Identification and Molecular Characterization of m3 Muscarinic Receptor Dimers*

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Several studies suggest, but do not prove directly, that muscarinic receptors may be able to form dimeric or oligomeric arrays. To address this issue in a more direct fashion, we designed a series of biochemical experiments using a modified version of the rat m3 muscarinic receptor (referred to as m3*) as a model system. When membrane lysates prepared from m3* receptor-expressing COS-7 cells were subjected to Western blot analysis under non-reducing conditions, several immunoreactive species were observed corresponding in size to putative receptor monomers, dimers, and oligomers. However, under reducing conditions, the monomeric receptor species represented the only detectable immunoreactive protein, consistent with the presence of disulfide-linked m3 receptor complexes. Similar results were obtained when native m3 muscarinic receptors present in rat brain membranes were analyzed. Control experiments carried out in the presence of high concentrations of the SH group alkylating agent, N-ethylmaleimide, suggested that disulfide bond formation did not occur artifactually during the preparation of cell lysates. The formation of m3* receptor dimers/multimers was confirmed in coimmunoprecipitation studies using differentially epitope-tagged m3* receptor constructs. In addition, these studies showed that m3* receptors were also able to form non-covalently associated receptor dimers and that m3* receptor dimer formation was receptor subtype-specific. Immunological studies also demonstrated that m3* receptor dimers/multimers were abundantly expressed on the cell surface. Site-directed mutagenesis studies indicated that two conserved extracellular Cys residues (Cys-140 and Cys-220) play key roles in the formation of disulfide-linked m3* receptor dimers. These results provide the first direct evidence for the existence of muscarinic receptor dimers and highlight the specificity and molecular diversity of G protein-coupled receptor dimerization/oligomerization.

Traditionally, molecular models describing the interaction of G protein-coupled receptors (GPCRs) with their G protein targets have been based on the assumption that the receptors exist as monomers and couple to G proteins in a 1:1 stoichiometry. However, several recent studies suggest that GPCRs are able to form dimeric or oligomeric arrays (1–9), indicating that classical models of receptor/G protein coupling may be oversimplified (for a recent review, see Ref. 10).

Hebert et al. (2), for example, provided the first direct evidence that β2-adrenergic receptors can exist in dimeric forms. These investigators also demonstrated that dimer formation occurred through non-covalent interactions and that receptor dimers were not formed artifactually during processing of samples for immunological studies. Moreover, dimer formation was shown to correlate well with the ability of the β2-adrenergic receptor to interact productively with G proteins (2). In addition, consistent with the notion that GPCR dimerization may be functionally relevant, a dimerization-defective mutant δ-opioid receptor lacked the ability to undergo agonist-dependent internalization (5).

Radioligand binding studies (11–14) suggest that muscarinic receptors, like other GPCRs, may also be arranged in dimeric or oligomeric complexes. Potter et al. (11), for example, showed that the complex agonist binding properties of muscarinic receptors expressed in rabbit heart and rat brain stem were consistent with the presence of two agonist binding sites located on dimeric receptor molecules. Likewise, computer simulations of the binding properties of the agonist, [3H]oxotremorine-M, at m2 muscarinic receptors expressed in cultured cells or porcine atria were consistent with the existence of receptor dimers as well as monomers (12). Similar findings were obtained in studies using purified muscarinic receptors (13), suggesting that the complex binding properties of muscarinic agonists are due to different states of the receptor proteins themselves and are not caused by the association of receptors with different classes of G proteins. However, although the results of these studies are consistent with the presence of muscarinic receptor dimers (or multimers), they do not provide direct physical evidence that such receptor species actually exist.

To examine potential intermolecular interactions between mutant muscarinic receptors, we previously generated two chimeric m3 muscarinic/β2-adrenergic receptors in which a region containing transmembrane domains (TM) VI and VII had been exchanged between the two wild-type receptors (15). Whereas none of the two-hybrid receptors showed ligand binding activity when expressed alone, significant numbers of muscarinic and adrenergic binding sites were detected after coexpression of the two mutant receptors (15). These data strongly suggested that the two chimeric proteins were able to interact functionally with each other, probably due to the ability of N- and C-terminal receptor regions (containing TM I-V and TM VII, inositol; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane domains.)
residues) to adopt the proper transmembrane topology (16–18), even when present in the structural context of the mutant receptors. It remains unclear, however, whether such intramolecular interactions can also occur among wild-type muscarinic receptors.

To demonstrate the existence of muscarinic receptor dimers or oligomers in a direct fashion, we used the rat m3 muscarinic receptor as a model system, employing a combined biochemical/molecular genetic approach. Initially, immunoblotting experiments were carried out under reducing and non-reducing conditions, using membrane lysates prepared from COS-7 cells transfected with a modified version of the m3 receptor (referred to as m3'; see Table I) as well as rat brain membranes expressing the native m3 muscarinic receptor. These studies strongly suggested that the m3 muscarinic receptor is capable of forming disulfide-linked dimers and oligomers. This finding was confirmed in coimmunoprecipitation experiments using two modified m3 receptor species that carried different epitope tags at their C termini. Moreover, additional experiments were carried out to investigate the receptor subtype specificity of m3 receptor dimerization and to verify that m3 receptor dimers preexisted in transfected cells prior to cell lysis.

We also examined whether m3 receptor dimers were present on the cell surface or whether they were retained intracellularly. To address this question, transfected COS-7 cells were incubated with a membrane-impermeable monoclonal antibody directed against an extracellular receptor epitope tag. Isolation and analysis of antibody-bound receptors via immunological techniques clearly demonstrated that m3 receptor dimers were abundantly expressed on the cell surface.

Finally, site-directed mutagenesis studies were performed to identify the Cys residue(s) involved in the formation of disulfide-linked m3 receptor dimers/oligomers. Mutant receptors were created in which specific Cys residues were replaced with either Ser or Ala and expressed in COS-7 cells. Subsequently, the expression pattern of the different mutant receptors was examined in immunoblotting studies under reducing and non-reducing conditions. These studies led to the identification of a conserved Cys pair (Cys-140/Cys-220) that is predicted to play a key role in the formation of disulfide-linked m3 receptor dimers.

**EXPERIMENTAL PROCEDURES**

**Materials**—CNBr-activated Sepharose 4B, proteinase inhibitors, carboxymethyl-choline chloride (carbachol), acetylecholine chloride, and atropine sulfate were purchased from Sigma. 4-Diphenylacetoxy-N-methylpiper- eridine methiodide ([DAMP]) was from Research Biochemicals Inc., and N-[3H]methylscopolamine ([3H]NMS; 79 Ci/mmol) and myo-[3H]inositol (20 Ci/mmol) were from NEN Life Science Products. All enzymes used for molecular cloning were obtained from New England Biolabs. Other sources of reagents were 12CA5 monoclonal antibody (Roche Molecular Biochemicals), anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech), and enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech). Nitrocellulose membranes (0.2 μm) were obtained from Schleicher & Schuell. All other chemicals used for SDS-PAGE and Western blotting were purchased from Bio-Rad.

**DNA Constructs**—All mutations were introduced into Rn3pcD-N-HA (also referred to as m3wt; Table I), a mammalian expression plasmid coding for the rat m3 muscarinic receptor (19) containing a 9-amino acid hemagglutinin (HA) epitope tag (YPYDVPDYA; Ref. 20) after the initiating methionine codon (18). The presence of the epitope tag had no significant effect on the ligand binding and G protein coupling properties of the wild-type m3 receptor (19). Mutations were introduced by using standard polymerase chain reaction mutagenesis techniques (21) or the QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The five N-terminal Asn residues that are predicted to serve as targets for N-linked glycosylation (Asn-6, Asn-15, Asn-41, Asn-48, and Asn-52; Fig. 1) were simultaneously replaced, using a two-step polymerase chain reaction procedure, with Gln residues. Moreover, the central portion (amino acids Ala-274 to Lys-469) of the third intracellular loop (13) was deleted from the glycosylation-free mutant construct as described previously (22). For the sake of simplicity, the encoded receptor was referred to as m3' (Table I). The construction and complete pharmacologic characterization of m3'-derived mutant receptors in which one or more Cys residues were preexisted in transfected cells prior to cell lysis.

**Cell Culture and Transfections**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2% glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO2 incubator. For transfections, 1 × 106 cells were seeded into 100-mm dishes. About 24 h later, cells were transfected with 4 μg of plasmid DNA/dish by using the LipofectAMINE® Plus kit (Life Technologies, Inc.) according to the manufacturer’s instructions.

**Radioligand Binding Studies**—Cells to be used for radioligand binding studies were harvested about 70 h after transfections. Binding assays were carried out with membrane homogenates prepared from transfected cells essentially as described (24). Incubations were carried out for 2–3 h at room temperature in a 0.25–0.5-ml volume. Binding buffer consisted of 25 mM sodium phosphate (pH 7.4) and 5 mM MgCl2. Six to eight different concentrations (15–20,000 pM) of the radioligand, [3H]NMS, were used in saturation binding assays. For competition binding studies, seven different concentrations of the cold inhibitors, 4-DAMP, carbachol, and acetylecholine, were used. The [3H]NMS concentration used in these experiments was 200 pM, except for studies with the m3’/C140A/C220A construct where an [3H]NMS concentration of 2 nM was employed. Nonspecific binding was determined in the presence of 10 μM (m3’/C140A/C220A) or 1 μM atropine (all other receptors).

Binding data were analyzed by nonlinear least squares curve-fitting procedures, using the computer program LIGAND (saturation binding data; Ref. 25) or KALEIDAGRAPH (competition binding data; Synergy software, respectively).

**Receptor-mediated Stimulation of Phosphatidylinositol (PI) Hydrolysis**—For PI assays, transfected COS-7 cells were washed into 6-well plates (approximately 0.5 × 105 cells/well) about 24 h after transfections, and 3 μCi/ml myo-[3H]inositol was added to the growth medium. The next day (20–24 h later), the labeled cells were washed once with 2 ml of Hanks’ balanced salt solution containing 20 mM HEPES and then incubated for 20 min (at room temperature) with 1 ml of the same medium containing 10 mM LiCl. Cells were then incubated in the same medium with different concentrations of carbachol for 1 h at 37 °C. After removal of the medium, reactions were terminated by addition of 0.75 ml of 20 mM formic acid, followed by a 30-min incubation at 4 °C. Samples were then neutralized with 0.25 ml of 60 mM ammonium hydroxide, and the inositol monophosphate fraction was isolated by anion exchange chromatography as described (26) and counted on a LKB liquid scintillation counter.

**Preparation of Membrane Extracts**—Membranes were prepared from total rat brain as described previously (13). For immunoblotting experiments, membranes were incubated with sample buffer (for composition, see below) at room temperature for 30 min prior to SDS-PAGE.

**Preparation of Rat Brain Membranes**—COS-7 cells were collected by centrifugation about 70 h after transfections. Cell pellets were incubated with 20 ml of reduction buffer for 20 min with the following composition: 10 mM PBS containing a mixture of proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). This step served to remove soluble as well as peripheral membrane proteins. Following centrifugation at 2,000 × g for 10 min, supernatants were...
disected, and cell pellets were treated with lysis buffer (PBS containing 1% digitonin, 0.5% deoxycholate, and a mixture of proteinase inhibitors (see above)) at 4 °C for 1 h. Cell lysates were then centrifuged in a refrigerated Eppendorf 5417R microcentrifuge at maximal speed for 30 min. Supernatants containing solubilized receptors were used either directly for SDS-PAGE or stored at −80 °C for later use.

Binding of m3 Muscarinic Receptors to ABT-Agarose Affinity Gels—Membrane extracts prepared from COS-7 cells transfected with the m3 receptor construct were subjected to affinity chromatography using 3-(2′-aminobenzylhydroxy)-tropane (ABT)-agarose (kindly provided by Dr. T. Haga, University of Tokyo, Japan), essentially as described by Haga et al. (25). Briefly, m3 receptor-expressing COS-7 cells were first treated with 0.2% digitonin to remove peripheral membrane and soluble-proteins, as described in the previous paragraph. Receptors were then solubilized in buffer A (10 mM Tris-HCl (pH 7.4), containing 1% digitonin, 0.1% sodium cholate, 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin). Following centrifugation in a refrigerated Eppendorf 5417R microcentrifuge at maximal speed for 30 min, supernatants (100 μl, about 20 μg protein/μl) containing solubilized receptors were incubated with 20 μl of ABT-agarose gel suspension at 4 °C overnight (about 16 h). Nonspecifically-bound proteins were washed out with 3 × 1 ml of buffer B (buffer A containing 0.2 mM NaCl and 0.1% digitonin). Specifically-bound receptor proteins were eluted using 50 μl of buffer B containing 100 μM atropine. Eluted receptor proteins were subjected to Western blotting analysis using the anti-m3 antibody (see next paragraph).

Antibodies—A polyclonal antibody was raised in rabbits against a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to the C-terminal 18 amino acids of the rat m3 muscarinic receptor (Fig. 1). This antibody (referred to as anti-m3) was purified by affinity chromatography on a peptide column according to standard procedures and specifically recognized the m3 muscarinic receptor protein. An affinity purified anti-V2 polyclonal antibody (raised in rabbits against a peptide corresponding to the C-terminal 29 amino acids of the human V2 vasopressin receptor) was kindly provided by Dr. Paul Goldsmith (National Institutes of Health). m1 and m2 muscarinic receptor-specific rabbit antisera were raised against i3 loop-glutathione S-transferase fusion proteins essentially as described by Leyev et al. (28). These antiserum was used directly for Western blotting experiments. The ID4 mouse monoclonal antibody (referred to as anti-Rh throughout this study) that specifically recognizes the C-terminal segment of bovine rhodopsin (29) was generously provided by Dr. Kevin Ridge (Center for Advanced Research in Biotechnology). The 12CA5 anti-HA monoclonal antibody that specifically detects the HA epitope tag was purchased from California Biochemicals. Immunoprecipitates, the anti-HA, anti-Rh, and anti-V2 antibodies were covalently conjugated to Sepharose 4B according to the manufacturer’s instructions (Sigma).

Immunoprecipitation Studies—Membrane extracts prepared from transfected COS-7 cells were incubated with Sepharose 4B-conjugated antibodies under gentle rotating at 4 °C for at least 2 h. Incubations were carried out in PBS containing 0.5% digitonin, 0.5% bovine serum albumin (BSA), and proteinase inhibitors (see above). Immunoprecipitates were collected by centrifugation, washed three times with PBS containing 0.1% digitonin, eluted with Laemmli buffer (see below), and directly subjected to SDS-PAGE. To eliminate non-covalent interactions among receptors, membrane extracts prepared from cells cotransfected with m3 receptor constructs containing different epitope tags were incubated with 1.5% SDS at 37 °C for 1 h. Prior to immunoprecipitation, the SDS concentration was reduced to 0.1% by dilution of samples with PBS containing 0.5% BSA.

Electrophoresis and Western Blotting—SDS-PAGE was performed essentially as described by Laemmli (30). Samples were mixed with an equal volume of 2-fold concentrated sample buffer (composition: 125 mM Tris-HCl (pH 6.8), 20% glycerol, 100 mM dithiothreitol (only if indicated), 4% SDS, and 0.01% (w/v) bromphenol blue) and incubated at room temperature for 30 min. Subsequently, proteins were resolved on 8 or 12% (w/v) acrylamide slab gels in the presence of 0.1% SDS. Proteins were electrophoretically transferred onto nitrocellulose membranes (0.2 μm) as described (31). In brief, membranes were blocked with 5% BSA in PBS and incubated with the primary antibody (1 μg/ml) at room temperature for 1 h. After washing in PBS containing 0.05% Tween 20 and 1% BSA (PBS-T/BSA), bound antibody was then probed with a secondary antibody (1:1000 dilution) conjugated to horseradish peroxidase in PBS-T/BSA for 1 h at room temperature. After extensive washing of blots with PBS containing 0.05% Tween 20, proteins were visualized using an ECL kit (Amersham Pharmacia Biotech).

Detection of m3 Receptor Dimers on the Cell Surface—COS-7 cells were transfected in 100-mm dishes with the m3 receptor construct as described above. About 70 h after transfections, cells were washed with ice-cold PBS and then incubated with 5 ml of PBS containing 3 μg/ml of the 12CA5 anti-HA antibody and 0.5% BSA label cell-surface m3 receptor species. Incubations were carried out at room temperature for 2 h. Subsequently, cells were washed three times with PBS and transferred into 1.5-ml Eppendorf tubes. Cells were then treated with 100 μl of lysis buffer (PBS containing 1% digitonin, 0.5% deoxycholate, and proteinase inhibitors) at 4 °C for 1 h. After centrifugation in a refrigerated Eppendorf 5417R microcentrifuge (maximal speed, 30 min), 10 μl of supernatant was subjected directly to non-reducing SDS-PAGE to examine total solubilized receptors. The remaining extract (90 μl) was incubated with protein A-Sepharose 4B beads (15 μl) at 4 °C for 1 h to immunoprecipitate antibody-bound surface receptors. Supernatants containing receptors that did not bind anti-HA antibody were subjected to non-reducing SDS-PAGE (15 μl/well) to examine the expression of intracellular receptors. Immunoprecipitates (containing cell surface receptors) were washed twice with PBS containing 0.1% digitonin, eluted with 30 μl of sample buffer (see above), and then subjected to SDS-PAGE (10 μl/well). After electrophoresis, receptor proteins were detected via Western-blotting analysis using the anti-m3 antibody as described above.

RESULTS

Detection of Disulfide-linked m3 Receptor Dimers and Oligomers via Western Blotting—COS-7 cells were transiently transfected with a modified version of the rat m3 muscarinic receptor (referred to as m3). The m3 receptor contained an N-terminal HA epitope tag and lacked all five potential N-glycosylation sites present in the N-terminal portion of the m3 receptor protein (N6Q, N15Q, N41Q, N48Q, and N52Q) as well as the central portion of the i3 loop (3aa–274 to Lys–469) (Fig. 1 and Table I). These structural modifications had little effect on the ligand binding and G protein coupling properties of the m3 muscarinic receptor (Table II), consistent with previous studies using the m3 (22, 32) as well as other muscarinic receptor subtypes (33–37). However, in contrast to the wildtype m3 receptor, the m3 receptor could be detected easily and reproducibly in immunoblotting experiments using antibodies directed against the N and C termini of the receptor protein (see below), probably due to the lack of heterogeneous glycosylation and increased receptor stability. For this reason, the m3 receptor was used throughout this study.

Initially, two different antibodies, the anti-m3 rabbit polyclonal antibody (which was raised against a peptide corresponding to the C-terminal 18 amino acids of the rat m3 muscarinic receptor) and the anti-HA 12C5A monoclonal antibody (which recognizes the N-terminal receptor epitope tag), were employed to detect the m3 receptor in immunoblotting experiments, using membrane lysates prepared from m3-expressing COS-7 cells (Fig. 2). When SDS-PAGE was performed under non-reducing conditions (DTT was omitted from the sample buffer), several immunoreactive bands were observed (Fig. 2, A and B, lane 1); a band migrating at around 45 kDa corresponding in size to the putative m3 receptor monomer, as well as several higher molecular mass forms of approximately 75, 90, and >120 kDa. The 75-kDa band was detected clearly only with the anti-m3 antibody (Fig. 2a, lane 1), since the anti-HA antibody cross-reacted with an endogenous COS-7 cell protein of similar size (Fig. 2B, lane V).

Interestingly, the 90-kDa band that was detected by both antibodies corresponded in size to a putative m3 receptor dimer. It is likely that the 75-kDa band was derived from the 90-kDa species via proteolytic degradation. In agreement with this notion, a weak 30-kDa band was detected only when blots were probed with the anti-m3 antibody (Fig. 2a, lane 1), suggesting that the 30-kDa band corresponded to a degraded m3 receptor species that lacked the N-terminal third (about 15 kDa).

3 F. Y. Zeng and J. Weiss, unpublished data.
kDa) of the receptor protein. Partial degradation of the putative 90-kDa receptor dimer is therefore a likely cause for the appearance of the 75-kDa band.

In parallel, Western blotting experiments were also performed under reducing conditions including the incubation (30 min at room temperature) of samples with 50 mM of the reducing agent, DTT, prior to SDS-PAGE. Under these conditions, the high molecular mass immunoreactive species were no longer observed, and the 45-kDa m3' receptor monomer became the only detectable band (Fig. 2, A and B, lane 3). Taken together, these findings are consistent with the concept that the m3' receptor can form disulfide-linked dimers. The >120-
TABLE II
Ligand binding and functional properties of the m3\(^{9}\), m3\(^{-V2}\), and m3\(^{-Rh}\) muscarinic receptors expressed in COS-7 cells

| Receptor | Antagonist binding, \([\text{H}]\text{NMS}\) | Agonist binding, carbachol | PI hydrolysis |
|---------|-----------------|-----------------|-----------------|
|         | \(b_{\text{max}}\) (fmol/mg) | \(K_D\) (\(\mu\)M) | \(K_{\text{ag}}\) (\(\mu\)M) | Maximum IP\(_3\) production |
| m3(wt)  | 1540 ± 300     | 62 ± 4     | 338 ± 64   | 1.0 ± 0.2 |
| m3\(^{-}\) | 900 ± 199     | 44 ± 7     | 107 ± 39   | 1.0 ± 0.3 |
| m3\(^{-V2}\) | 1050 ± 250   | 78 ± 14    | 160 ± 48   | 2.9 ± 0.3 |
| m3\(^{-Rh}\) | 850 ± 95     | 60 ± 13    | 105 ± 25   | 1.2 ± 0.3 |

Specifically bound m3\(^{9}\) receptor species were eluted from the affinity gel with a buffer containing 100 \(\mu\)M atropine (for details, see "Experimental Procedures"). Atropine-eluted receptors were subjected to non-reducing SDS-PAGE (12\%) and Western blotting analysis using the anti-m3 antibody (Fig. 3, lane 2). As shown in Fig. 3, the observed pattern of high molecular mass m3\(^{9}\) receptor species detected with the atropine eluates was qualitatively similar to that found with membrane extracts that had not been subjected to affinity chromatography (Fig. 3, lane 1). This finding suggests that both monomeric and dimeric/oligomeric forms of the m3\(^{9}\) receptor are capable of binding muscarinic ligands.

Western Blot Analysis of Native m3 Muscarinic Receptors Expressed in Rat Brain—We next wanted to examine whether DTT-sensitive m3 receptor aggregates also exist in native tissues. Toward this goal, membranes were prepared from total rat brain and were subjected to Western blot analysis using the anti-m3 antibody. When these experiments were carried out under reducing conditions, the m3 receptor protein migrated as a single band of about 95 kDa (Fig. 4, lane 1). However, under non-reducing conditions, at least two additional high molecular mass products (about 170–190 kDa in size) could be observed (Fig. 4, lane 2), consistent with the pattern found with m3\(^{-}\)-transfected COS-7 cells. Similar results were obtained when samples were prepared for SDS-PAGE in the continued presence of NEM (10 mM) (data not shown). Generally, the sizes of immunoreactive bands observed in these experiments were considerably larger than those seen with the use of m3\(^{-}\)-transfected COS-7 cells, probably due to extensive glycosylation of the brain m3 receptor and the presence of the full-length i3 loop in the native receptor.

Coimmunoprecipitation Studies Confirm the Formation of m3 Receptor Dimers—Although the Western blotting experiments outlined above suggested the formation of m3 receptor dimers/oligomers, the possibility cannot be excluded that the high molecular mass species observed under non-reducing conditions were caused by cross-linking of m3\(^{-}\) receptors to other membrane proteins. To examine the potential formation of m3 receptor dimers in a more direct fashion, we therefore carried out a series of immunoprecipitation experiments using two m3\(^{-}\) receptor constructs (referred to as m3\(^{-V2}\) and m3\(^{-Rh}\); Table I) that carried different epitope tags at their C termini. In the m3\(^{-Rh}\) receptor, the last nine amino acids of bovine rhodopsin were added to the C terminus of m3\(^{9}\). Analogously, the m3\(^{-V2}\) receptor was obtained by attaching the last 29 amino acids of the human V2 vasopressin receptor to the C terminus of the m3\(^{9}\) construct. Radioligand binding studies showed that the m3\(^{-V2}\) and m3\(^{-Rh}\) receptors showed agonist (carbachol) and antagonist (NMS) binding properties similar to the m3\(^{9}\) receptor (Table II). Likewise, PI assays measuring the ability of the agonist, carbachol, to induce increases in intracellular inositol phosphate levels showed that the m3\(^{-V2}\) and m3\(^{-Rh}\) receptors

FIG. 2. Detection of disulfide-linked m3\(^{9}\) receptor dimers/oligomers via Western blotting analysis. COS-7 cells were transiently transfected with the m3\(^{9}\) receptor construct (lanes 1–3) or with vector (V) DNA (pcD-P5) as a control. Transfected cells were incubated in PBS with (lane 2) or without (lanes 1 and 3) NEM (10 mM) at room temperature for the last 1 h of culture. Membrane extracts (20 \(\mu\)g/lane) were mixed with sample buffer in the absence (lanes 1 and 2) or presence of 50 mM DTT (lane 3) and subjected to 12% SDS-PAGE as described under "Experimental Procedures." Receptor bands were visualized via Western blotting using the anti-m3 antibody (Fig. 3, lane 2). As shown in Fig. 3, the observed pattern of high molecular mass m3\(^{9}\) receptor species detected with the atropine eluates was qualitatively similar to that found with membrane extracts that had not been subjected to affinity chromatography (Fig. 3, lane 1). This finding suggests that both monomeric and dimeric/oligomeric forms of the m3\(^{9}\) receptor are capable of binding muscarinic ligands.

kDa immunoreactive species are likely to represent disulfide-linked oligomeric aggregates of the m3\(^{9}\) receptor.

To exclude the possibility that the high molecular mass m3\(^{9}\) receptor bands were artifacts due to disulfide bond exchange reactions during the preparation of samples for SDS-PAGE, m3\(^{9}\)-expressing COS-7 cells were incubated with the membrane-permeable, SH group alkylating reagent, NEM (10 mM), for 1 h at room temperature immediately before harvesting. In addition, cells were processed for SDS-PAGE in the continued presence of NEM (10 mM) starting from the point of cell lysis. NEM (10 mM) was also present in the loading buffer. As shown in Fig. 2, A and B (lane 2), NEM treatment of samples (cells) had no effect on the observed pattern of high molecular mass immunoreactive species, when Western blotting experiments were carried out under non-reducing conditions. This result indicated that the putative m3\(^{9}\) receptor dimers/oligomers preexisted in COS-7 cells prior to processing of samples for SDS-PAGE.

Binding of Solubilized m3\(^{9}\) Muscarinic Receptors to ABT-Agarose Affinity Gels—To study whether the putative m3\(^{9}\) receptor dimers/oligomers were able to bind muscarinic ligands, we examined the ability of these high molecular mass receptor species to bind specifically to an ABT-agarose affinity gel (ABT is an antagonist ligand that binds to muscarinic receptors with nanomolar affinity; Ref. 27). For these experiments, membrane extracts prepared from COS-7 cells transfected with the m3\(^{9}\) receptor construct were subjected to ABT-agarose affinity chromatography essentially as described by Haga and Haga (27).
anti-m3 antibody. Protein molecular mass standards (in kDa) are indicated. Two separate experiments gave similar results.

**FIG. 5.** Immunoblotting and coimmunoprecipitation studies with m3 receptor constructs carrying different C-terminal epitope tags. COS-7 cells were transfected/cotransfected with the indicated m3 receptor constructs. The anti-Rh antibody was used to detect the 9-amino acid rhodopsin epitope tag present at the C terminus of the m3-Rh receptor. Analogously, the anti-V2 antibody was employed to visualize the m3-V2 receptor that contained the last 28 amino acids of the human V2 vasopressin receptor at its C terminus. A, specificity of the anti-V2 and anti-Rh antibodies. Membrane extracts (20 μg/lane) were mixed with sample buffer in the absence of reducing agents and then subjected to 12% SDS-PAGE. Immunoreactive bands were visualized via Western blotting using the indicated anti-Rh (lanes 1-3) or anti-V2 (lanes 4 and 4) antibodies. B, detection of m3-V2/m3-Rh receptor dimers/multimers using a coimmunoprecipitation strategy. Membrane extracts (containing about 100 μg protein) prepared from COS-7 transfected/cotransfected with the indicated m3 receptor constructs were subjected to immunoprecipitation (IP) by using the anti-Rh (lanes 1-3) or anti-V2 (lanes 4 and 6) antibodies conjugated to Sepharose 4B beads, as described under “Experimental Procedures.” Immunoprecipitated receptors were subjected to 12% SDS-PAGE (non-reducing conditions) and detected via Western blotting using the indicated antibodies. C, to examine whether receptor dimers preexisted in cells (in vivo) or were formed during cell lysis (in vitro), equal volumes of membrane extracts prepared from COS-7 cells individually transfected with the m3-Rh or m3-V2 receptor constructs were mixed before immunoprecipitation by the anti-Rh antibody. D, to eliminate non-covalent interactions among receptors, membrane extracts prepared from COS-7 cells cotransfected with the m3-V2 and m3-Rh receptor constructs were incubated with 1.5% SDS (1 h, 37 °C) and then diluted with PBS to reduce the SDS concentration to 0.1% prior to immunoprecipitation with the anti-Rh antibody. Protein molecular mass standards (in kDa) are indicated. Two additional experiments gave similar results.

Immunoblotting studies (Fig. 5A) demonstrated that the m3-V2 and m3-Rh receptors could be detected specifically with the anti-Rh monoclonal antibody (1D4; this antibody specifically recognizes the C-terminal segment of bovine rhodopsin (29)) and the anti-V2 polyclonal antibody (which is directed against the C terminus of the human V2 vasopressin receptor (23)), respectively. The anti-Rh antibody did not recognize the m3-V2 receptor (Fig. 5A, lane 1) but was able to interact specifically with the m3-Rh receptor when cotransfected with the m3-V2 construct (Fig. 5A, lane 2) or expressed alone (data not shown). Analogously, the anti-V2 antibody was unable to react with the m3-Rh receptor (Fig. 5A, lane 3) but efficiently recognized the m3-V2 receptor when cotransfected with the m3-Rh construct (Fig. 5A, lane 4) or expressed alone (data not shown).

The specificity of the anti-V2 and anti-Rh antibodies was also preserved in immunoprecipitation studies. As shown in Fig. 5B (lane 2), the anti-Rh antibody was unable to precipitate detectable amounts of m3-V2 receptors using membrane lysates prepared from m3-V2-expressing cells. Similarly, no detectable amounts of m3-Rh receptors could be immunoprecipitated by the anti-V2 antibody using membrane lysates prepared from m3-Rh-expressing cells (Fig. 5B, lane 4). In addition, m3-Rh receptors immunoprecipitated by the anti-Rh antibody were not recognized by the anti-V2 antibody (Fig. 5B, lane 1), and m3-V2 receptors immunoprecipitated by the anti-V2 antibody did not cross-react with the anti-Rh antibody (Fig. 5B, lane 5).

Immunoprecipitation studies using cell lysates prepared from cells cotransfected with the m3-V2 and m3-Rh constructs showed that the anti-Rh antibody was able to coprecipitate m3-V2 receptors (Fig. 5B, lane 3). Analogously, the anti-V2 antibody was capable of coprecipitating m3-Rh receptors from lysates prepared from cotransfected cells (Fig. 5B, lane 6). In both cases, immunoblotting experiments (carried out under non-reducing conditions) revealed the presence of receptor monomers (estimated molecular masses: 46 kDa for m3-Rh and 49 kDa for m3-V2, respectively) as well as several higher molecular mass species, including two intense bands of approximately 80 and 95 kDa (Fig. 5B, lanes 3 and 6). The 95-kDa band corresponded in size to a putative m3-V2/m3-Rh heterodimer. As already discussed above, the 80-kDa species is most likely derived from the 95-kDa product by partial proteolytic degradation.

We next wanted to examine whether m3-V2/m3-Rh heterodimers were already preformed in cotransfected cells (in vivo) or generated artifically during processing of cells for coimmunoprecipitation studies. To address this issue, membrane extract prepared from m3-V2-expressing cells was mixed with an equal volume of membrane lysate prepared from...
m3-Rh-expressing cells, followed by immunoprecipitation of receptors using the anti-Rh antibody. In this case, the anti-Rh antibody failed to coprecipitate m3-V2 receptors (Fig. 5C). Similarly, the anti-V2 antibody was unable to coimmunoprecipitate m3-Rh receptors from such membrane lysate mixtures (data not shown). These data strongly suggest that m3-V2/m3-Rh heterodimers preexisted in cells prior to cell lysis.

As described above, coimmunoprecipitation studies using lysates derived from m3-V2/m3-Rh-cotransfected cells did not only result in the appearance of putative dimeric or oligomeric receptor forms but also of receptor monomers (Fig. 5B, lanes 3 and 6). This observation suggests that m3-V2/m3-Rh dimers can also form via non-covalent (SDS-sensitive) interactions, in addition to disulfide cross-linking. To test further this concept, membrane extracts prepared from cells coexpressing m3-V2 and m3-Rh receptors were denatured by incubation (1 h at 37 °C) with a high concentration of SDS (1.5%) prior to immunoprecipitation with the anti-Rh antibody. In this case, m3-V2 receptor monomers could no longer be detected in the immunoprecipitates (Fig. 5D). In contrast, the characteristic pattern of high molecular mass immunoreactive species (putative disulfide-linked m3-V2/m3-Rh dimers/oligomers) remained unaffected (compare Fig. 5B, lane 3).

Receptor Subtype Specificity of m3 Receptor Dimer Formation—Our next goal was to investigate whether m3 receptor dimer formation was receptor subtype-specific. To address this issue, we examined the ability of the m3 receptor to associate with the structurally closely related m1 and m2 muscarinic receptor subtypes (human) as well as with the V2 vasopressin receptor (human). [3H]NMS binding studies with singly transfected COS-7 cells showed that the m1 and m2 receptors were expressed at levels similar to those found with the m3 construct (B_max: about 1,000 fmol/mg). Similar receptor densities were found with cells transfected with the V2 receptor plasmid, as determined in [3H]arginine vasopressin binding studies (data not shown).

To detect the expression of the m1 and m2 muscarinic receptors via Western blotting, rabbit antisera were raised against non-conserved regions of the i3 loops of the two receptors, essentially as described by Levey et al. (28). Initially, immunoblotting experiments were performed (under non-reducing conditions) to examine the receptor subtype specificity of the m1 and m2 antisera. The m1 antiserum detected several immunoreactive species ranging in size from 65 to 140 kDa using membrane extracts prepared from cells cotransfected with the m1 and m3'-plasmids (Fig. 6A, lane 2) or transfected with the m1 construct alone (data not shown). This pattern was not observed with m3'-expressing cells, which yielded only two faint background bands (Fig. 6A, lane 1). Similarly, immunoblotting experiments with the m2 antiserum revealed a series of bands ranging in size from 70 to 160 kDa only when m2/m3'-cotransfected cells (Fig. 6B, lane 2) or cells transfected with the m2 construct alone (data not shown) were used. This characteristic pattern of bands was not observed with m3'-transfected cells (Fig. 6B, lane 1). In this case, the m2 antiserum detected a major band of 85 kDa (Fig. 6B, lane 1), which, however, was also observed with vector-transfected cells (data not shown), indicative of cross-reactivity of the m2 antiserum with a native COS-7 cell protein. Analogous experiments also verified the specificity of the anti-V2 antibody which also recognized multiple V2 receptor species (Fig. 6C, lanes 1 and 2) (also see Ref. 2). The appearance of high molecular mass m1, m2, and V2 receptor species is again consistent with the formation of receptor dimers or oligomers. In agreement with the findings obtained with the m3' receptor, the m1 and m2 antisera recognized only one major 65–75-kDa product when immunoblotting studies were carried out under reducing conditions (data not shown).

We next performed a series of coimmunoprecipitation experiments using lysates prepared from cotransfected COS-7 cells to examine whether or not the m3' receptor was able to form heterodimers with the m1, m2, or V2 receptors. In these experiments, m3' receptors were immunoprecipitated with the anti-HA antibody, followed by Western blot analysis (under non-reducing conditions) of immunoprecipitated proteins using the anti-m3, anti-m1, anti-m2, and anti-V2 antibodies. These studies showed that the anti-HA antibody was unable to coprecipitate m1, m2, or V2 receptors (Fig. 6D, lanes 4–6). However, in all cases, the expected pattern of immunoreactive m3' receptor species was observed when blots were probed with the anti-HA antibody.
m3 antibody (Fig. 6D, lanes 1–3), indicating that m3 receptor dimerization/oligomerization was receptor subtype-specific.

Disulfide-linked m3 Receptor Dimers Are Present on the Cell Surface—To investigate whether disulfide-linked m3 receptor dimers were present on the cell surface or retained intracellularly, intact m3-transfected COS-7 cells were labeled with the anti-HA monoclonal antibody which recognizes the extracellular N terminus of the m3 receptor protein. Previous immunofluorescence studies showed that the anti-HA antibody does not penetrate the plasma membrane barrier of intact COS-7 cells (18, 23). Thus, the anti-HA antibody should bind only to cell-surface m3 receptors.

Following a 2-h incubation of transfected cells with the anti-HA antibody (20 μg/ml), antibody-bound surface receptors were separated from unbound cytoplasmic receptors by using protein A-Sepharose 4B beads. The two resulting protein fractions were then analyzed by immunoblotting using the anti-m3 antibody (Fig. 7). Arrows indicate the positions of putative receptor monomers (about 45 kDa) and dimers (about 75 and 90 kDa). Protein molecular mass standards (in kDa) are indicated. Two additional experiments gave similar results.

Identification of Cys Residues Critical for the Formation of Disulfide-linked m3 Receptor Dimers—Our next goal was to identify the Cys residue(s) that are critically involved in the formation of disulfide-linked m3 receptor dimers. The wild-type rat m3 muscarinic receptor contains 12 Cys residues, four of which (Cys-140, Cys-220, Cys-516, and Cys-519) are located on the extracellular receptor surface (Fig. 1). Cys-140 and Cys-220 are located on the first and second extracellular loops, respectively, whereas Cys-516 and Cys-519 are both contained within the third extracellular loop. Two Cys residues (Cys-289 and Cys-419) located within the central portion of the i3 loop were deleted during the construction of the m3 receptor. Since disulfide bridges primarily occur on the extracellular surface of integral membrane proteins, we speculated that one or more of the four extracellular Cys residues might participate in the formation of disulfide-linked m3 receptor dimers.

To test this hypothesis, we generated a series of mutant m3 receptors in which specific Cys residues were replaced with Ala or Ser residues. The precise structures of these mutant receptors are given in Table I (see also Fig. 1). All investigated Cys → (Ala/Ser) mutant m3 receptors were still able to mediate carbachol-dependent increases in intracellular inositol phosphate levels (E_{max} = 65–100% as compared with m3 (100%)). A detailed pharmacologic analysis of these and other mutant m3 receptors carrying different Cys → Ala or Cys → Ser point mutations will be presented elsewhere.

We first examined the expression pattern of a mutant m3 receptor, referred to as m3(6C → A/S), in which Cys-140, Cys-220, Cys-516, and Cys-519 were the only remaining Cys residues (Table I). Western blot analysis showed that this mutant receptor was capable of forming disulfide-linked dimers in a fashion similar to the m3 receptor (Fig. 9A, lane 2). In contrast, a mutant m3 receptor, referred to as m3(4C → A), in which Cys-140, Cys-220, Cys-516, and Cys-519 were replaced with Ala residues, no longer yielded dimeric or oligomeric receptor species (Fig. 9A and B, lane 3). Simultaneous replacement of Cys-516 and Cys-519 (resulting in m3(C516A/C519A)) had little effect on the ability of the m3
receptor to form disulfide-linked dimers (Fig. 9, A and B, lane 4). However, a mutant m3 receptor in which Cys-140 and Cys-220 were converted to Ala (yielding m3(C140A/C220A)) migrated almost exclusively as a monomeric species (Fig. 9, A and B, lane 5), indicating that Cys-140 and/or Cys-220 are critically involved in the formation of covalently linked m3 receptor dimers. Based on this result, we examined the expression pattern of two additional mutant m3 receptors containing either the C140A or the C220A single point mutation. These studies showed that the monomer/dimer ratio was significantly increased for both mutant m3 receptors (Fig. 10). However, this effect was clearly more pronounced in the case of the m3(C220A) construct in which case most of the higher molecular mass receptor forms were converted to monomeric receptor species (Fig. 10, lane 3).

![Fig. 9. Identification of Cys residues involved in the formation of disulfide-linked m3 receptor dimers. COS-7 cells were transfected with the following m3 receptor constructs: m3 (lane 1), m3(C140A/C220A) (lane 2), m3(C140A) (lane 3), m3(C516A/C519A) (lane 4), and m3(C140A/C220A) (lane 5). Lanes 1, 2, 3, 4, and 5 correspond to the following m3 receptors: m3(C516A/C519A), m3(C140A/C220A), m3(C140A) and m3(C220A), respectively. The covalent receptor dimers are indicated by arrows.](image)

**Fig. 9.** Identification of Cys residues involved in the formation of disulfide-linked m3 receptor dimers. COS-7 cells were transfected with the following m3 receptor constructs: m3 (lane 1), m3(C140A/C220A) (lane 2), m3(C140A) (lane 3), m3(C516A/C519A) (lane 4), and m3(C140A/C220A) (lane 5). Lanes 1, 2, 3, 4, and 5 correspond to the following m3 receptors: m3(C516A/C519A), m3(C140A/C220A), m3(C140A) and m3(C220A), respectively. The covalent receptor dimers are indicated by arrows.

**Further Biochemical and Pharmacologic Analysis of the m3(C140A/C220A) Mutant Receptor**—Since the m3(C140A/C220A) construct almost completely lost the ability to form disulfide-linked receptor dimers, the biochemical and pharmacologic properties of this mutant receptor were studied in more detail. We first wanted to examine whether or not the m3(C140A/C220A) construct still retained the ability to form non-covalently linked receptor dimers. To address this question, we carried out a set of immunoprecipitation experiments, in a fashion analogous to that described above for the m3 receptor. For these studies, we generated two m3(C140A/C220A)-derived mutant receptors that carried different epitope tags at their C termini. The m3(C140A/C220A)-Rh receptor contained the last nine amino acids of bovine rhodopsin, whereas the m3(C140A/C220A)-V2 construct contained the last 29 amino acids of the human V2 vasopressin receptor (Table I).

Membrane extracts prepared from COS-7 cells cotransfected with m3(C140A/C220A)-V2 and m3(C140A/C220A)-Rh were subjected to immunoprecipitation using the anti-Rh antibody. Subsequently, immunoprecipitates were subjected to 12% SDS-PAGE (non-reducing conditions) and Western blotting analysis using the anti-V2 antibody (Fig. 11, lane 1), indicating that the m3(C140A/C220A) construct retained the ability to form non-covalent receptor dimers or multimers.

Radioligand binding studies demonstrated that the m3(C140A/C220A) receptor displayed >50-fold reduced binding affinities for the antagonists [3H]NMS (K<sub>app</sub> = 2,300 ± 190 pm) and 4-DAMP (K<sub>app</sub> = 62 ± 0.9 pm) and the agonists carbachol (K<sub>app</sub> = 10,000 μM) and acetylcholine (K<sub>app</sub> = 2,420 ± 220 μM), as compared with the m3 receptor (Table II, K<sub>app</sub> = 1.55 ± 0.05 nM, K<sub>app</sub> = 17 ± 1 μM (n = 3)).

PI assays showed that the m3(C140A/C220A) receptor was able to mediate carbachol- or acetylcholine-dependent increases in intracellular inositol phosphate levels to a maximum extent similar to that observed with the m3 receptor (6–8-fold above basal; n = 3). However, the m3(C140A/C220A) construct displayed a pronounced increase (>10,000-fold) in agonist EC<sub>50</sub> values (carbachol, 19,500 ± 3,300 nM; acetylcholine, 22,200 ± 2,000 nM). The corresponding EC<sub>50</sub> values for the m3 receptor construct were 1.0 ± 0.3 nM (carbachol) and 1.4 ± 1.0 nM (acetylcholine), respectively (n = 3).

**Fig. 10.** Role of Cys-140 and Cys-220 in the formation of disulfide-linked m3 receptor dimers. COS-7 cells were transfected with the following m3 receptor constructs: m3 (lane 1), m3(C140A) (lane 2), and m3(C220A) (lane 3). Membrane extracts prepared from transfected COS-7 cells were subjected to non-reducing SDS-PAGE (12%). Receptor proteins were visualized via Western blotting analysis using the anti-m3 antibody. Protein molecular mass standards (in kDa) are indicated. Two additional experiments gave similar results.

**Fig. 11.** The m3(C140A/C220A) receptor retains the ability to form non-covalently linked aggregates. Membrane extracts prepared from COS-7 cells transfected with m3(C140A/C220A)-V2 (lane 2), m3(C140A/C220A)-Rh (lane 3), or cotransfected with m3(C140A/C220A)-V2 and m3(C140A/C220A)-Rh (lane 4) were subjected to immunoprecipitation (IP) using the anti-Rh antibody conjugated to Sepharose 4B beads, as described under “Experimental Procedures” (for receptor structures, see Table I). Immunoprecipitates were subjected to 12% SDS-PAGE (non-reducing conditions) and Western blotting analysis using the anti-V2 antibody. The anti-Rh and anti-V2 antibodies interact with the C-terminal rhodopsin and V2 vasopressin receptor tags present in the m3(C140A/C220A)-Rh and m3(C140A/C220A)-V2 constructs, respectively. Lane 1 was included for control purposes and shows the expression of the m3(C140A/C220A)-V2 receptor using membrane extracts prepared from cells cotransfected with m3(C140A/C220A)-V2 and m3(C140A/C220A)-Rh (no immunoprecipitation). Lanes 2 and 3 confirm the specificity of the anti-V2 and anti-Rh antibodies. Lane 4 indicates that the anti-Rh antibody was able to coprecipitate m3(C140A/C220A)-V2 receptors.

**DISCUSSION**

In this study, we demonstrated that the m3 muscarinic receptor is capable of forming disulfide-linked as well as non-covalent receptor dimers/multimers. We showed that dimer formation was receptor subtype-specific and that disulfide-linked dimers were present at the cell surface. Moreover, site-directed mutagenesis studies were carried out to identify the Cys residue(s) that participate in covalent receptor cross-linking. Our findings confirm and considerably extend previous studies examining the dimerization/oligomerization of other GPCR subtypes (1–10).

Most studies were carried out with a modified version of the m3 muscarinic receptor, referred to as m3′, that could be de-
ected more easily and reproducibly via immunological techniques (for m3 receptor, see Table I and Fig. 1). Western blotting analysis of membrane lysates prepared from m3- transfected COS-7 cells showed that the m3 receptor migrated as a 45-kDa monomer band under reducing conditions (Fig. 2). However, when immunoblotting experiments were carried out under non-reducing conditions, several additional higher molecular mass forms corresponding in size to putative receptor dimers and multimers were observed (Fig. 2). Similar results were obtained when analogous studies were carried out in the presence of the membrane-permeable, sulphhydril-alkylating reagent, NEM (Fig. 2), ruling out the possibility that the observed high molecular mass m3 receptor species were artifacts due to disulfide bond exchange reactions during preparation of samples for SDS-PAGE.

Interestingly, biochemical analysis of membrane lysates prepared from m3-,transfected COS-7 cells via ABT-agarose affinity chromatography and immunoblotting analysis showed that the high molecular mass m3 receptor species were capable of binding muscarinic ligands (Fig. 3).

To study the potential oligomerization of m3 receptors expressed in physiological tissues, membranes prepared from total rat brain were subjected to SDS-PAGE and Western blotting analysis, in a fashion similar to lysates prepared from m3-expressing COS-7 cells (Fig. 4). The observed pattern of immunoreactive bands indicated that native m3 muscarinic receptors are also able to form disulfide-linked receptor dimers/oligomers.

The results of the immunoblotting experiments did not completely rule out the possibility that the high molecular mass m3 receptor species observed in these studies were caused by cross-linking of the m3 receptor to other membrane proteins. To study m3 receptor dimerization/oligomerization in a more direct fashion, we therefore designed a coimmunoprecipitation strategy using two different versions of the m3 receptor (m3-V2 and m3-Rh) carrying two different C-terminal epitope tags. The m3-V2 and m3-Rh receptors were coexpressed in COS-7 cells, immunoprecipitated with the anti-V2 or anti-Rh antibodies, subjected to SDS-PAGE, and then probed on Western blots with one or the other antibody. These studies clearly showed that the anti-V2 antibody was able to coimmunoprecipitate m3-Rh receptors and, vice versa, that the anti-Rh antibody was capable of coimmunoprecipitating m3-V2 receptors (Fig. 5).

Interestingly, probing of coimmunoprecipitates with the anti-V2 or anti-Rh antibody did not only yield the expected pattern of high molecular mass m3 receptor species but also resulted in the appearance of the 45–50-kDa band characteristic for the m3 receptor monomer (Fig. 5b, lanes 3 and 6). This observation suggested that m3-V2/m3-Rh receptor complexes are not due exclusively to disulfide cross-linking but can also be formed by non-covalent (SDS-sensitive) interactions. In agreement with this notion, SDS (1.5%) treatment of cell lysates prepared from COS-7 cells cotransfected with the m3-V2 and m3-Rh receptor constructs prevented the appearance of m3-V2 receptor monomers when receptors were coimmunoprecipitated with the anti-Rh antibody (Fig. 5d). In contrast to this finding, it has been shown that other GPCRs of the rhodopsin family such as the β2-adrenergic receptor (2), V2 vasopressin receptor (2), or different dopamine receptor subtypes (3, 6) form homodimers that are SDS-resistant.

To exclude the possibility that m3-V2/m3-Rh receptor complexes were formed artificiually during the preparation of cell lysates and processing of samples for SDS-PAGE, coimmunoprecipitation studies were also carried out using mixtures of cell lysates prepared from COS-7 cells individually expressing either the m3-V2 or the m3-V2 receptor. In this case, however, the anti-Rh antibody failed to coimmunoprecipitate m3-V2 receptors (Fig. 5), strongly suggesting that m3-V2/m3-Rh receptor dimers or multimers were formed in vivo prior to cell lysis.

To study the receptor subtype selectivity of m3 receptor dimerization/oligomerization, coimmunoprecipitation studies were also performed using COS-7 cells cotransfected with the m3 receptor and the structurally closely related m1 or m2 muscarinic receptor subtypes or the V2 vasopressin receptor. In these experiments, the anti-HA antibody was able to precipitate m3 receptors but failed to coimmunoprecipitate m1, m2, or V2 receptors (Fig. 6), clearly demonstrating the specificity of m3 receptor dimerization/oligomerization.

To examine whether m3 receptor dimers were expressed on the cell surface, intact m3-expressing COS-7 cells were labeled with the membrane-impermeable anti-HA antibody (18, 23), followed by immunoprecipitation of antibody-bound surface receptors and Western blotting analysis. These experiments demonstrated that disulfide-linked m3 receptor dimers were abundantly expressed on the cell surface (Fig. 7).

Western blotting experiments also showed that carbachol treatment of intact m3 receptor-expressing COS-7 cells had little effect on m3 receptor dimerization (Fig. 8), suggesting that agonist stimulation neither promotes nor destabilizes receptor dimer formation. Consistent with these results, dimerization of the Ca²⁺-sensing receptor expressed in HEK293 cells (see below) was also found to be agonist-insensitive (9). In contrast, biochemical studies using membrane preparations derived from COS-7 cells transfected with the β2-adrenergic receptor showed that agonist stimulation stabilized the dimeric state of this receptor subtype (2). On the other hand, the proportion of δ-opioid receptor dimers detected in stably transfected Chinese hamster ovary cells was found to be decreased upon incubation of intact cells with several opioid agonists (5). It remains to be clarified whether the observed differences in agonist regulation of receptor dimerization reflect intrinsic differences in the structural properties of the studied receptor proteins or are due to variations in experimental conditions.

To identify the Cys residues that are involved in the covalent cross-linking of m3 muscarinic receptor monomers, a series of Cys → Ala (or Cys → Ser) mutant receptors were examined for their ability to form disulfide-linked dimers/oligomers (Figs. 9 and 10). Since disulfide bonds are usually found only on the extracellular surface of integral membrane proteins, we focused our attention on the four extracellular Cys residues, Cys-140, Cys-220, Cys-516, and Cys-519 (Fig. 1). Immunoblotting experiments showed that the m3(C140A/C220A) mutant receptor migrated almost exclusively as a monomeric species (under reducing and non-reducing conditions; Fig. 9, lane 5), strongly suggesting that Cys-140 and/or Cys-220 are involved in the formation of covalently linked m3 receptor dimers. Additional mutagenesis and immunoblotting studies demonstrated that the presence of Cys-220 is of primary importance for intermolecular m3 receptor cross-linking (Fig. 10).

Interestingly, coimmunoprecipitation studies indicated that the m3(C140A/C220A) construct retained the ability to form non-covalent receptor aggregates (Fig. 11). This observation is consistent with the concept that the formation of non-covalent receptor complexes precedes disulfide cross-linking of receptor monomers.

It should be noted that the Cys-140/Cys-220 pair is not only conserved among all GPCRs of the rhodopsin family (38, 39). Several studies suggest (see, for example, Refs. 40–42) that the two conserved Cys residues are likely to form an intramolecu-
lar disulfide bond, thus covalently linking the first and second extracellular loops. However, our findings indicate that the two conserved extracellular Cys residues can also participate in the formation of intermolecular disulfide bonds.

Radioligand binding and functional studies showed that the m3(C140A/C220A) construct displayed pronounced reductions in ligand binding affinities (>50-fold) and functional agonist potencies (>10,000-fold; PI assays), as compared with the m3 receptor. It remains to be explored whether these functional deficits are due to the absence of intramolecular or intermolecular disulfide bonds.

Interestingly, disulfide-linked GPCR dimers have first been identified among members of a unique GPCR subfamily including the metabotropic glutamate receptors (mGluRs) and the Ca2+-sensing receptor (4). However, our findings indicate that the lack of functional complementation seen in the coexpressed m3, m4, and m5 receptors showed that the two chimeric proteins were able to exhibit dimerization (5).

Moreover, cotransfection studies with two m3 muscarinic/α2-adrenergic hybrid receptors in which a region containing TM VI and TM VII were exchanged between the two wild-type receptors showed that the two chimeric proteins were able to functionally complement each other (22). Interestingly, functional complementation was no longer observed when most of the i3 loop was deleted from the two mutant receptor constructs (22), suggesting that the proposed interaction between the two chimeric receptors requires i3 loops of sufficient length. However, since the experiments reported here were carried out with a mutant m3 receptor carrying the identical i3 loop deletion, our data clearly indicate that the presence of the full-length i3 loop is not required for the formation of disulfide-linked as well as SDS-sensitive m3 receptor dimers. One possibility is that the lack of functional complementation seen in the coexpression experiments described by Maggio et al. (22) is caused by steric hindrance due to drastic shortening of the i3 loops, preventing the N- and C-terminal m3 (or α2) receptor domains from associating with each other.

In conclusion, this study highlights the structural diversity through which GPCR dimerization can be achieved. The relative functional importance of muscarinic receptor monomers and dimers remains to be explored in future studies.

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