Use of an in Situ Disulfide Cross-Linking Strategy To Map Proximities between Amino Acid Residues in Transmembrane Domains I and VII of the M_3 Muscarinic Acetylcholine Receptor

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ABSTRACT: In this study, we employed an in situ disulfide cross-linking strategy to gain insight into the structure of the inactive and active state of the M_3 muscarinic acetylcholine receptor. Specifically, this study was designed to identify residues in TM I that are located in close to Cys532 (position 7.42), an endogenous cysteine residue present in the central portion of TM VII. Cysteine residues were substituted, one at a time, into 10 consecutive positions of TM I (Ala71−Val80) of a modified version of the M_3 muscarinic receptor that lacked most endogenous cysteine residues and contained a factor Xa cleavage site within the third intracellular loop. Following their expression in COS-7 cells, the 10 resulting cysteine mutant receptors were oxidized in their native membrane environment, either in the absence or in the presence of muscarinic ligands. Disulfide cross-link formation was monitored by examining changes in the electrophoretic mobility of oxidized and factor Xa-digested receptors on SDS gels. When molecular iodine was used as the oxidizing agent, the L77C receptor (position 1.42) was the only mutant receptor that displayed significant disulfide cross-linking, either in the absence or in the presence of muscarinic agonists or antagonists. On the other hand, when the Cu(II)−(1,10-phenanthroline)$_3$ complex served as the redox catalyst, muscarinic ligands inhibited disulfide cross-linking of the L77C receptor, probably because of impaired access of this relatively bulky oxidizing agent to the ligand binding crevice. The iodine cross-linking data suggest that M_3 muscarinic receptor activation is not associated with significant changes in the relative orientations of the outer and/or central segments of TM I and VII. In bovine rhodopsin, the residues present at the positions corresponding to Cys532 and Leu77 in the rat M_3 muscarinic receptor are not located directly adjacent to each other, raising the possibility that the relative orientations of TM I and VII are not identical among different class I GPCRs. Alternatively, dynamic protein backbone fluctuation may occur, enabling Cys532 to move within cross-linking distance of Leu77 (Cys77).

G protein-coupled receptors (GPCRs)[1] constitute by far the largest group of cell surface receptors found in nature (*1*−*3*). Despite the remarkable diversity of their activating ligands, all GPCRs are predicted to share a conserved molecular architecture consisting of a bundle of seven transmembrane helices (TM I−VII) connected by three extracellular and three intracellular loops (Figure 1). Until recently, predictions of GPCR structure were based primarily on low-resolution structural information of rhodopsin and systematic sequence analyses, aided by a vast body of mutagenesis and biochemical data (*4*, *5*). The recent elucidation of the high-resolution crystal structure of the inactivated state of bovine rhodopsin therefore represents a major breakthrough in the field (*6*, *7*). Most GPCRs share a considerable degree of sequence homology with rhodopsin (rhodopsin-type or class I GPCRs) (*1*, *2*, *8*, *9*) and are therefore predicted to fold in a fashion similar to that of rhodopsin. However, the extent of structural similarity between rhodopsin and other class I GPCRs remains unknown at present (*9*).

Following their binding to the extracellular surface of GPCRs, activating ligands are thought to induce changes in the arrangement of the TM helical bundle which are propagated to the intracellular surface of the receptor, thus enabling the receptor to productively couple to heterotrimeric G proteins (*8*−*12*). Since all GPCRs are likely to share a conserved overall three-dimensional fold, the structural changes required for receptor activation may be similar in all receptors. Recent evidence suggests that GPCR activation triggers a reorientation of the cytoplasmic end of TM VI as

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[1] Abbreviations: BMOE, bismaleimidoethane; Cu−Phen, Cu(II)−(1,10-phenanthroline)$_3$ complex; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GPCR, G protein-coupled receptor; HA tag, hemagglutinin epitope tag; HBSS, Hank's balanced salt solution; i3 loop, third intracellular loops of GPCRs; IP_1, inositol monophosphate; [^{3}H]NMS, *N*-[^{3}H]methylscopolamine; MTSEA, methanethiosulfonate ethylammonium; NEM, *N*-ethylmaleimide; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; *p*-PDM, *N*,*N*′-*p*-phenylenedimaleimide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-T, PBS (pH 7.4) containing 0.05% Tween 20; PI, phosphatidylinositol; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; TMA, tetramethylammonium; TM, transmembrane domain.

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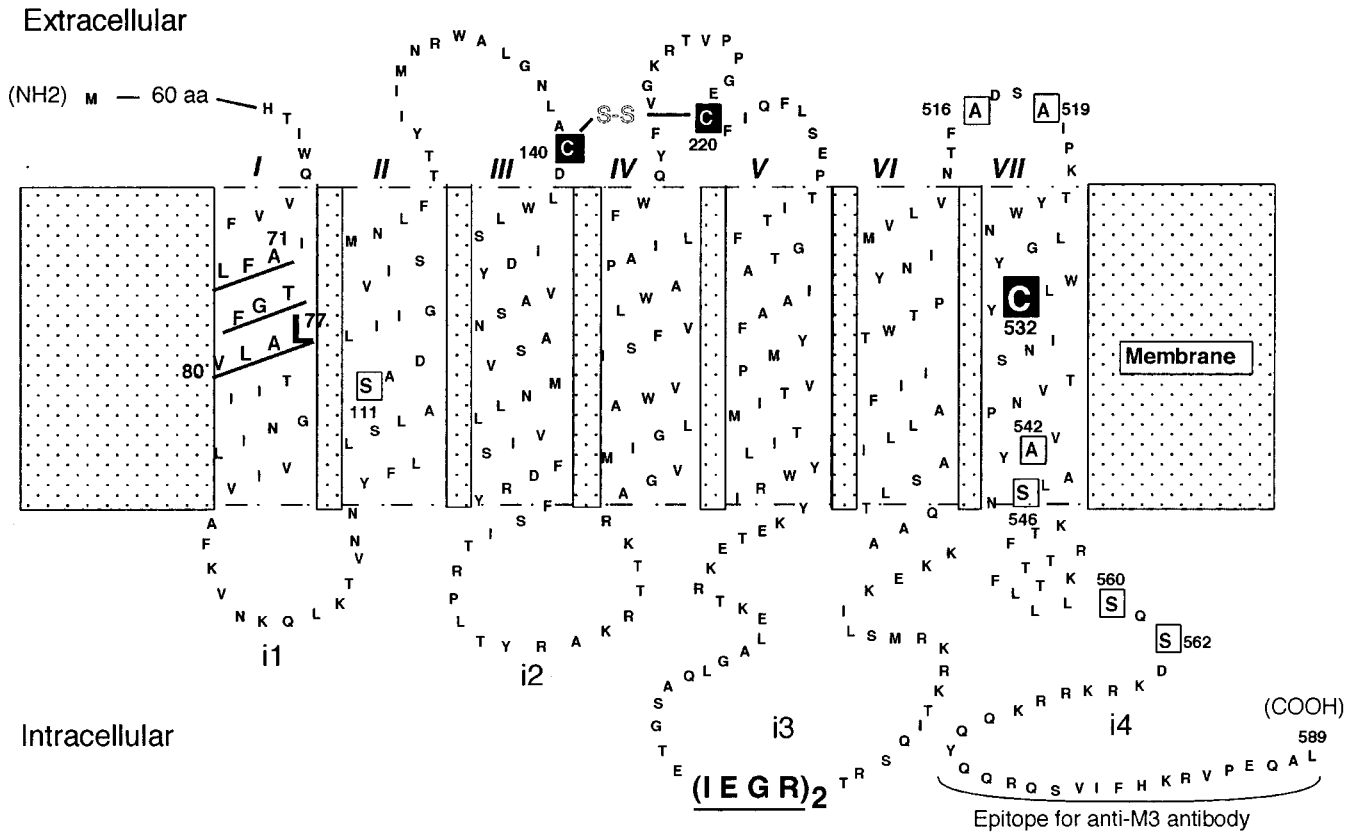

FIGURE 1: Structure of Cys mutant M_3 muscarinic receptors used in this study. Ten consecutive residues in TM I (Ala71−Val80, underlined) were individually replaced with Cys residues. All Cys substitutions were introduced into a modified version of the rat M_3 muscarinic receptor termed M3′(3C)-Xa (*37*). The M3′(3C)-Xa construct contains only three remaining Cys residues, Cys140, Cys220, and Cys532 (white C in black square). All other native Cys residues (Cys111, Cys516, Cys519, Cys542, Cys546, Cys560, and Cys562) were replaced with serine or alanine residues (black letters in white squares). In addition, the central portion of the i3 loop (Ala274−Lys469) was replaced with two factor Xa cleavage sites [$(IEGR)_2$, underlined]. All mutant receptors contained an N-terminal HA epitope tag and the N-terminal Asn6Gln, Asn15Gln, Asn41Gln, Asn48Gln, and Asn52Gln point mutations to prevent N-glycosylation of the receptors (not shown; *37*). To detect the different mutant M_3 muscarinic receptors via Western blotting, we used a rabbit polyclonal antibody (anti-M3) directed against the indicated C-terminal receptor sequence (*48*). Numbers refer to amino acid positions in the rat M_3 muscarinic receptor sequence (*42*).

well as changes in the relative disposition of TM VI and III (*13*−*20*). Site-directed spin labeling studies (*14*) carried out with the photoreceptor rhodopsin predict that rhodopsin activation involves a rigid body movement of the cytoplasmic end of TM VI, away from the C-terminus of TM III. In agreement with this concept, cross-linking of the cytoplasmic ends of TM III and TM VI, either via disulfide bonds in rhodopsin (*14*) or via metal ion bridges in rhodopsin and other GPCRs (*15*, *16*), prevented receptor activation. Moreover, spectroscopic analysis of the β2-adrenergic receptor (*21*) and disulfide cross-linking studies with the M_3 muscarinic receptor (*22*) indicate that agonist stimulation also leads to conformational changes that move the cytoplasmic end of TM VI closer to that of TM V. The currently available evidence suggests that GPCR activation opens a cleft on the intracellular side of the receptor that increases the accessibility of functionally critical residues located on the inner surfaces of TM II, III, VI, and VII (reviewed in ref *12*). However, despite these recent findings, our knowledge of the molecular nature of the structural changes involved in GPCR activation is still very incomplete.

Most studies examining activity-dependent structural changes in GPCRs have focused on changes in the relative disposition of residues located on the intracellular receptor surface. The most detailed investigations have been carried out with the photoreceptor rhodopsin, involving systematic site-directed spin labeling (*23*−*27*) and disulfide cross-linking (*28*−*33*) studies. However, little is known about activity-dependent changes in the relative orientation of TM residues located closer to the extracellular surface of the receptors where ligand binding is predicted to initiate the receptor activation process. To address this issue, we employed a disulfide cross-linking strategy using the M_3 muscarinic acetylcholine receptor as a model system. Specifically, we used a recently developed in situ cross-linking protocol that promotes the formation of disulfide bonds with receptors being present in their native membrane environment (*22*).

Residues located within the extracellular segment of TM VII of the muscarinic receptors are known to be critically involved in ligand binding (*34*−*36*), consistent with studies on many other classes of GPCRs. We therefore decided to test the hypothesis that agonist binding to the M_3 muscarinic receptor may lead to changes in the relative orientation of residues present in TM VII. To shed light on this issue, we used a mutant version of the M_3 muscarinic receptor [M3′-(3C)-Xa (*37*)] that contained only a single cysteine (Cys) residue within the TM receptor core. This Cys residue, Cys532 [position 7.42 according to the nomenclature of Ballesteros and Weinstein (*38*)], is located in the central portion of TM VII, close to the ligand biding site (Figure 1; *34*−*36*) and is predicted to face the interior of the TM receptor core (*5*, *6*).

Since TM VII lies adjacent to TM I in the three-dimensional structure of GPCRs, our initial goal was to identify TM I residues that are located in the proximity of Cys532 in the inactive form of the M_3 receptor. Toward this aim, we mutated, to Cys, 10 consecutive residues in TM I [Ala71−Val80 (positions 1.36−1.45); Figure 1], one at a time, and subsequently examined the ability of the introduced Cys residues to form disulfide bonds with Cys532. In the second part of the study, cross-linking experiments were carried out in the presence of agonist ligands to monitor activity-dependent changes in receptor structure.

EXPERIMENTAL PROCEDURES

Materials. Copper sulfate ($CuSO_4$), 1,10-phenanthroline, iodine, *N*-ethylmaleimide (NEM), *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM), *N*,*N*′-*p*-phenylenedimaleimide (*p*-PDM), carbamylcholine chloride (carbachol), acetylcholine bromide, tetramethylammonium (TMA) bromide, oxotremorine M methiodide, atropine sulfate, *N*-methylatropine bromide, dithiothreitol (DTT), and mammalian protease inhibitor cocktail were purchased from Sigma. Bismaleimidoethane (BMOE) and the Micro BCA protein assay kit were obtained from Pierce. *N*-[^{3}H]Methylscopolamine ([^{3}H]NMS, 79−83 Ci/mmol) and [^{3}H]myoinositol (20 Ci/mmol) were from Perkin-Elmer Life Sciences and American Radiolabeled Chemicals, respectively. Factor Xa protease and digitonin were purchased from Roche Molecular Biochemicals. Precast Novex tris-glycine polyacrylamide gels and SeeBlue Plus 2 prestained molecular mass standards were obtained from Invitrogen. Hybond ECL nitrocellulose membranes, anti-rabbit IgG antibody conjugated to horseradish peroxidase, ECL detection reagents, and Hyperfilm ECL chemiluminescence film were from Amersham Pharmacia Biotech. Methanethiosulfonate ethylammonium (MTSEA) was obtained from Toronto Research Chemicals. Laemmli loading buffer was from Bio-Rad. All other reagents were of the highest grade commercially available.

Preparation of Cu(II)−(1,10-Phenanthroline)$_3$ and Iodine Solutions. Copper sulfate ($CuSO_4$) was mixed with 1,10-phenanthroline at a molar ratio of 1:3, as recommended previously (*39*). Throughout the text, the concentrations indicated for the Cu(II)−(1,10-phenanthroline)$_3$ complex (Cu−Phen) refer to molar copper concentrations. Iodine was first dissolved in ethanol (50 mg/mL) and then diluted in dH_2O to the required working concentration (5 mM).

Expression Plasmid Constructs. All mutant receptors used for disulfide cross-linking studies were derived from a modified version of the rat M_3 muscarinic receptor termed M3′(3C)-Xa (*37*). Mutations were introduced into a pCD-based expression plasmid (*40*) encoding the M3′(3C)-Xa construct (*37*). The M3′(3C)-Xa receptor contained an N-terminal hemagglutinin (HA) epitope tag (YPYDVPDYA; following the initiating methionine codon), and several Asn → Gln point mutations to prevent receptor glycosylation (*37*). In addition, the M3′(3C)-Xa receptor lacked most endogenous Cys residues, except for Cys140, Cys220, and Cys532, and contained two adjacent factor Xa cleavage sites ($IEGR_2$) in the central portion of the third intracellular loop (i3 loop; Figure 1). Cys substitutions, one at a time, were introduced into the M3′(3C)-Xa receptor at positions Ala71−Val80 using standard PCR mutagenesis techniques. For the MTSEA labeling experiments, point mutations were introduced into a pCD-based expression plasmid encoding the wild-type rat M_3 muscarinic receptor [M3(wt)] containing an N-terminal HA epitope tag (*41*). Specifically, the C532A single- and L77C/C532A double-point mutations were introduced into the M3(wt) receptor background. The identity of all mutant receptor constructs was verified by DNA sequencing. Residues are numbered according to their positions in the wild-type rat M_3 muscarinic receptor sequence (*42*) and, where relevant, according to the "generic" numbering system proposed by Ballesteros and Weinstein (*38*).

Transient Expression of Receptor Constructs. COS-7 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% CO_2 incubator. Approximately 24 h prior to transfections, 1×10^6 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. Where indicated, 1 μM atropine was added to the incubation medium for the last 24 h of culture to boost mutant receptor expression levels.

Crude Membrane Preparations. Transfected cells were harvested ∼48 h after transfections. To ensure the removal of previously added atropine, the cells were washed twice (10 min each wash) with 10 mL of ice-cold phosphate-buffered saline (PBS; pH 7.4). Subsequently, 1 mL of ice-cold buffer A [25 mM sodium phosphate and 5 mM $MgCl_2$ (pH 7.4)] was added to each 100 mm dish, followed by a 15 min incubation at 4 °C. Cells were then scraped off the plates and homogenized using a Polytron tissue homogenizer (setting 5; 20 s), followed by a 15 min centrifugation at 20000*g* at 4 °C. The membrane pellets were then resuspended in buffer A (1 mL/100 mm dish), rehomogenized, frozen on dry ice, and stored at −70 °C until they were needed. Protein concentrations were measured using the Micro BCA protein assay reagent kit with bovine serum albumin as a standard.

Receptor Binding Assays. Radioligand binding assays were performed using membrane homogenates prepared from transfected COS-7 cells essentially as described previously (*43*). All incubations were carried out in 1 mL of buffer A (∼10−20 μg of membrane protein per tube) for 2 h at room temperature (22 °C). In saturation binding assays, six different concentrations of the radioligand, [^{3}H]NMS, ranging from 20 to 5000 pM, were tested. In competition binding assays, incubations were carried out with a fixed concentration of [^{3}H]NMS (500 pM) in the presence of 10 different concentrations of the competing ligand, carbachol. Reactions were terminated by rapidly filtering the mixture on water-presoaked 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).

Stimulation of Phosphatidylinositol (PI) Hydrolysis. Approximately 24 h after transfections, COS-7 cells were trypsinized and seeded into six-well plates (∼0.75×10^6 cells/well), and 3 μCi/mL of [^{3}H]myoinositol and 1 μM atropine (where indicated) were added to the growth medium.

Following a 24 h labeling period, the cells were washed twice for 10 min at room temperature with 2 mL of Hank's balanced salt solution (HBSS) to remove the previously added atropine. After preincubation of cells for 20 min at room temperature with 1 mL of HBSS containing 20 mM HEPES (pH 7.4) and 10 mM LiCl, the cells were incubated with various carbachol concentrations (final concentration of $10^{-9}-10^{-5}$ M) for 1 h at 37 °C. Reactions were terminated by removing the assay medium and adding 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 (*44*) and counted on a liquid scintillation counter. Concentration−response curves were analyzed using the nonlinear curve-fitting program Prism 3.0 (GraphPad).

Cross-Linking Studies. Membrane preparations were thawed at room temperature and then briefly homogenized. To induce the formation of disulfide bonds, membranes prepared from one 100 mm dish of transfected COS-7 cells (containing ~0.6−0.8 mg of protein) were incubated with the Cu(II)−(1,10-phenanthroline)$_3$ complex (25−250 μM) or iodine (500 μM). Incubations were carried out in the presence or absence of muscarinic ligands (1 μM atropine or 1 mM carbachol) for 10−12 min at room temperature (22 °C) with end-over-end rotation (30 rpm). Reactions were terminated by adding EDTA and *N*-ethylmaleimide (10 mM each) to the reaction mixture, followed by a 10 min incubation on ice. Membranes were then lysed, followed by treatment of membrane lysates with factor Xa as described in the following paragraph. In a separate set of studies, membrane preparations were incubated with homobifunctional thiol-specific cross-linking agents (*o*-PDM, *p*-PDM, or BMOE; 0.5 mM each) for 20 min at room temperature with end-over-end rotation (30 rpm). Reactions were terminated by adding DTT at a final concentration of 9 mM (*45*). After a 10 min incubation on ice, the membrane preparations were washed twice with 1 mL of ice-cold PBS (pH 7.4) and then solubilized with digitonin (see below).

Membrane Solubilization and Factor Xa Digestion. Following treatment with oxidizing agents or chemical cross-linkers, membranes were centrifuged at 8000*g* for 10 min at 4 °C. The pellets were then incubated with 250 μL of 0.2% digitonin in PBS (pH 7.4) for 20 min on ice. After recentrifugation (8000*g* for 10 min at 4 °C), the pellets were incubated with 100 μL of 1.2% digitonin in factor Xa digestion buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM $CaCl_2$] for ~16 h at 4 °C with end-over-end rotation (30 rpm). The samples were then centrifuged at 20000*g* for 30 at 4 °C, and the supernatants were either used directly for SDS−PAGE or frozen on dry ice and stored at −70 °C until they were needed. Protein concentrations were determined using the Micro BCA protein assay reagent kit with bovine serum albumin as a standard. To proteolytically cleave solubilized mutant M_3 muscarinic receptors, membrane lysates (~15 μg of protein) were incubated with factor Xa protease (final concentration of ~0.1 μg/μL) at room temperature for 16−20 h. The reactions were then terminated by adding 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 they were needed.

Western Blotting. SDS−PAGE was performed essentially as described by Laemmli (*46*). Samples were incubated for 30 min at 37 °C with Laemmli loading buffer, in the absence (nonreducing conditions) or presence (reducing conditions) of 50 mM DTT. The samples were then loaded on 10 to 20% tris-glycine polyacrylamide gels which were run at 125 V in the presence of 0.1% SDS. Proteins were electroblotted onto nitrocellulose membranes using the procedure described by Burnette (*47*). Membranes were then 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 (5 min, room temperature), the membranes were incubated with 1 μg/mL anti-M3 antibody (*48*) in PBS-T for 1 h at room temperature. The blots were then washed three times with PBS-T (10 min/wash) and incubated for 1 h at room temperature in PBS-T with anti-rabbit IgG (1:3000) antibody conjugated to horseradish peroxidase. After three 15 min washes with PBS-T, proteins were visualized by using ECL detection reagents and autoradiography. The intensities of immunoreactive bands were quantitated by scanning densitometry using the program NIH Image (National Institutes of Health).

Modification of Receptors with MTSEA. COS-7 cells were transfected with plasmids encoding the M3(wt) receptor or M3(wt)-derived mutant receptors containing the C532A single- or C532A/L77C double-point mutation. Since all three receptors were expressed at high levels (as determined in [^{3}H]NMS saturation binding studies), transfected cells were grown in the absence of atropine. Membrane homogenates (180 μL aliquots containing ~0.6 μg/μL protein) prepared from transfected COS-7 cells were incubated with different concentrations of freshly prepared MTSEA (0.5−5 mM) at room temperature for 10 min. Reactions were stopped by adding 8 mL of buffer A, followed by radioligand binding studies using a saturating concentration (3 nM) of [^{3}H]NMS, as described above.

For antagonist protection assays, membrane homogenates (180 μL aliquots containing ~0.6 μg/μL protein) prepared from transfected COS-7 cells were incubated with 10 μM *N*-methylatropine (total volume of 190 μL) for 20 min at room temperature. Subsequently, MTSEA (10 μL) was added to give a final concentration of 2.5 mM, and the mixture was allowed to incubate at room temperature for an additional 10 min. The reaction was terminated by 9.5-fold dilution with buffer A, followed by a 20 min centrifugation at 14000*g* and 4 °C. Membrane pellets were then resuspended in 8 mL of buffer A, briefly homogenized, and used for [^{3}H]NMS (3 nM) radioligand binding assays as described above. Protection was calculated as 1 − (inhibition in the presence of *N*-methylatropine)/(inhibition in the absence of *N*-methylatropine).

RESULTS

A modified version of the rat M_3 muscarinic receptor, termed M3′(3C)-Xa (Figure 1; *37*), was used as a template for Cys scanning mutagenesis. Besides other modifications, the M3′(3C)-Xa construct lacks most native Cys residues and contains a factor Xa cleavage site that replaces the central portion of the i3 loop. The M3′(3C)-Xa construct contains only three remaining Cys residues, Cys140, Cys220, and Cys532. The presence of these Cys residues was found to

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

	[^{3}H]NMS binding		carbachol binding		carbachol-induced IP_1 production		
	pK_d	B_{max} (pmol/mg of protein)	pK_{app}	n_H	pEC_{50}	E_{max} (fold IP_1 above basal)	basal IP_1 (dpm)
M3′(3C)-Xa	10.05 ± 0.01	2.49 ± 0.1	4.66 ± 0.04	0.63 ± 0.05	7.33 ± 0.11	7.5 ± 1.1	3503 ± 562
A71C	10.00 ± 0.02	2.18 ± 0.2	4.51 ± 0.04	0.62 ± 0.04	7.23 ± 0.10	7.0 ± 1.0	2971 ± 860
F72C	9.94 ± 0.13	2.54 ± 0.4	4.60 ± 0.03	0.67 ± 0.07	7.03 ± 0.06	7.4 ± 1.0	2146 ± 216
L73C	10.00 ± 0.07	1.83 ± 0.2	4.64 ± 0.06	0.68 ± 0.02	7.30 ± 0.20	7.7 ± 1.4	2449 ± 641
T74C	10.05 ± 0.05	2.27 ± 0.5	4.53 ± 0.02	0.83 ± 0.08	7.20 ± 0.32	7.8 ± 1.9	2846 ± 1112
G75C	9.85 ± 0.03	1.86 ± 0.2	4.60 ± 0.06	0.66 ± 0.03	7.21 ± 0.21	7.2 ± 2.9	3151 ± 1055
F76C	9.78 ± 0.15	2.43 ± 0.6	4.54 ± 0.06	0.61 ± 0.12	7.43 ± 0.20	6.7 ± 0.5	3715 ± 283
L77C	9.84 ± 0.06	2.68 ± 0.6	4.56 ± 0.06	0.64 ± 0.04	7.12 ± 0.20	7.0 ± 1.8	3934 ± 466
A78C	9.80 ± 0.02	1.59 ± 0.2	4.30 ± 0.07[b]	0.60 ± 0.07	7.49 ± 0.50	6.4 ± 1.8	3579 ± 512
L79C	9.64 ± 0.08	1.91 ± 0.4	4.11 ± 0.01[b]	0.64 ± 0.02	7.42 ± 0.38	6.5 ± 1.3	3350 ± 318
V80C	9.83 ± 0.08	1.96 ± 0.2	4.40 ± 0.04[b]	0.89 ± 0.04	7.20 ± 0.07	7.2 ± 1.5	4179 ± 274

[a] COS-7 cells transfected with the indicated M3′(3C)-Xa-derived Cys mutant M_3 receptors were incubated with atropine (1 μM) for the last 24 h of culture. Radioligand binding and PI assays were performed and analyzed as described in Experimental Procedures. Carbachol binding data were corrected for the Cheng−Prusoff shift (*56*). Data are presented as means ± SEM from at least three independent experiments, each done in duplicate. [b] $p < 0.05$ [one-way ANOVA, vs the M3′(3C)-Xa value].

be essential for M3′(3C)-Xa receptor function [substitution, with serine or alanine, of either of these three Cys residues in the M3′(3C)-Xa background completely abolished ligand binding activity (*37*)]. Cys532 is located in the exofacial segment of TM VII and is thought to face the inside of the receptor protein (*4*, *5*). Cys140 and C220 are predicted to be linked via a disulfide bond (Figure 1; *48*) which is conserved in virtually all class I GPCRs. Cys532 is therefore the only "free" Cys residue present in the M3′(3C)-Xa receptor. Previous studies showed that the M3′(3C)-Xa mutant receptor retained the ability to bind muscarinic ligands and to induce agonist-dependent PI hydrolysis in a manner similar to that of the wild-type M_3 muscarinic receptor (*37*).

Since TM VII lies adjacent to TM I in the three-dimensional structure of GPCRs (*4*−*7*), our goal was to identify TM I residues located in the proximity of Cys532 in the inactive and active state of the M3′(3C)-Xa receptor. To address this issue, we first mutated to Cys, one at a time, 10 consecutive residues in TM I [Ala71−Val80 (positions 1.36−1.45); Figure 1], and subsequently examined the ability of the introduced Cys residues to form disulfide bonds with Cys532, either in the inactive state of the receptor or in the presence of agonists (see below).

Expression and Ligand Binding Properties of Mutant M_3 Muscarinic Receptors. All mutant receptors were transiently expressed in COS-7 cells and initially examined for their ability to bind the muscarinic antagonist, [^{3}H]NMS. To increase the sensitivity of the in situ disulfide cross-linking assays, transfected COS-7 cells were grown in the presence of atropine (1 μM) for the last 24 h of culture to boost mutant receptor expression levels. As reported previously (*22*), the inclusion of atropine in the culture medium resulted in pronounced increases (4−6-fold) in mutant receptor densities (B_{max}), as determined in [^{3}H]NMS saturation binding studies [shown for the M3′(3C)-Xa base mutant and a representative Cys mutant receptor, L77C, in Figure 2A]. As shown in Table 1, all mutant receptors were expressed at similar levels, ranging from ∼1.6 to 2.7 pmol/mg of membrane protein. Moreover, all Cys mutant receptors, similar to the M3′(3C)-Xa construct, were able to bind [^{3}H]NMS with high affinity (range of K_D values of ∼0.1−0.2 nM; Table 1). Similarly, [^{3}H]NMS competition binding studies showed that the TM I Cys substitutions had little effect on carbachol binding affinities (range of K_{app} values of ∼10−20 μM; Table 1).

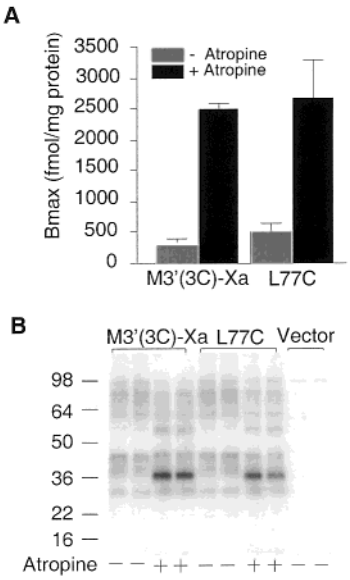

FIGURE 2: Effect of atropine treatment of transfected COS-7 cells on expression levels of mutant M_3 muscarinic receptors. Results obtained with the M3′(3C)-Xa "base mutant" and a representative M3′(3C)-Xa-derived Cys mutant receptor (L77C) are shown. Transfected COS-7 cells were incubated with or without 1 μM atropine during the last 24 h of culture. (A) Saturation binding analysis. [^{3}H]NMS saturation binding assays revealed pronounced increases in the maximum number of receptor binding sites (B_{max}) in membranes prepared from cells grown in the presence of atropine. Data are given as means ± SEM of three independent experiments, each performed in duplicate. (B) Western blot analysis. Membrane lysates (∼3 μg of protein/sample) were subjected to SDS−PAGE and Western blotting under reducing conditions (50 mM DTT in loading buffer) using the anti-M3 antibody. Samples prepared from atropine-treated cells expressing the M3′(3C)-Xa or L77C mutant receptors yielded a pronounced ∼38 kDa band. In contrast, samples prepared from cells grown in the absence of atropine gave only very faint 38 kDa bands that were visible on the original film but were no longer visible after the scanning process. Assays were run in duplicate. Protein molecular mass standards (in kilodaltons) are indicated at the left.

Agonist-Induced PI Hydrolysis Mediated by Mutant M_3 Muscarinic Receptors. To examine whether the different mutant receptors retained the ability to interact with G proteins, we next studied their ability to mediate carbachol-induced stimulation of PI hydrolysis. The different TM I Cys substitutions had no significant effect on basal inositol

phosphate levels (Table 1). In the presence of increasing concentrations of the agonist, carbachol, all mutant receptors induced a pronounced increase in the level of PI hydrolysis. As shown in Table 1, the 10 Cys mutant receptors displayed maximum responses similar to that of the M3′(3C)-Xa base mutant from which they were derived (E_{max} ∼ 6−8-fold stimulation of IP_1 above basal levels). Similarly, carbachol EC_{50} values were not significantly affected by the different Cys substitutions (range of EC_{50} values of ∼30−90 nM). These results indicated that the introduced Cys residues did not interfere with the agonist-induced conformational changes required for productive coupling to G proteins.

Disulfide Cross-Linking Studies. To examine the potential proximity of Cys532 to Cys residues introduced into TM I, we investigated the ability of the 10 Cys mutant receptors to form intramolecular disulfide bonds. Specifically, we employed a recently developed disulfide cross-linking strategy that allows monitoring of the formation of disulfide cross-links with receptors being present in their native membrane environment (in situ; *22*). Membranes prepared from transfected COS-7 cells were incubated with oxidizing agents, the Cu(II)−(1,10-phenanthroline)$_3$ complex (25 and 100 μM) or iodine (500 μM), for ∼10−12 min at room temperature, either in absence or in the presence of the antagonist, atropine (1 μM), or the agonist, carbachol (1 mM). Following a solubilization step (for details, see Experimental Procedures), receptors were digested to completion with factor Xa (0.1 μg/μL, 16−20 h at room temperature), subjected to SDS−PAGE, and then detected via Western blotting using the anti-M3 antibody which is directed against the C-terminus of the M_3 receptor protein (Figure 1; *37*). In the case of successful disulfide cross-linking between Cys532 and specific Cys residues present in TM I, this strategy is predicted to yield an ∼38 kDa full-length receptor band under nonreducing but not under reducing conditions, as reported previously for a different set of M3′(3C)-Xa-derived Cys mutant receptors (*22*). In control experiments in which samples were not treated with factor Xa, the intensity of the ∼38 kDa band increased dramatically when cells were incubated with atropine (1 μM) for the last 24 h of culture, in agreement with the pronounced increase in the number of [^{3}H]NMS binding sites [shown for the M3′(3C)-Xa construct and a representative Cys mutant receptor, L77C, in Figure 2]. This observation, together with the results of previous cell surface biotinylation experiments (*22*), strongly suggests that the 38 kDa immunoreactive species corresponds to properly folded cell surface receptors. As shown in Figure 2B, atropine treatment not only led to the appearance of a pronounced 38 kDa receptor band but also yielded an additional, fainter band ∼55 kDa in size. Since a putative receptor dimer is predicted to migrate at ∼76 kDa, the 55 kDa band may correspond to a complex of the 38 kDa receptor species with another membrane protein (this, however, is purely speculative). The additional weak immunoreactive bands visible in Figure 2B may represent misfolded receptors, partially degraded receptors (receptor fragments), or aggregates of receptors and/or receptor fragments.

Initially, membranes prepared from transfected COS-7 cells were incubated with the oxidizing agent, Cu−Phen (100 μM, 10−12 min at room temperature), followed by receptor solubilization, cleavage of receptors with factor Xa, and Western blotting analysis using the anti-M3 antibody (for details, see Experimental Procedures). Under these experimental conditions, only one of the 10 analyzed Cys mutant receptors, L77C, exhibited significant disulfide cross-linking, as indicated by the appearance of a prominent 38 kDa band on Western blots (nonreducing conditions; Figure 3A). Identical results were obtained when the Cu−Phen concentration was reduced to 25 μM (data not shown). When Western blotting studies were carried out under reducing conditions (incubation of samples with 50 mM DTT), the prominent 38 kDa band (L77C receptor) was no longer observed (data not shown), indicative of the involvement of disulfide bonds.

Besides the L77C receptor which gave a pronounced cross-linking signal, several other mutant receptors gave very faint 38 kDa bands (Figure 3). However, these bands were not observed consistently throughout different assays. One possibility therefore is that these faint 38 kDa bands were caused by incomplete factor Xa digestion.

Factor Xa cleaves all mutant receptors within the i3 loop, generating an N-terminal and a C-terminal cleavage product. The resulting C-terminal receptor fragment, which can be detected by the C-terminal antibody, has a molecular mass of ∼15 kDa. In Figure 3, this C-terminal receptor fragment corresponds to the 15 kDa band at the bottom of the blots (this band is absent in samples not treated with factor Xa; see Figure 2B).

As outlined above, oxidized and factor Xa-treated samples prepared from cells expressing the L77C mutant receptor yielded a distinct 38 kDa band, indicative of disulfide cross-linking. However, these samples still yielded a pronounced 15 kDa immunoreactive species which corresponds to the C-terminal receptor fragment generated by factor Xa digestion (Figure 3). This observation clearly indicates that the L77C mutant receptor underwent only partial disulfide cross-linking under the chosen experimental conditions. To quantitate the degree of disulfide cross-linking displayed by the L77C mutant receptor, we determined the intensities of the 38 and 15 kDa bands by using scanning densitometry (NIH Image). This analysis indicated that ∼10−20% of the L77C receptor protein underwent disulfide cross-linking under the experimental conditions used in this study.

We next examined whether incubation of the Cys mutant receptors with an agonist or antagonist ligands resulted in an altered disulfide cross-linking pattern. When receptor-containing membrane preparations were incubated with Cu−Phen (100 μM) in the presence of the antagonist, atropine (1 μM), the L77C mutant receptor no longer underwent disulfide cross-linking (Figure 3A). Similarly, as observed in the absence of ligands, none of the remaining nine Cys mutant receptors formed intramolecular disulfide bridges in the presence of atropine. The observation that atropine treatment completely prevented the appearance of the 38 kDa receptor band (L77C mutant receptor) clearly indicates that the receptors subjected to disulfide cross-linking in the absence of atropine are functionally competent (capable of ligand binding) and do not represent misfolded receptor species.

When membranes prepared from L77C-expressing cells were incubated with Cu−Phen (100 μM) in the presence of the agonist carbachol (1 mM), the intensity of the 38 kDa band was reduced by ∼3−6-fold (Figure 3A), indicative of

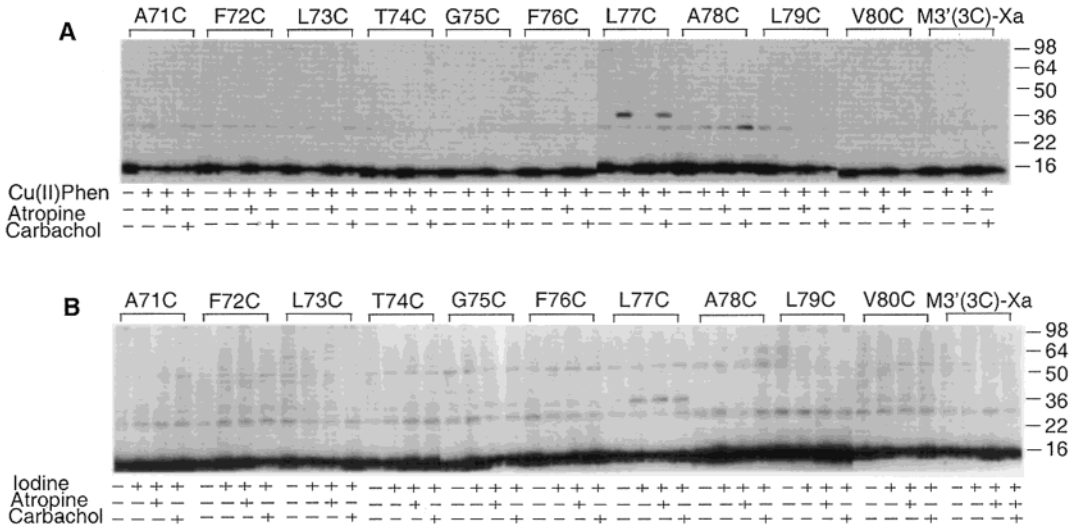

FIGURE 3: Detection of disulfide cross-linking in mutant M_3 muscarinic receptors via a Western blotting strategy. The indicated Cys substitutions were introduced into the M3′(3C)-Xa mutant receptor background (Figure 1). Membranes prepared from COS-7 cells expressing the indicated mutant receptors were incubated with 100 μM Cu−Phen (A) or 500 μM iodine (B) for 10−12 min at room temperature, either in the absence or in the presence of muscarinic ligands (1 μM atropine or 1 mM carbachol). Receptors were then solubilized and digested to completion with factor Xa (see Experimental Procedures for details). Samples containing equal amounts of protein (~3 μg) were run on 10 to 20% tris-glycine polyacrylamide gels, followed by Western blotting analysis using the anti-M3 antibody (nonreducing conditions). In both panels A and B, only preparations containing the L77C mutant receptor yielded a prominent ~38 kDa receptor band, indicative of successful disulfide cross-linking. In the Cu−Phen experiments (A), this signal was either abolished or reduced in magnitude when incubations were carried out in the presence of atropine or carbachol, respectively. In the iodine experiments (B), incubation with atropine or carbachol had no significant effect on the intensity of the 38 kDa receptor band observed with the L77C mutant receptor. The pronounced 15 kDa band at the bottom of the blots corresponds to the C-terminal receptor fragment resulting from cleavage of the receptors with factor Xa (this band is absent in samples not treated with factor Xa; see Figure 2B). Several other weak immunoreactive bands (e.g., at ~30 kDa) are visible in most lanes. The precise nature of these band remains unclear. Representative blots are shown. Two additional experiments gave similar results. Protein molecular mass standards (in kilodaltons) are indicated at the right.

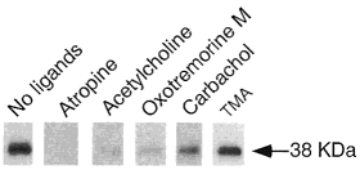

FIGURE 4: Effect of different muscarinic ligands on Cu−Phen-induced cross-linking of the L77C mutant M_3 receptor. Membranes prepared from COS-7 cells expressing the L77C mutant receptor were oxidized with Cu−Phen (100 μM), either in the absence of ligands or in the presence of the muscarinic antagonist, atropine (1 μM), and the muscarinic agonists, acetylcholine (1 mM), oxotremorine M (1 mM), carbachol (1 mM), or TMA (50 mM). Receptors were then solubilized and digested to completion with factor Xa (see Experimental Procedures for details). Samples containing equal amounts of protein (~3 μg) were subjected to SDS−PAGE and Western blotting using the anti-M3 antibody (nonreducing conditions). Note that the intensity of the 38 kDa receptor band, which is indicative of disulfide cross-linking, decreased to different extents in the presence of different muscarinic ligands. Data shown here are representative of two or three independent experiments which gave similar results.

a reduced level of disulfide cross-linking. As observed in the absence of ligands, carbachol (1 mM) did not induce the formation of disulfide bonds in any of the remaining nine Cys mutant receptors (Figure 3A). Qualitatively similar results were obtained when L77C-containing membrane preparations were oxidized in the presence of muscarinic agonists other than carbachol, including acetylcholine (1 mM), oxotremorine M (1 mM), and TMA (50 mM) (Figure 4). However, the degree of inhibition of disulfide bond formation was most pronounced with acetylcholine and oxotremorine M and least prominent with TMA, a low-affinity ligand characterized by a relatively small molecular size.

Since both agonist and antagonist ligands strongly inhibited or abolished Cu−Phen-dependent cross-linking of the L77C mutant receptor, we considered it likely that muscarinic ligands prevent Cu−Phen, a rather complex and bulky molecule, from accessing the central hydrophilic receptor cavity. To further explore this possibility, we carried out an additional set of cross-linking experiments using molecular iodine (I_2) as the oxidizing agent. We speculated that iodine, due to its relatively small molecular size (<2.7 Å), might be able to access the central binding crevice and promote disulfide bond formation between adjacent Cys residues even in the presence of agonist or antagonist ligands.

The results of a representative cross-linking experiment using iodine (500 μM) as the oxidizing agent are shown in Figure 3B. In the presence of iodine and the absence of ligands, L77C was the only Cys mutant receptor that was able to form disulfide cross-links, as observed with the use of Cu−Phen as the redox catalyst. In this case, however, the presence of muscarinic ligands (1 μM atropine or 1 mM carbachol) had no significant effect on the efficiency of disulfide bond formation in the L77C mutant receptor (Figure 3B), in contrast to the pronounced inhibition of disulfide cross-linking observed when Cu−Phen was used as the oxidizing agent (Figure 3A). In the iodine experiments, as observed in the Cu−Phen studies, the presence of atropine or carbachol did not lead to the appearance of a significant cross-linking signal in any of the remaining nine Cys mutant receptors (Figure 3B).

To confirm that the cross-linking between Cys77 and Cys532 occurred intramolecularly rather than intermolecularly (between two or more receptor molecules), we carried

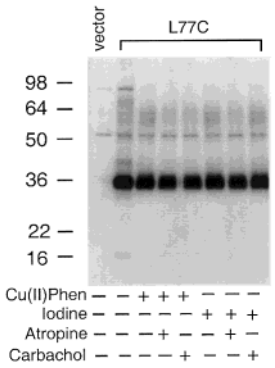

FIGURE 5: Lack of effect of oxidizing agents on the electrophoretic mobility of the L77C mutant M_3 receptor. Membrane extracts prepared from COS-7 cells transfected with vector DNA or the L77C mutant receptor were treated with oxidizing agent Cu−Phen (100 μM) or iodine (500 μM) for 10−12 min at room temperature, in either the absence or presence of ligands (1 μM atropine or 1 mM carbachol). Samples were processed in a fashion identical to the one described in the legend of Figure 3, except that the factor Xa digestion step was omitted in this set of experiments (see Experimental Procedures for details). After solubilization of receptors with digitonin, samples (~3 μg of protein) were subjected to SDS−PAGE and Western blotting using the anti-M3 antibody (nonreducing conditions). Note that incubation of the L77C mutant receptor with oxidizing agents did not result in the formation of a significant amount of dimeric or oligomeric receptor species.

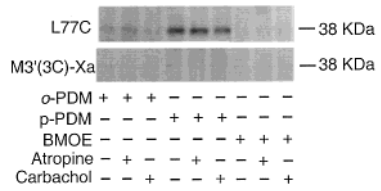

FIGURE 6: Cross-linking experiments using mutant M_3 muscarinic receptors and homobifunctional cross-linkers. Membranes were prepared from COS-7 cells transfected with the L77C mutant receptor or the M3′(3C)-Xa construct from which it was derived. Membrane preparations (~0.6−0.8 mg of protein/mL) were incubated for 20 min at room temperature with 0.5 mM homobifunctional, thiol-specific cross-linkers *o*-PDM, BMOE, and *p*-PDM (distances between the reactive maleimido groups of 6, 8, and 10 Å, respectively). Incubations were carried out either in the absence or in the presence of muscarinic ligands (1 μM atropine or 1 mM carbachol). Reactions were stopped by addition of DTT (9 mM). After solubilization of receptors with digitonin and digestion of receptors with factor Xa (see Experimental Procedures for details), samples (~3 μg of protein/sample) were subjected to SDS−PAGE and Western blotting using the anti-M3 antibody (nonreducing conditions). Note that treatment of L77C-containing membranes with *p*-PDM but not with *o*-PDM or BMOE resulted in the appearance of a prominent 38 kDa receptor band, indicative of successful receptor cross-linking. The presence of ligands had no significant effect on the intensity of the observed 38 kDa receptor species. As expected, no cross-linking was detected with the M3′(3C)-Xa base mutant. Representative blots are shown. Two additional experiments gave similar results.

out an additional set of Western blotting studies. Specifically, membranes prepared from L77C-expressing COS-7 cells were treated with the oxidizing agent Cu−Phen (100 μM) or iodine (500 μM), either in the absence or in the presence of atropine (1 μM) or carbachol (1 mM). Samples were then processed for SDS−PAGE and Western blotting in the same fashion as described above (nonreducing conditions), except that the factor Xa cleavage step was omitted. Under these conditions, the formation of intermolecular disulfide bonds would be expected to result in dimeric or oligomeric receptor species (>75 kDa in size). However, as shown in Figure 5, Western blotting experiments failed to reveal significant amounts of high-molecular mass receptor species under the experimental conditions used in this study. This observation indicated that Cu−Phen- or iodine-catalyzed disulfide bond formation between Cys77 and Cys532 involved intramolecular rather than intermolecular disulfide cross-links.

Cross-Linking Studies Using Homobifunctional Thiol-Specific Cross-Linking Agents. To provide an experimental estimate of the distance between Cys77 and Cys532 in the L77C mutant receptor, we carried out additional cross-linking experiments using the noncleavable, homobifunctional, thiol-specific reagents, *o*-PDM, *p*-PDM, and BMOE, as "molecular rulers". *o*-PDM and *p*-PDM are rigid thiol cross-linkers in which the two reactive maleimido groups are linked to benzene rings in the ortho and para positions at distances of ~6 and ~10 Å, respectively (*49*). On the other hand, the two maleimido groups of BMOE are joined by a two-carbon chain, allowing a maximum distance of ~8 Å between the two reactive groups. For these experiments, membranes prepared from L77C-expressing COS-7 cells were incubated with *o*-PDM, *p*-PDM, or BMOE (0.5 mM each) for 20 min at room temperature, either in the absence of ligands or in the presence of atropine (1 μM) or carbachol (1 mM). Receptors were then solubilized, digested to completion with factor Xa, and subjected to SDS−PAGE and Western blotting (nonreducing conditions). As shown in Figure 6, treatment of L77C-containing membranes with *o*-PDM or BMOE did not result in a significant degree of cross-linking. In contrast, incubation with *p*-PDM resulted in the appearance of a prominent 38 kDa receptor band, indicative of the formation of a cross-link between Cys77 and Cys532. Figure 6 also shows that the presence of ligands (atropine or carbachol) had no significant effect on the efficiency of *p*-PDM-dependent cross-linking.

Effect of MTSEA on [^{3}H]NMS Binding to Wild-Type and Mutant M_3 Receptors. To examine whether Leu77 (Cys77) and Cys532 project into the central, hydrophilic ligand binding crevice, we next used a strategy termed the substituted cysteine-accessibility method (SCAM; *50*, *51*). Initially, we treated membranes prepared from COS-7 cells expressing different M_3 receptor constructs with increasing concentrations of the positively charged sulfhydryl-specific agent, MTSEA, which preferentially interacts with Cys residues present in a hydrophilic environment (*50*, *51*). After the MTSEA incubation step, we carried out [^{3}H]NMS saturation binding studies to examine the effect of MTSEA treatment on ligand binding. The ability of MTSEA treatment to interfere with ligand binding usually indicates that the Cys residue(s) modified by MTSEA projects into the central binding crevice, as discussed in detail elsewhere (*51*).

Incubation of membranes containing the wild-type rat M_3 muscarinic receptor [M3(wt)] with MTSEA (0.5−5 mM, 10 min at room temperature) resulted in a concentration-dependent loss of [^{3}H]NMS binding sites (72 ± 2% decrease in B_{max} at 5 mM MTSEA; Figure 7A), suggesting that modification by MTSEA of one or more water-accessible Cys residues interfered with [^{3}H]NMS binding. Interestingly, Cys532 is the only Cys residue in the M_3 muscarinic receptor located in the extracellular half of the TM receptor core (Figure 1) where the binding of muscarinic ligands is thought

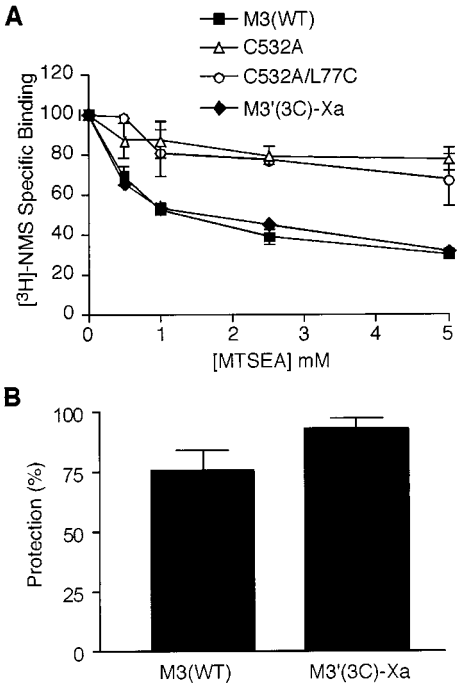

FIGURE 7: Reaction of MTSEA with wild-type and mutant M_3 muscarinic receptors. (A) Effect of MTSEA treatment on [^{3}H]NMS binding to wild-type and mutant M_3 muscarinic receptors. The C532A single- or the C532A/L77C double-point mutation was introduced into the wild-type rat M_3 muscarinic receptor [M3(wt)]. Membranes (~10−20 μg of protein) prepared from COS-7 cells transiently expressing the indicated receptor constructs were incubated for 10 min at room temperature with increasing concentrations (from 0.5 to 5 mM) of MTSEA, followed by [^{3}H]NMS saturation binding studies. For each receptor, the number of [^{3}H]-NMS binding sites (B_{max}) assessed in the absence of MTSEA was set equal to 100% (see the text for absolute receptor densities). (B) *N*-Methylatropine protection experiments carried out with the M3(wt) and M3′(3C)-Xa receptors. Membranes (~10−20 μg of protein) generated from transfected COS-7 cells were first incubated for 20 min at room temperature in the absence or presence of 10 μM *N*-methylatropine and then reacted with MTSEA (2.5 mM) for 10 min at room temperature. After a washing step, samples were processed for [^{3}H]NMS binding assays as described in Experimental Procedures. Protection was calculated as 1 − (inhibition in the presence of *N*-methylatropine)/(inhibition in the absence of *N*-methylatropine). The data are presented as means ± SEM of at least three independent experiments, each carried out in duplicate or triplicate.

to occur (*34*−*36*). We therefore considered it likely that modification of Cys532 by MTSEA was responsible for the detrimental effect of MTSEA on [^{3}H]NMS binding to the M_3 receptor. Consistent with this concept, the M3′(3C)-Xa construct exhibited essentially the same degree of MTSEA reactivity as the wild-type M_3 receptor (Figure 7A). Moreover, the inhibitory effect of MTSEA on [^{3}H]NMS binding was greatly reduced in the C532A mutant M_3 receptor (23 ± 6% decrease in B_{max} at 5 mM MTSEA; Figure 7A). The effect of the C532A point mutation on MTSEA sensitivity could not be analyzed in the M3′(3C)-Xa receptor background since the resulting mutant receptor lacked the ability to bind muscarinic ligands (*37*). One possible explanation for this latter observation is that the C532A point mutation destabilizes the structure of the M3′(3C)-Xa receptor, probably due to the multiple mutations already present in this construct.

As shown in Figure 7B, the reversible muscarinic antagonist, *N*-methylatropine (10 μM), retarded the reaction of MTSEA (2.5 mM) with the M3(wt) and M3′(3C)-Xa receptors. The degree of protection was similar for the two receptors [77 ± 9% for M3(wt) and 93 ± 6% for M3′(3C)-Xa].

To investigate whether Leu77 (Cys77), like Cys532, also projects into the hydrophilic receptor binding crevice, we introduced the L77C point mutation into the M_3(wt) receptor containing the C532A substitution. However, the resulting C532A/L77C mutant receptor did not show an increase in MTSEA sensitivity, as compared with the C532A receptor (30 ± 8% decrease in the [^{3}H]NMS B_{max} value at 5 mM MTSEA; Figure 7A).

[^{3}H]NMS saturating binding studies showed that introduction of the C532A single- or the C532A/L77C double-point mutation into the M3(wt) receptor had no significant effect on receptor expression levels (B_{max} in picomoles per milligram): 2.9 ± 0.7 for M3(wt), 4.2 ± 0.9 for C532A, and 2.5 ± 0.2 for C532A/L77C (n = 3). The C532A mutant receptor exhibited ~5−6-fold reduced [^{3}H]NMS and carbachol binding affinities, as compared with the M3(wt) receptor [[^{3}H]NMS pK_d, 10.23 ± 0.04 for M3(wt) and 9.44 ± 0.08 for C532A; carbachol pK_{app}, 4.24 ± 0.05 for M3(wt) and 3.51 ± 0.09 for C532A]. The corresponding values for the C532A/L77C mutant receptor were as follows: [^{3}H]NMS pK_d, 9.52 ± 0.40; and carbachol pK_{app}, 4.80 ± 0.30.

DISCUSSION

This study was designed to provide insight into the three-dimensional structure of the inactive and active state of the M_3 muscarinic receptor. Specifically, we used a recently developed disulfide cross-linking scanning strategy that allows monitoring of the formation of disulfide bonds between adjacent Cys residues with receptors being present in their native membrane environment (*22*). During the past few years, several groups have employed different biophysical and biochemical approaches to map helix−helix contact sites and to study activity-dependent changes in GPCR structure. Most of these studies were carried out with mutant versions of rhodopsin (*23*−*33*) or the β2-adrenergic receptor (*17*, *19*, *21*) studied after detergent solubilization and additional purification steps. Thus, there is a clear need for additional studies designed to examine GPCR structure and agonist-dependent changes in receptor conformation with receptors being present in their native membrane environment (in situ).

The specific aim of this study was to monitor proximities between amino acid residues present in TM I and TM VII of the M_3 muscarinic receptor, both in the absence and in the presence of activating ligands. Toward this goal, we initially generated 10 mutant M_3 receptors all of which contained one free (non-disulfide-bonded) endogenous Cys residue, Cys532 (position 7.42), located close to the center of TM VII, and a second free Cys residue within TM I at positions Ala71−Val80 (positions 1.36−1.45).

All 10 Cys mutant receptors were able to bind the antagonist, [^{3}H]NMS, and the agonist, carbachol, with high affinities, similar to those obtained with the M3′(3C)-Xa receptor from which they were derived (Table 1). Moreover, PI assays showed that none of the 10 TM I Cys substitutions had a significant effect on the efficiency of agonist-induced

receptor−G protein coupling (Table 1). These findings demonstrated that none of Cys substitutions interfered with the proper folding of the resulting mutant M_3 receptor proteins. The ligand binding data also indicated that the targeted TM I residues do not make a significant contribution to the binding of muscarinic ligands, consistent with previous findings demonstrating that the key residues involved in the recognition of muscarinic ligands are located predominantly on TM III and V−VII (*34*−*36*).

To induce disulfide bond formation between adjacent Cys residues, receptor-containing membrane preparations were initially exposed to the oxidizing agent, Cu−Phen (25 and 100 μM). Successful disulfide cross-linking was monitored via Western blotting by studying the appearance of a 38 kDa full-length receptor band after factor Xa digestion of oxidized receptors (*22*). In the absence of ligands, the receptor containing the L77C point mutation in TM I was the only mutant receptor displaying significant disulfide cross-linking (Figure 3), suggesting that the Cys residues present at positions 77 and 532 in this mutant receptor can face each other in the three-dimensional structure of the receptor.

To estimate the approximate distance between Cys77 and Cys532, we carried out additional cross-linking studies using the rigid, thiol-specific cross-linking agents *o*-PMD, BMOE, and *p*-PMD (distances between the reactive maleimido groups of 6, 8, and 10 Å, respectively). Among these three agents, only *p*-PMD was able to cross-link Cys77 and Cys532 (Figure 6). This observation indicated that the two residues were separated by a distance of ~10 Å which is still in the range of disulfide cross-linking (*52*). In agreement with this finding, the α-carbon atoms of the residues present at the corresponding positions (1.42 and 7.42) in bovine rhodopsin (Leu47 and Ala295, respectively) are ~11 Å apart, as predicted by the high-resolution X-ray structure (*6*). However, the high-resolution rhodopsin structure also indicates that Leu47 and Ala295 (Leu77 and Cys532, respectively, in the M_3 muscarinic receptor) do not face each other at the TM I−TM VII interface but that residue 7.42 projects into the central ligand binding cavity away from TM I (Figure 8). One possibility therefore is that the relative orientation of TM I and TM VII in the M_3 muscarinic receptor differs from that of bovine rhodopsin. Consistent with this concept, several other experimental observations also suggest that the structure of biogenic amine GPCRs is probably not identical to that of bovine rhodopsin (reviewed in ref *9*).

In contrast to rhodopsin, the M_3 muscarinic receptor and most other class I GPCRs do not contain a proline residue within TM I (*9*). In rhodopsin, the presence of the TM I proline residue at position 1.48 results in a significant kink that bends TM I inward toward the TM receptor core (*6*, *9*). In the absence of the proline residue, the TM I region of the M_3 muscarinic receptor may form a more ideal α-helix which may affect the orientation of specific TM I residues relative to adjacent TM helices. A characteristic feature of the structure of TM VII in bovine rhodopsin is the presence of an unusual 3_{10} helix involving residues 7.43−7.46 (*6*). As has been discussed elsewhere (*6*, *9*), this unusual feature may be stabilized by the covalent attachment of the aldehyde group of the 11-*cis*-retinal ligand to the ϵ-amino group of Lys296 (position 7.43). Since other GPCRs, including the M_3 muscarinic receptor, do not share this unique feature, it is possible that the orientation of specific TM VII residues relative to TM I differs between rhodopsin and other class I receptors. One possibility therefore is that Cys532 (position 7.42) is located more closely at the TM I−TM VII interface in the M_3 muscarinic receptor which would allow the formation of a disulfide bond between Cys532 and a Cys residue present at position 1.42.

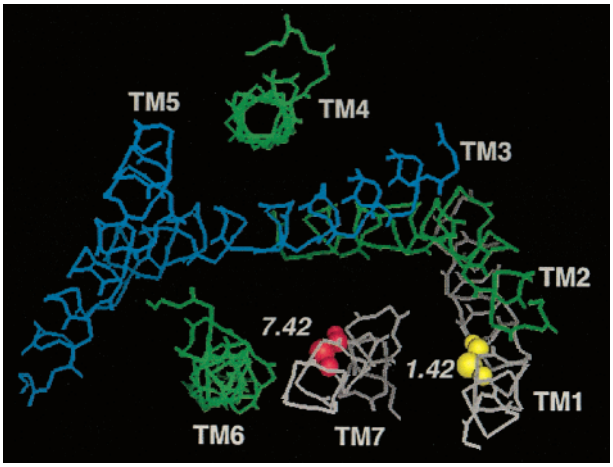

FIGURE 8: Predicted location of amino acid residues present at positions 1.42 and 7.42 in the three-dimensional structure of bovine rhodopsin. Only the seven TM helices are shown ("top" view). The depicted structure is based on the high-resolution crystal structure of bovine rhodopsin in its inactive state (Protein Data Bank entry 1F88) (*6*). In bovine rhodopsin, positions 1.42 and 7.42 correspond to Leu47 and Ala295, respectively. The corresponding residues in the rat M_3 muscarinic receptor are Leu77 and Cys532, respectively. As described in the text, our cross-linking studies suggest that Leu77 and Cys532 are facing each other in the three-dimensional structure of the M_3 muscarinic receptor. However, in the structure of bovine rhodopsin, Ala295 (position 7.42) projects away from Leu47 (position 1.42), suggesting that the relative orientation of TM I and VII may not be identical in rhodopsin and in the M_3 muscarinic receptor.

An alternative explanation for the observed formation of a disulfide bond between Cys residues present at positions 1.42 and 7.42 in the M_3 muscarinic receptor is that the extracellular segments of TM I and VII are able to undergo considerable dynamic movements. Such a model is supported by the finding that disulfide cross-linking between Cys77 and Cys532 did not occur with high efficiency (Figure 3), suggesting that the two residues may not be located very close to each other. Moreover, in bovine rhodopsin (*6*), the β-carbon atom of residue 1.42 makes the closest approach to TM VII of any of the TM I residues that were substituted in this study. Again, this observation would be more consistent with a model in which a dynamic rotation of TM VII could bring Cys532 within disulfide cross-linking distance of a Cys residue present at position 1.42. Such conformational flexibility of the extracellular portions of the TM bundle may be a prerequisite for the proper docking of GPCR ligands.

However, the above observations do not necessarily rule out the possibility that the relative orientations of TM I and TM VII somewhat differ between rhodopsin and the M_3 muscarinic receptor. For example, it is possible that a combination of both factors, dynamic fluctuations in receptor structure and minor changes in the relative orientation of TM I and TM VII, are responsible for the observed disulfide cross-linking pattern. Clearly, additional TM I−TM VII

contact sites need to be identified to distinguish between these two possibilities.

To examine whether receptor activation was associated with changes in the orientation of Cys532 relative to residues in TM I, we carried out additional cross-linking experiments in the presence of the antagonist, atropine, and the agonist, carbachol. Interestingly, when Cu−Phen served as the oxidizing agent, the ability of the L77C mutant receptor to undergo disulfide cross-linking was abolished in the presence of atropine and was significantly worsened in the presence of carbachol and other muscarinic agonists. The extent of inhibition of disulfide cross-linking correlated with the affinity of the ligands for the M_3 receptor: atropine > acetylcholine = oxotremorine M > carbachol ≫ tetramethylammonium (*53*). The observation that atropine treatment completely abolished the appearance of the L77C receptor 38 kDa band (which is indicative of disulfide cross-linking) convincingly demonstrated that cross-linking involved functional (atropine-sensitive) L77C receptors rather than misfolded or partially denatured receptor species.

Since both atropine and agonist ligands abolished or greatly decreased the level of Cu−Phen-dependent disulfide cross-linking in the L77C receptor (Figure 3A), we considered it likely that this phenomenon was caused by competition between muscarinic ligands and the relatively bulky Cu−Phen moiety for access to the hydrophilic binding crevice. Although the mechanism by which Cu−Phen catalyzes disulfide bond formation is not well-understood, previous work (*39*) suggests that Cu−Phen-catalyzed disulfide bond formation does not require the generation of hydrogen peroxide as an oxidizing intermediate. This observation is in agreement with the concept (but does not prove it) proposed here that Cu−Phen must be present in the direct vicinity of the sulfhydryl groups to be cross-linked. Consistent with this notion, the efficiency of disulfide cross-linking displayed by the L77C mutant receptor was not significantly affected by atropine or agonist ligands when iodine, which has a molecular size of <2.7 Å, served as the oxidizing agent (Figure 3B). Iodine cross-linking experiments also showed that none of the remaining nine TM I Cys mutant receptors showed a significant degree of disulfide cross-linking, either in the absence or in the presence of carbachol (Figure 3B). Similarly, carbachol treatment of L77C-containing membrane preparations had no significant effect on the ability of the bifunctional thiol-specific cross-linker, *p*-PMD, to cross-link Cys77 and Cys532 (Figure 6). This observation was somewhat surprising, given that *p*-PMD is a relatively bulky molecule (though much smaller than Cu−Phen) and that the antagonist ligand, *N*-methylatropine, was able to retard the reaction of MTSEA with Cys532 (Figure 7B; see the discussion below). One possible explanation for these seemingly discrepant findings is that *p*-PMD and MTSEA differ in the nature of their reactive groups and in their overall molecular geometries, perhaps allowing these two agents to approach Cys532 by somewhat different routes.

Taken together, the iodine cross-linking data support the concept that the TM VII region surrounding Cys532 does not undergo significant structural changes during receptor activation. However, the occurrence of minor conformational changes in this region, which may be difficult to detect by the use of a disulfide cross-linking strategy, cannot be completely excluded. In addition, the possibility exists that the more cytoplasmic segment of TM VII which contains the highly conserved Asn-Pro motif (positions 7.49 and 7.50) undergoes more pronounced structural changes during receptor activation. In fact, the concept that the C-terminal segment of TM VII may undergo activity-dependent conformational changes is supported by both site-directed spin labeling (*26*) and immunological studies (*54*) carried out with bovine rhodopsin.

Replacement of Cys532 in the M3(wt) receptor with alanine resulted in a 5−6-fold decrease in [^{3}H]NMS and carbachol binding affinities, in agreement with previous mutagenesis studies carried out with the M_1 muscarinic receptor subtype (*36*, *54*). Moreover, while the level of [^{3}H]-NMS binding to the M3(wt) and M3′(3C)-Xa receptors could be greatly reduced by incubation of receptor-containing membranes with the positively charged sulfhydryl-specific agent, MTSEA, [^{3}H]NMS binding to the M3(wt)-C532A mutant receptor was only slightly impaired by MTSEA treatment (Figure 7A). In addition, the reversible muscarinic antagonist, *N*-methylatropine, significantly retarded the reaction of MTSEA with the M3(wt) and M3′(3C)-Xa receptors (Figure 7B). Taken together, these observations provide strong experimental evidence that Cys532 (position 7.42) faces the hydrophilic ligand binding crevice where it can covalently react with the charged MTSEA agent.

To study whether Leu77 also projects into the hydrophilic ligand binding cavity, we also examined the ability of MTSEA to inhibit [^{3}H]NMS binding to an M3(wt)-derived mutant receptor containing the C532A/L77C double mutation [the C532A mutation was required to render the M3(wt) receptor MTSEA-insensitive]. However, MTSEA treatment of the M3(wt)-C532A/L77C mutant receptor did not lead to reduced [^{3}H]NMS binding activity (Figure 7). In agreement with this observation, Shi et al. (*55*) recently showed that charged MTS reagents were unable to inhibit antagonist binding to mutant human D2 dopamine receptors containing single Cys substitutions within TM I (at positions 1.37−1.49). There are two possible scenarios that could explain the observation that MTSEA treatment had no significant effect on [^{3}H]NMS binding to the M3(wt)-C532A/L77C mutant receptor. First, Leu77 may not face the hydrophilic ligand binding crevice and may therefore not be accessible to the MTSEA reagent. Second, L77C may indeed be located in a hydrophilic environment and be modified by MTSEA, but MTSEA modification of Cys77 may still allow ligand binding. Clearly, this second scenario is the more likely one since Cys77 can form cross-links with Cys532 which faces the hydrophilic receptor binding crevice. If it is assumed that Cys77 does in fact react with the MTS reagent, the increased size of the Cys side chain may not be sufficient to interfere with ligand binding because of the rather peripheral location of TM I.

In conclusion, we have used a novel in situ disulfide cross-linking strategy to map the proximity of amino acid residues present in TM I and TM VII of the M_3 muscarinic receptor. The iodine cross-linking data suggest that agonist activation of the M_3 receptor does not lead to significant changes in the relative orientation of the outer segments of TM I and VII. Since the M_3 muscarinic receptor is closely structurally homologous to many other class I GPCRs, our findings should be of broad general relevance.

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