links was limited to only a small number of amino acids.

Additional experiments showed that carbachol induced the formation of intramolecular disulfide bonds rather than promoting cross-links between different receptor molecules (intermolecular disulfide bonds) in the I169C/K484Cand I169C/A488C receptors (Figure 4). In another set of control experiments, cell membranes prepared from transfected COS-7 cells were pretreated with 5 M urea prior to disulfide cross-linking (Figure 5). Previous work has shown that this concentration of urea greatly reduces the activity of membrane-attached heterotrimeric G proteins, probably due to denaturation of heterotrimeric G proteins and other nonintegral membrane proteins (*26*, *32*, *39*). We found that urea treatment had essentially no effect on the extent of agonist-dependent disulfide cross-linking displayed by the I169C/K484C and I169C/A488C mutant receptors (Figure 5). These findings strongly support the concept that the observed agonist-dependent disulfide cross-links reflect conformational changes intrinsic to the I169C/K484C and I169C/A488C receptor proteins rather than being caused by receptor/G protein precoupling.

We recently established a 3D model of the TM core of the rat M_3 muscarinic receptor by using homology modeling based on the high-resolution X-ray structure of the inactive state of bovine rhodopsin (*12*, *28*). Overall, this M_3 receptor model shows high structural similarity with that of the rhodopsin template (*28*). The M_3 receptor model predicts that the Cα atoms of $I169^{3.54}$ and $K484^{6.29}$ or $A488^{6.33}$ are ∼10 Å apart in the inactive state of the receptor. The distance between the Cα carbons of two Cys residues engaged in a

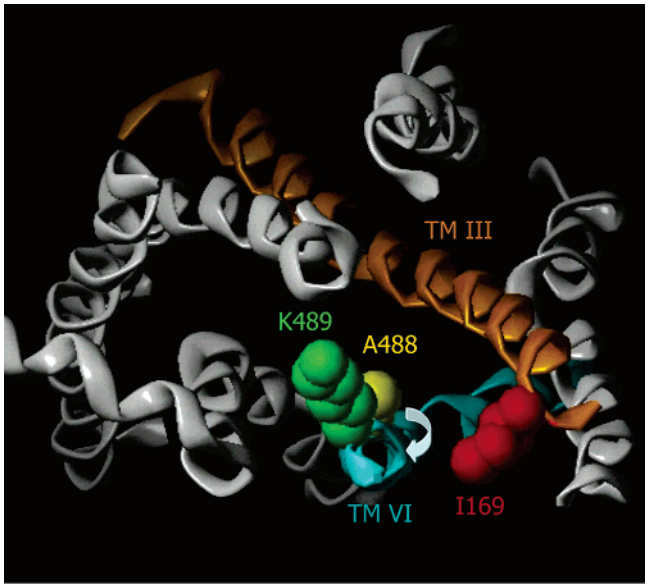

FIGURE 6: Predicted location of $I169^{3.54}$, $K484^{6.29}$, and $A488^{6.33}$ in the 3D structure of the M_3 muscarinic receptor, as viewed from the cytoplasm. A 3D model of the inactive state of the rat M_3 muscarinic receptor was built via homology modeling using the high-resolution X-ray structure of bovine rhodopsin as a template (*12*, *28*). In the inactive state of the receptor, $K484^{6.29}$ and $A488^{6.33}$ which are located at the cytoplasmic end of TM VI are projecting away from $I169^{3.54}$ present at the bottom of TM III. Our disulfide cross-linking data are therefore consistent with the concept that carbachol binding to the receptor leads to conformational changes that include a rotational movement (clockwise as viewed from the cytoplasm) of the cytoplasmic segment of TM VI.

disulfide bond usually ranges from about 3.8 to 6.8 Å **(***10*, *41***)**, providing a possible explanation as to why I169C does not readily form a disulfide cross-link with K484C or A488C in the resting state of the M_3 receptor. The weak but reproducible cross-linking signal displayed by the I169C/K488C mutant receptor in the absence of ligands may be due to thermal motions of the polypeptide backbone of the TM helical bundle (*42*).

Similarly, our M_3 receptor model predicts that the Cα atoms of all other residues within the $K484^{6.29}$−$S493^{6.38}$ sequence and the Cα atom of $I169^{3.54}$ are ∼7−14 Å apart in the inactive state of the receptor. Since the distance between the Cα carbons of two Cys residues engaged in a disulfide bond is usually <6.8 Å **(***10*, *41***)**, the inability of $I169C^{3.54}$ to readily form disulfide cross-links with Cys residues present at positions $K484^{6.29}$−$S493^{6.38}$ in the absence of carbachol may be primarily due to the fact that none of these residues are located sufficiently close to position 3.34 in the inactive state of the receptor.

Figure 6 shows that, in the inactive state of the M_3 muscarinic receptor, K484 and A488 which are located at the cytoplasmic end of TM VI are projecting away from $I169^{3.54}$ present at the bottom of TM III. The ability of the muscarinic agonist, carbachol, to promote the formation of disulfide cross-links in the I169C/K484C and I169C/A488C mutant receptors is therefore consistent with the concept that activation of the M_3 muscarinic receptor in its native membrane environment is associated with a clockwise rotation of the cytoplasmic end of TM VI (cytoplasmic view) which increases the proximity between I169C and K484C or A488C, thus, facilitating the formation of disulfide cross-links.

Our findings are in agreement with the results of a site-directed spin-labeling study carried out with Cys-substituted mutant versions of bovine rhodopsin. In this study, Farrens et al. (*16*) provided evidence that light-induced rhodopsin activation triggers an "outward" movement of the cytoplasmic portion of TM VI, accompanied by a clockwise rotation of ∼30°, as viewed from the cytoplasm. A similar activity-dependent change in receptor confirmation is also supported by biochemical and biophysical studies carried out with mutant versions of the β_2-adrenergic receptor (*18*−*22*). Biochemical studies with Cys- or His-substituted mutant versions of bovine rhodopsin or other GPCRs have also demonstrated that cross-linking of the cytoplasmic ends of TM III and VI, either via disulfide bonds (*16*) or via binding of metal ions (*17*, *21*), disrupts productive rhodopsin/transducin coupling. This observation indicates that the activity-dependent reorientation of the cytoplasmic end of TM VI plays a fundamental role in allowing productive receptor/G protein coupling.

As discussed above, our cross-linking data obtained with Cys-substituted mutant M_3 muscarinic receptors suggest that agonist activation leads to a conformational change that moves $K484C^{6.29}$ and $A488C^{6.33}$ closer to $I169C^{3.54}$, consistent with an agonist-induced rotation of the cytoplasmic end of TM VI. In agreement with these findings, site-directed spin-labeling studies carried out with bovine rhodopsin (*16*) indicate that rhodopsin activation also causes a decrease in the distance between the corresponding residues present at positions 6.33 and 3.54 (V250 and V139, respectively; residue 6.29 was not included in this previous study). Thus, our data do not contradict the proposed "outward" movement of the cytoplasmic end of TM VI (*16*).

The findings obtained in the present study greatly differ from those in a previous disulfide cross-linking study in which similar Cys substitutions were introduced into bovine rhodopsin (*16*). In the inactive (dark) state of rhodopsin, a Cys residue introduced at the bottom of TM III ($V139C^{3.54}$) was found to efficiently cross-link with Cys residues present at positions 247, 248, 249, 250, or 251 (positions 6.30−6.34) within the cytoplasmic segment of TM VI (note that the corresponding residues in the M_3 muscarinic receptor were also targeted in the present study). Since cross-link formation was virtually complete for all five mutant receptors in the absence of light, the effect of light-induced activation of rhodopsin on disulfide bond formation was not studied. The results of the cross-linking study by Farrens et al. (*16*) suggested that the cytoplasmic end of TM VI is conformationally highly flexible in the inactive state of rhodopsin. In contrast, the results of the present study suggest that this is not the case for the resting state of the M_3 muscarinic receptor. A likely observation for this discrepancy is that the rhodopsin cross-linking studies were carried out with rhodopsin proteins solubilized in dodecyl maltoside micelles. In contrast, the present cross-linking study was carried out with double Cys mutant receptors present in their native membrane environment.

The concept that receptor solubilization increases the conformational flexibility of the cytoplasmic segment of TM VI is strongly supported by a previous study in which we studied the formation of disulfide cross-links in the same

set of double Cys M_3 mutant receptors analyzed in the present study following their solubilization with digitonin (*25*). In this previous study, we found a very high degree of cross-linking promiscuity in the inactive state of the M_3 muscarinic receptor, similar to the cross-linking pattern observed by Farrens et al. (*16*) with Cys-substituted mutant versions of rhodopsin. In the absence of ligands, $I169C^{3.54}$ was able to form disulfide bonds with Cys residues introduced at positions $484^{6.29}-491^{6.36}$ within the cytoplasmic segment of TM VI (*25*), a pattern not observed in the present study. The study by Zeng et al. (*25*) did not examine whether agonist ligands led to changes in the observed cross-linking patterns.

Taken together, the results of the present study, combined with the outcome of previous disulfide cross-linking studies, strongly suggest that the degree of structural flexibility of the cytoplasmic segment of TM VI is limited when receptors are present in their native membrane environment but rather pronounced when receptors are solubilized in detergent micelles. Similar differences in conformational flexibility have been observed previously in site-directed spin-labeling studies analyzing activity-dependent changes in the orientation of a helix 8 Cys residue (C316) in bovine rhodopsin (*43*, *44*). When experiments were carried out with solubilized receptors present in lauryl maltoside micelles, rhodopsin activation was associated with a pronounced change in the electron paramagnetic resonance (EPR) spectrum of the nitroxide attached to C316 (*43*). In contrast, when analogous studies were performed with modified versions of rhodopsin present in the native disc membrane, the EPR spectrum of the nitroxide attached to C316 showed little change following rhodopsin activation (*44*).

A characteristic feature of the plasma membrane is that the ~ 30 Å hydrophobic core region is surrounded on both sides by ~15 Å regions enriched in phospholipid headgroups (*45*). The cytoplasmic extension of TM VI of the M_3 muscarinic receptor and most other GPCRs contains several amino acids with positively charged side chains (Figure 1; *8*, *45*). These residues are primarily found on one face of the helix and are predicted to be engaged in ionic interactions with the negatively charged phospholipid headgroups of the lipid bilayer (*45*). It is therefore likely that these interactions reduce the conformational flexibility of the cytoplasmic end of TM VI of GPCRs present in lipid bilayers.

The Cu−Phen concentration (2.5 μM) used in the present study was chosen because we had shown previously (*26*) that this relatively low concentration was highly effective in promoting the formation of disulfide cross-links between Cys residues introduced into the cytoplasmic ends of TM V and VI. Disulfide cross-linking studies with Cys-substituted, solubilized versions of bovine rhodopsin (*16*) and the M_3 muscarinic receptor (*25*) were carried out by using a considerably higher Cu−Phen concentration (50 μM). We therefore cannot completely rule out the possibility that this relatively high Cu−Phen concentration also contributed to the cross-linking promiscuity observed in these previous studies (*16*, 25).

It is also possible that the environment surrounding the Cys residues introduced into the cytoplasmic ends of TM III and VI is different in detergent solution as compared to the lipid bilayer. We therefore cannot completely rule out the possibility that impaired access of the oxidizing agent to the intracellular receptor surface may have contributed to the rather restricted cross-linking pattern observed in the present study.

Since the M_3 muscarinic receptor shares a significant degree of structural homology with many other class I GPCRs, our findings should be of considerable general relevance.

ACKNOWLEDGMENT

We thank Dr. Soo-Kyung Kim (NIDDK) for helpful discussions and for preparing Figure 6..

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BI051503Q