

Molecular Aspects of Muscarinic Receptor Dimerization

Fu-Yue Zeng, Ph.D., and Jürgen Wess, Ph.D.

The five muscarinic acetylcholine receptors (M_1 – M_5) are prototypical members of the superfamily of G-protein-coupled receptors (GPCRs). During the past decade, this laboratory has used different members of this receptor subfamily as model systems to study the molecular mechanisms involved in GPCR function. This article reviews recent investigations dealing with molecular aspects of M_3 muscarinic receptor assembly and dimerization/oligomerization. Studies with coexpressed M_3 receptor fragments and M_3 muscarinic/ α_{2C} -adrenergic hybrid receptors showed that muscarinic receptors, like other GPCRs, are composed of multiple autonomous folding domains. Moreover, biochemical studies have provided

direct evidence for the formation of M_3 receptor dimers/oligomers. These high molecular mass receptor species are expressed on the cell surface and can bind muscarinic ligands. M_3 receptor dimerization/oligomerization appears to be receptor subtype-selective and involves both non-covalent interactions as well as disulfide-crosslinking of receptor monomers. These studies add to the growing number of reports suggesting the existence of GPCR dimers or multimers. The precise functional characteristics of such receptor aggregates remain to be elucidated.

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Traditionally, the interaction of ligand-activated G-protein-coupled receptors (GPCRs) with their respective G-protein targets has been described by the use of models that assume that the receptors exist as monomers and couple to G-proteins in a 1:1 stoichiometry. However, recent evidence suggests that such classical models of receptor/G-protein coupling may be oversimplified (for a recent review, see Hebert and Bouvier 1998). Most notably, a series of elegant studies has shown that the GABA_BR1 and GABA_BR2 receptor subtypes form heterodimers *in vivo* that are required for proper cell

surface localization of the GABA_BR1 subtype and GABA_B receptor function (Jones et al. 1998; White et al. 1998; Kaupmann et al. 1998; Kuner et al. 1999). In another groundbreaking study, Jordan and Devi (1999) demonstrated that κ and δ opioid receptors can form heterodimers with distinct ligand binding and functional properties, raising the possibility that heterodimerization may represent a more general mechanism to modulate GPCR function.

In this article, we review work dealing with the identification and molecular characterization of muscarinic acetylcholine receptor dimers (or oligomers), with primary emphasis on studies carried out in our own laboratory. The five muscarinic receptors (M_1 – M_5) are prototypical members of the GPCR superfamily (Wess 1996). To analyze the molecular properties of muscarinic receptor dimers/multimers in greater detail, we have used the M_3 muscarinic receptor as a model system. The M_3 receptor, similar to the M_1 and M_5 muscarinic receptor subtypes, is preferentially coupled to G-proteins of the G_q/G₁₁ family (Wess 1996). At a biochemical level, acti-

From the Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, MD.

Address correspondence to: Jürgen Wess, Ph.D., Laboratory of Bioorganic Chemistry, NIH-NIDDK, Bldg. 8A, Room B1A-05, Bethesda, MD 20892. Tel.: (301) 402-3589; Fax: (301) 402-4182; E-mail: jwess@helix.nih.gov

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vation of these G-proteins mediates the stimulation of phospholipase C β , resulting in the hydrolysis of phosphoinositide (PI) lipids.

COEXPRESSION STUDIES INVOLVING MUTANT M₃ MUSCARINIC RECEPTORS OR M₃ RECEPTOR FRAGMENTS

Several years ago, in an attempt to learn more about GPCR folding and assembly, we carried out a series of

cotransfection experiments involving two M₃ muscarinic/ α_{2C} -adrenergic hybrid receptors (Figure 1A) (Maggio et al. 1993a). In the α_2 /M₃ hybrid receptor, the first five transmembrane domains (TM I–IV) were derived from the rat α_{2C} -adrenergic receptor and the segment containing the last two TM domains (TM VI and VII) consisted of rat M₃ receptor sequence. On the other hand, in the M₃/ α_2 mutant receptor, the bulk of the receptor (containing TM I–V) consisted of M₃ receptor sequence and the C-terminal portion (containing TM VI and VII) was derived from the α_{2C} -adrenergic receptor

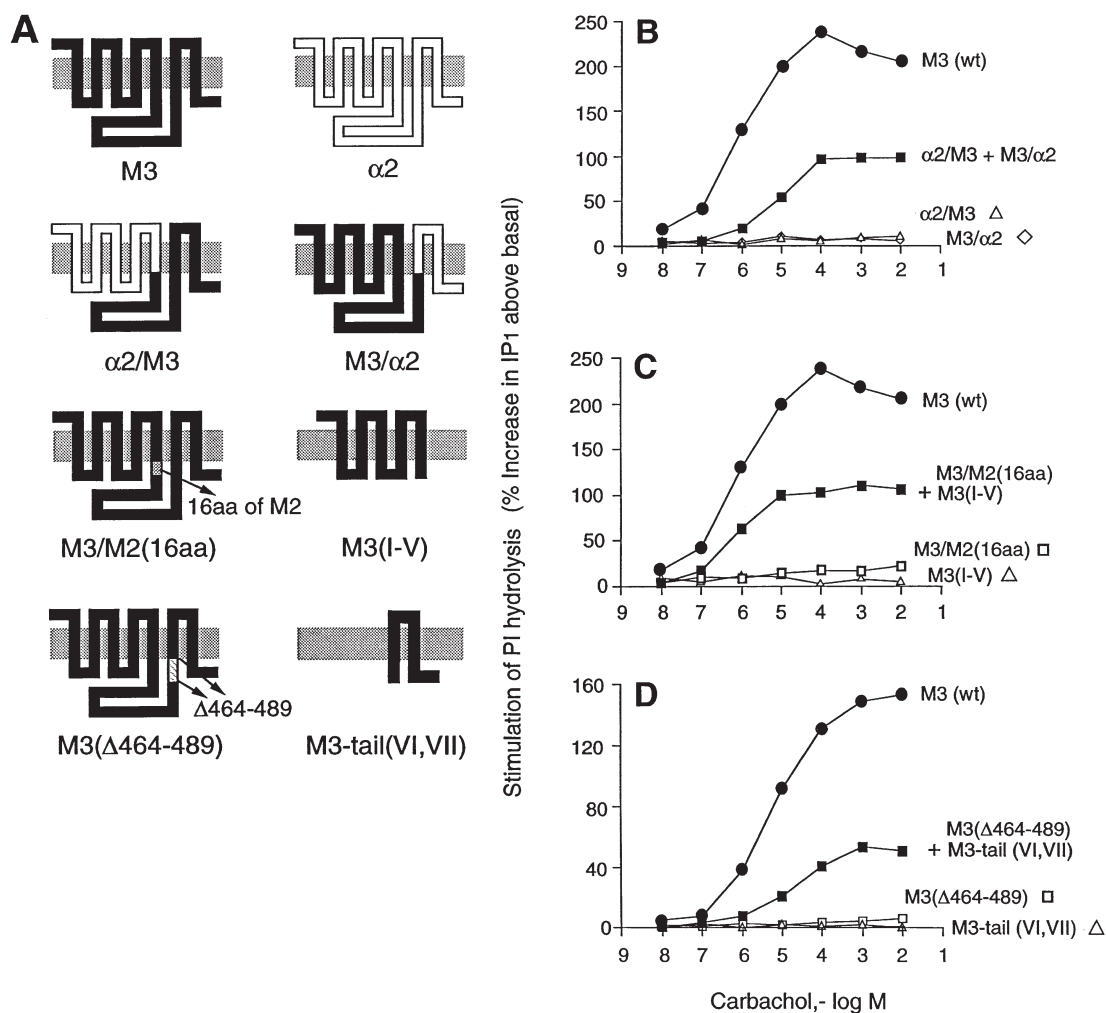


Figure 1. (A) Structure of mutant M₃ muscarinic receptors and receptor fragments. The α_2 /M₃ and M₃/ α_2 hybrid receptors were generated from rat M₃ muscarinic and rat α_{2C} -adrenergic receptor sequences as described (Maggio et al. 1993a). In M₃/M₂(16aa), the first 16 amino acids of the i3 loop of the M₃ receptor were replaced with the corresponding segment of the human M₂ muscarinic receptor (Wess et al. 1989). In M₃(Δ 464–489), 26 amino acids were deleted from the C-terminal portion of the i3 loop of the M₃ receptor. M₃(I–V) represents a M₃ receptor which has been truncated after the first 21 amino acids of the i3 loop (Schöneberg et al. 1995). M₃-tail(VI,VII) represents a C-terminal M₃ receptor fragment (amino acids 464–589, preceded by an initiating methionine). Numbers refer to amino acid positions within the rat M₃ muscarinic receptor sequence (Bonner et al. 1987; also see Figure 3). (B–D) Agonist-dependent stimulation of PI hydrolysis following coexpression of functionally inactive mutant M₃ muscarinic receptors or receptor fragments. Transfected COS-7 cells were incubated with increasing concentrations of the muscarinic agonist, carbachol, and the resulting increases in intracellular inositol monophosphate (IP₁) levels were determined as described (Maggio et al. 1993a). Responses are expressed as percent increase in IP₁ above basal levels determined in the absence of carbachol. Representative concentration-response curves are shown.

(the third intracellular (i3) loops of these receptors consisted of M₃ receptor sequence in both cases).

The individual chimeras, when transiently expressed in COS-7 cells, were unable to mediate stimulation of PI hydrolysis in an agonist-dependent fashion (Figure 1B) and to bind adrenergic or muscarinic radioligands (Maggio et al. 1993a). However, coexpression of the two hybrid receptors resulted in the appearance of a small but significant number (30–35 fmol/mg) of muscarinic ([³H]N-methylscopolamine, [³H]NMS), and adrenergic ([³H]rauwolscine) binding sites (Maggio et al. 1993a). Moreover, incubation of cotransfected cells with the muscarinic agonist, carbachol, led to a pronounced increase in the breakdown of PI lipids (Figure 1B). It is likely that this rescue in receptor activity is due to direct interactions between the two hybrid receptors, resulting in the reconstitution of functional receptor units (Maggio et al. 1993a).

Maggio et al. (1996) recently reported that functional complementation was no longer observed when most of the i3 loop was deleted from the α 2/M3 and M3/ α 2 mutant receptor constructs. However, as described in more detail below, we found that the identical deletion did not interfere with the ability of the M₃ muscarinic receptor to oligomerize, as determined in coimmunoprecipitation and immunoblotting studies (Zeng and Wess 1999). Thus, one possibility is that the lack of functional complementation seen in the coexpression experiments involving i3 loop-shortened versions of α 2/M3 and M3/ α 2 is due to steric hindrance, preventing “domain exchange” between the two hybrid receptors.

As indicated above, neither α 2/M3 nor M3/ α 2 were able to bind ligands, leaving open the question whether these mutant receptors were properly folded and transported to the cell surface. We therefore carried out additional cotransfection studies using mutant M₃ receptors that still retained ligand-binding activity but were defective in G-protein coupling. In one case, we used a mutant M₃ receptor in which the first 16 amino acids of the i3 loop were replaced with the corresponding M₂ receptor sequence (Figure 1A). This hybrid receptor was capable of binding muscarinic ligands with high affinity (Wess et al. 1989) but was no longer able to mediate agonist-dependent PI hydrolysis (Figure 1C). We next coexpressed this M₃/M₂ chimera with a binding- and coupling-defective mutant M₃ receptor [referred to as M3(I–V) in Figure 1A] that was truncated within the i3 loop (after the first 21 amino acids of the i3 loop). Interestingly, PI assays showed that carbachol stimulation of cells coexpressing these two mutant receptors resulted in the appearance of considerable functional activity (Figure 1C), as had been observed with the two coexpressed muscarinic/adrenergic hybrid receptors.

A similar experiment is depicted in Figure 1D (J. Wess, unpublished results). In this case, we generated an M₃ receptor deletion mutant in which amino acids

464–489 were removed from the C-terminal segment of the i3 loop (Figure 1A). This construct retained the ability to bind muscarinic ligands with high affinity (J. Wess, unpublished results), but was no longer able to mediate agonist-dependent G-protein coupling (Figure 1D). However, agonist-induced functional coupling could be partially restored when the M3 Δ (464–489) construct was coexpressed with a C-terminal M₃ receptor fragment [referred to as M3-tail(VI,VII) in Figure 1A]. This fragment included TM VI and VII as well as the region that had been deleted in the M3 Δ (464–489) mutant receptor. These experiments indicate that intermolecular interactions between (mutant) receptors can occur even when one of the receptors retains the ability to bind muscarinic ligands when expressed alone.

Similar rescue experiments involving the use of coexpressed mutant receptors or receptor fragments have also been reported for other classes of GPCRs including the V2 vasopressin receptor (Schöneberg et al. 1996, 1997), the AT1 angiotensin II receptor (Monnot et al. 1996), the LH/CG receptor (Osuga et al. 1997), and, more recently, the calcium-sensing receptor (Bai et al. 1999). The latter study convincingly demonstrates that functional rescue requires the direct physical association between coexpressed mutant receptors.

Although other scenarios are conceivable, one possibility is that the success of such rescue experiments involving the coexpression of functionally impaired mutant receptors is based on the multidomain structure of GPCRs (Kobilka et al. 1988; Maggio et al. 1993b; Schöneberg et al. 1995, 1996, 1997; Ridge et al. 1995; Yu et al. 1995; Grosse et al. 1997; Nielsen et al. 1998). Schöneberg et al. (1995) showed, for example, that N- and C-terminal M₃ muscarinic receptor fragments generated by “splitting” this receptor in all three intracellular and all three extracellular loops could be stably expressed in cultured cells, apparently in the proper transmembrane topology. Whereas none of the fragments displayed any functional activity when expressed alone, coexpression of three of the six polypeptide pairs led to the restoration of ligand-binding activity (Figure 2). Interestingly, recent studies with the yeast GPCR, Ste2p, have shown that “splitting” this receptor in any intracellular or extracellular loop is functionally tolerated (Martin et al. 1999). Taken together, these data are consistent with the concept that GPCRs, like most other polytopic membrane proteins (Popot and Engelman 1990), are composed of multiple autonomous folding units containing at least one TM domain. The current view is that these protein subdomains can adopt their proper transmembrane topologies independently of each other and are able to recognize each other via specific helix–helix interactions to assemble into functional GPCRs.

Based on both theoretical and experimental data, Gouldson et al. (1998) recently proposed a model that

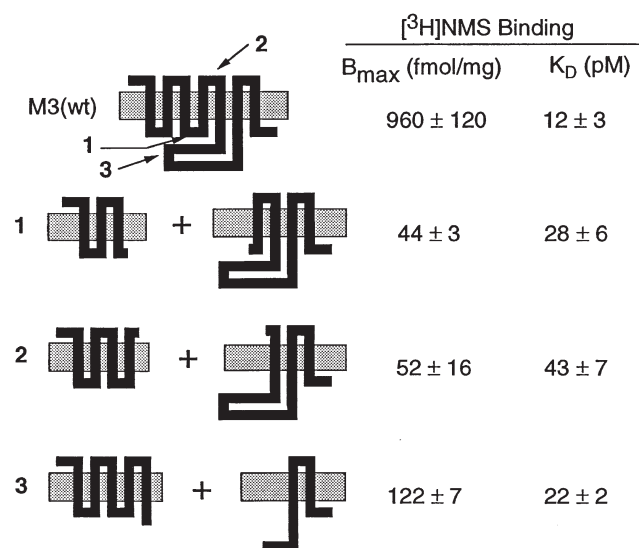


Figure 2. Coexpression of N- and C-terminal M₃ muscarinic receptor fragments in COS-7 cells. The indicated fragment pairs were generated by “splitting” the rat M₃ muscarinic receptor at the indicated positions (for precise amino acid compositions of the different receptor fragments, see Schöneberg et al. 1995). Whereas none of the receptor fragments displayed ligand-binding activity when expressed alone, coexpression of the indicated polypeptide pairs restored radioligand ([³H]NMS) binding (Schöneberg et al. 1995).

suggests that the active form of GPCRs is a “TM V/TM VI-domain swapped dimer,” thus providing a molecular basis for the success of the rescue experiments described above. According to this model, agonists are predicted to cause dimer formation by inducing conformational changes which promote interactions of residues located at the TM V/TM VI interface. However, this intriguing model needs to be tested by further experimentation.

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF M₃ RECEPTOR DIMERS/MULTIMERS

The “rescue studies” described above involving the use of different mutant receptors suggested that wild-type (wt) muscarinic receptors might also be able to form dimeric or oligomeric arrays. In fact, the existence of high molecular mass muscarinic receptor aggregates had been postulated earlier by several investigators (Potter et al. 1991; Hirschberg and Schimerlik 1994; Wreggett and Wells 1995; Chidiac et al. 1997). For example, Potter et al. (1991) showed that the complex agonist binding properties of muscarinic receptors expressed in rabbit heart and rat brain stem could be explained best by assuming the presence of two agonist-binding sites located on dimeric receptor mole-

cules. Similarly, systematic computational analysis of the binding properties of the agonist, [³H]oxotremorine-M, to M₂ muscarinic receptors expressed in cultured cells or porcine atria suggested the existence of receptor dimers as well as monomers (Hirschberg and Schimerlik 1994). Studies with purified muscarinic receptors (Wreggett and Wells 1995) revealed that the complex binding properties of muscarinic agonists are in fact due to different states of the receptor proteins themselves and are not dependent on the interaction with receptor-associated proteins such as G-proteins.

Based on these findings, we initiated a set of experiments in order to provide more direct physical evidence for the existence of muscarinic receptor dimers or oligomers. For these studies, the M₃ muscarinic receptor subtype served as a model system. To facilitate the detection of the M₃ receptor protein (expressed in COS-7 cells) via Western blotting analysis, we modified the receptor protein as follows (Figure 3)(Zeng et al. 1999):

1. A hemagglutinin (HA) epitope tag was added to the N-terminus of the receptor protein.
2. The five N-terminal asparagine residues that can serve as sites for N-linked glycosylation were replaced with glutamines. This modification was made to prevent the appearance of multiple immunoreactive M₃ receptor species on immunoblots caused by heterogeneous glycosylation.
3. The central portion of the i3 loop, altogether 196 amino acids, was deleted. This modification greatly facilitated the detection of M₃ receptor protein via immunoblotting by an as yet unknown mechanism.

This construct is referred to as M3' receptor in the following. As described below, the M3' receptor is efficiently recognized by a mouse monoclonal antibody directed against the N-terminal HA tag as well as a rabbit polyclonal antibody (anti-m3) raised against the C-terminus of the M₃ receptor protein (Figure 3; Zeng et al. 1999). Following its transient expression in COS-7 cells, the M3' receptor was able to mediate agonist-dependent PI hydrolysis in a fashion analogous to that observed with the wt M₃ receptor. In addition, the ligand-binding properties of the two receptors were also very similar (Zeng et al. 1999).

Detection of DTT-sensitive M₃ Receptor Aggregates

Figure 4 shows typical Western blots that were obtained when the M3' receptor was expressed in COS-7 cells. When SDS-PAGE was performed under non-reducing conditions (in the absence of dithiothreitol, DTT), both the anti-HA and the anti-m3 antibodies detected a similar set of multiple immunoreactive species. A major band migrated at around 45 kDa and corresponded in size to a putative M3' receptor monomer. In addition, several higher molecular mass species of ap-

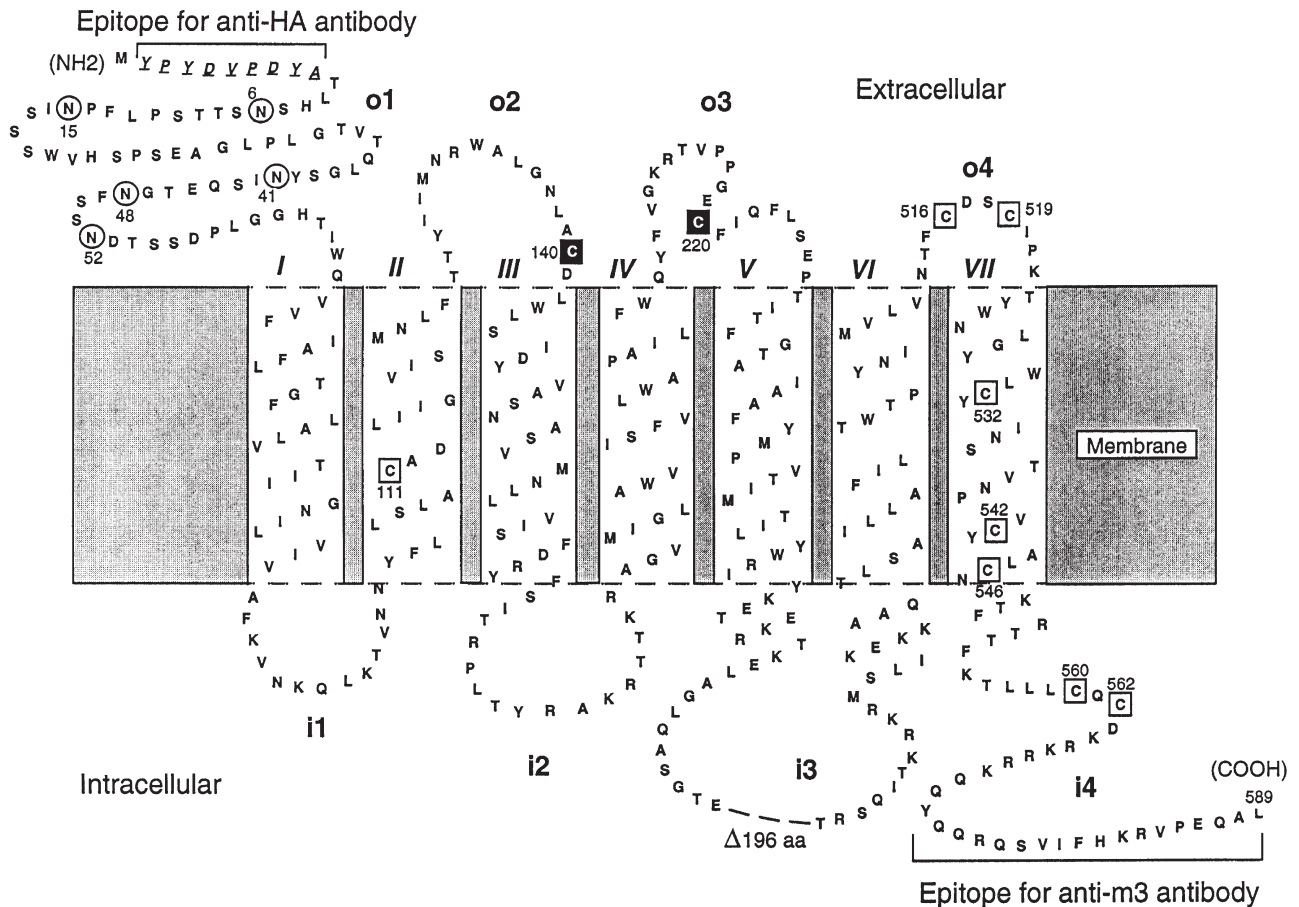


Figure 3. Structural modification of the rat M₃ muscarinic receptor. A modified version of the rat M₃ muscarinic receptor, referred to as M3', was generated as follows: An HA epitope tag was added to the N-terminus of the receptor protein, the five potential N-glycosylation sites present in the N-terminal portion of the receptor protein (Asn6, Asn15, Asn41, Asn48, and Asn52; shown circled) were replaced with Gln residues, and the central portion of the i3 loop (Ala274-Lys469) was deleted. Cys residues are shown boxed. The two extracellular Cys residues that are conserved among most GPCRs are highlighted in black. A rabbit polyclonal antibody (referred to as anti-m3) was raised against the C-terminal 18 amino acids. Taken from Zeng and Wess (1999).

proximately 75, 90, and >120 kDa were observed. Interestingly, the 90-kDa band corresponded in size to a putative M3' receptor dimer. Most likely, the 75-kDa band was derived from the 90-kDa species via proteolytic degradation (Zeng and Wess 1999). A completely different pattern was observed when Western blotting experiments were carried out under reducing conditions (Figure 4, lanes 3). In this case, the high molecular mass immunoreactive species were no longer observed, and the 45-kDa receptor monomer represented the only detectable specific band.

To rule out the possibility that the high molecular mass receptor bands observed under non-reducing conditions represented artifacts created by disulfide bond exchange reactions during the preparation of samples for SDS-PAGE, COS-7 cells expressing the M3' receptor were incubated with the membrane-permeable, SH-group alkylating reagent, N-ethylmaleimide (NEM; 10 mM) for one h immediately before harvesting. Subse-

quently, cells/samples were processed for SDS-PAGE in the continued presence of NEM (10 mM) which was also present in the loading buffer. Figure 4 (lanes 2) shows that NEM treatment had essentially no effect on the observed pattern of high molecular mass receptor bands. Taken together, these observations suggest the existence of disulfide-linked M3' receptor dimers or oligomers (in the case of the >120 kDa immunoreactive species) that preexist in COS-7 cells prior to cell lysis.

To examine whether DTT-sensitive M₃ receptor aggregates also exist in native tissues, membrane proteins prepared from total rat brain were subjected to Western blotting analysis using the anti-m3 antibody. As shown in Figure 5, the M₃ receptor protein migrated as a single band of about 90–100 kDa when experiments were carried out under reducing conditions, whereas at least two additional high molecular mass products (about 170–190 kDa in size) were detected under non-reducing conditions. Thus, the overall pattern of bands observed

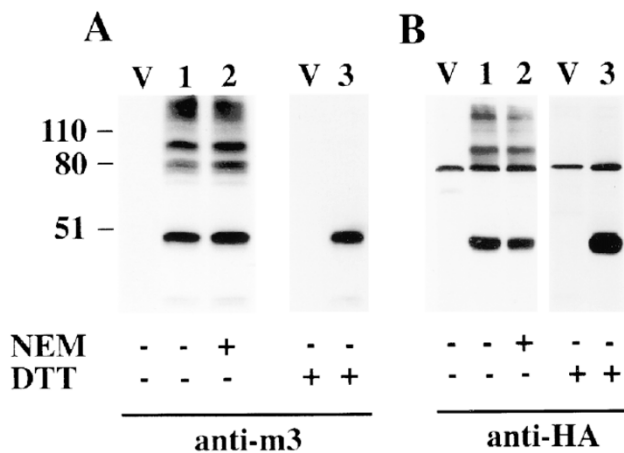


Figure 4. Detection of M3' muscarinic receptors expressed in COS-7 cells via immunoblotting. COS-7 cells were transiently transfected with vector DNA (V; pcD-PS) or the M3' construct (lanes 1–3). Membrane lysates were prepared from transfected cells and subjected to 12% SDS-PAGE and immunoblotting using the indicated antibodies (see Figure 1) as described (Zeng and Wess 1999). Panel B (lane V) indicates that the anti-HA antibody also detects a nonspecific band migrating at about 75 kDa. Transfected cells were incubated with (lane 2) or without (lanes 1 and 3) NEM (10 mM) for the last 1 h of culture. Before SDS-PAGE, membrane extracts (20 µg/lane) were mixed with sample buffer in the absence (lanes 1 and 2) or presence of 50 mM DTT (lane 3). Protein molecular mass standards (in kDa) are indicated. Taken from Zeng and Wess (1999).

in this set of experiments resembled that found with M3'-transfected COS-7 cells. The observed differences in band sizes were most probably due to the i3 loop deletion present in the M3' construct and the fact that the native M₃ receptor is extensively glycosylated.

Coimmunoprecipitation Studies

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 crosslinking of the M₃ receptor to other membrane proteins. We therefore carried out a series of coimmunoprecipitation experiments in order to study the potential formation of M₃ receptor dimers/oligomers in a more direct fashion. For these studies, we generated two additional M3' receptor constructs, referred to as M3'-V2 and M3'-Rh (Zeng and Wess 1999). In the M3'-Rh receptor, the last nine amino acids of bovine rhodopsin were added to the C-terminus of M3'. 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' construct. Radioligand binding and PI assays showed that incorporation of these epitope tags had no significant effect on M3' receptor function (Zeng and Wess 1999).

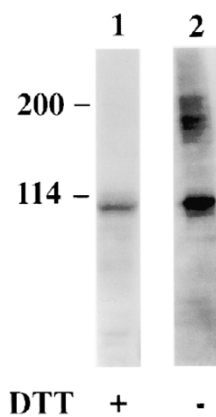


Figure 5. Detection of rat brain M₃ muscarinic receptors via immunoblotting. Rat brain membranes (50 µg protein/lane) were incubated with sample buffer in the presence (lane 1) or absence of 50 mM DTT (lane 2) and subjected to 8% SDS-PAGE and immunoblotting using the anti-m3 antibody (Zeng and Wess 1999). Protein molecular mass standards (in kDa) are indicated. Taken from Zeng and Wess (1999).

Figure 6A shows that the anti-rhodopsin (anti-Rh) monoclonal antibody (also known as 1D4 antibody; Ridge et al. 1995) specifically recognized the M3'-Rh construct and that the anti-V2 rabbit polyclonal antibody (Schöneberg et al. 1996) specifically detected the M3'-V2 receptor protein.

We next initiated a set of coimmunoprecipitation experiments (followed by SDS-PAGE and Western blotting) using membrane lysates prepared from cells cotransfected with the M3'-Rh and M3'-V2 constructs. These studies showed that immunoprecipitation with the anti-Rh antibody resulted in the appearance of M3'-V2 receptors in the immunoprecipitate, as revealed by the anti-V2 antibody (Figure 6B, lane 3). Analogously, immunoprecipitation with the anti-V2 antibody resulted in the appearance of M3'-Rh receptors in the immunoprecipitate, as detected by the anti-Rh antibody (Figure 6B, lane 6). These findings therefore provided direct evidence for the formation of M₃ receptor dimers/oligomers.

In the coimmunoprecipitation experiments, both antibodies did not only visualize the expected higher molecular mass receptor aggregates but also monomeric receptor species (Figure 6B, lanes 3 and 6). This observation suggested that M₃ receptor oligomerization also involves non-covalent, SDS-sensitive interactions (in addition to disulfide crosslinking) and that disulfide receptor crosslinking of receptor monomers is not quantitative. To study this phenomenon further, membrane lysates were prepared from cells cotransfected with the M3'-V2 and M3'-Rh constructs and then incubated with a high concentration of SDS (1.5%) prior to immunoprecipitation. Following dilution of samples and incubation with the anti-Rh antibody, immunopre-

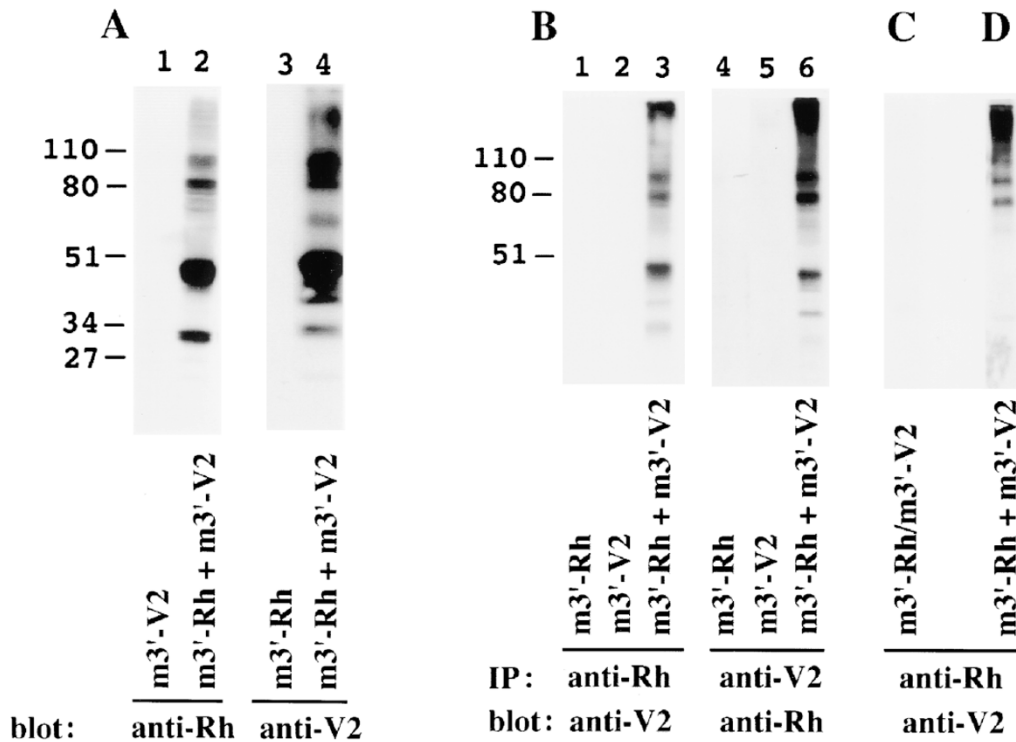


Figure 6. Coimmunoprecipitation studies with M3' receptor constructs carrying different C-terminal epitope tags. To generate the M3'-Rh receptor, the last nine amino acids of bovine rhodopsin (which are recognized by the anti-Rh antibody; Ridge et al. 1995) were added to the C-terminus of the M3' receptor. Analogously, the M3'-V2 construct was obtained by attaching the last 29 amino acids of the human V2 vasopressin receptor (which are recognized by the anti-V2 antibody; Schöneberg et al. 1996) to the C-terminus of the M3' receptor. COS-7 cells were transfected/cotransfected with the indicated receptor constructs. **(A)** Specificity of the anti-V2 and anti-Rh antibodies. Membrane extracts (20 μ g/lane) prepared from transfected COS-7 cells were subjected to 12% SDS-PAGE and Western blotting using the indicated antibodies (non-reducing conditions). **(B)** Coimmunoprecipitation of M3'-V2/M3'-Rh receptor complexes. Membrane extracts prepared from COS-7 transfected with the indicated M3' receptor constructs were subjected to immunoprecipitation (IP) by using the indicated antibodies conjugated to Sepharose 4B beads (Zeng and Wess 1999). Immunoprecipitated receptors were subjected to 12% SDS-PAGE and detected via Western blotting using the indicated antibodies (non-reducing conditions). **(C)** Equal volumes of membrane extracts prepared from COS-7 cells individually transfected with the M3'-Rh or M3'-V2 receptor constructs were mixed and subjected to immunoprecipitation and Western blotting as indicated. The absence of immunoreactive bands indicates that M3'-V2/M3'-Rh receptor complexes did not form artifactually during or after cell lysis. **(D)** Membrane extracts prepared from COS-7 cells cotransfected with the M3'-V2 and M3'-Rh receptor constructs were incubated with 1.5% SDS for 1 h at 37°C. The SDS concentration was then reduced to 0.1% via dilution with phosphate-buffered saline, and samples were subjected to immunoprecipitation and Western blotting as indicated (non-reducing conditions). The absence of a receptor monomer band suggests the existence of non-covalently linked, SDS-sensitive M3'-V2/M3'-Rh receptor complexes (see text for details). Protein molecular mass standards (in kDa) are indicated. Taken from Zeng and Wess (1999).

cipitated receptors were subjected to Western blotting analysis (Figure 6D). In this case, Western blotting with the anti-V2 antibody failed to reveal M3'-V2 receptor monomers; however, the characteristic pattern of high molecular mass immunoreactive species remained unaffected. These observations strongly support the notion that M₃ receptors can form complexes through non-covalent SDS-sensitive interactions, in addition to or prior to disulfide crosslinking. In contrast, it has been reported that other GPCRs of the rhodopsin family such as the β_2 -adrenergic receptor (Hebert et al. 1996), V2 vasopressin receptor (Hebert et al. 1996), or different

dopamine receptor subtypes (Ng et al. 1996; Nimchinsky et al. 1997) are able to form homodimers that are SDS-resistant.

We also mixed membrane extract prepared from M3'-V2-expressing cells with an equal volume of extract 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, as shown in Figure 6C. This finding confirmed that M3' receptor complexes were already preformed in cells prior to lysis and not generated artifactually during sample processing.

Subtype-specificity of M₃ Receptor Dimerization/Oligomerization

To investigate whether M₃ receptor oligomerization is receptor subtype-specific, we next studied whether or not the M₃' receptor was able to form heterodimers with the M₁, M₂, or V₂ receptors. To detect the M₁ and M₂ muscarinic receptors via Western blotting, we generated rabbit antisera (anti-m1 and anti-m2) directed against i3 loop-GST fusion proteins (Zeng and Wess 1999). Control experiments showed that the anti-m1, anti-m2, and anti-V₂ antibodies were able to detect their respective target receptors with high sensitivity (Figure 7A–C).

We next performed a set of coimmunoprecipitation experiments using lysates prepared from COS-7 cells cotransfected with the M₃' construct and M₁, M₂, or V₂ receptor expression plasmids. In this case, M₃' receptors were immunoprecipitated with the anti-HA antibody (note that only the M₃' receptor contained an HA epitope tag), followed by Western blot analysis (non-reducing conditions) of immunoprecipitated proteins using the anti-m3, anti-m1, anti-m2, and anti-V₂ antibodies. As shown in Figure 7D (lanes 4–6), the anti-HA

antibody failed to coprecipitate M₁, M₂, or V₂ receptors, indicating that M₃' receptor dimerization/oligomerization is receptor subtype-specific.

Consistent with our findings, receptor subtype specificity of GPCR dimerization has also been observed for individual members of the metabotropic glutamate receptor (mGluR) family (Romano et al. 1996; Robbins et al. 1999). However, recent work examining the possible association of different opioid (Jordan and Devi 1999) and GABA_B receptor subtypes (Jones et al. 1998; White et al. 1998; Kaupmann et al. 1998; Kuner et al. 1999) has clearly demonstrated the existence of receptor heterodimers. Moreover, Maggio et al. (1999) recently reported that coexpression of a mutant M₂ muscarinic receptor (containing the Asn404Ser point mutation) with the wt M₃ muscarinic receptor resulted in the appearance of muscarinic binding sites with novel pharmacological properties. Based on this observation, the authors proposed the existence of M₂/M₃ muscarinic receptor heterodimers. However, since no data showing a direct association between the two receptor species were presented, the reasons underlying the discrepant conclusions reached by Zeng et al. (1999) (see above) and Maggio et al. (1999) remain to be elucidated.

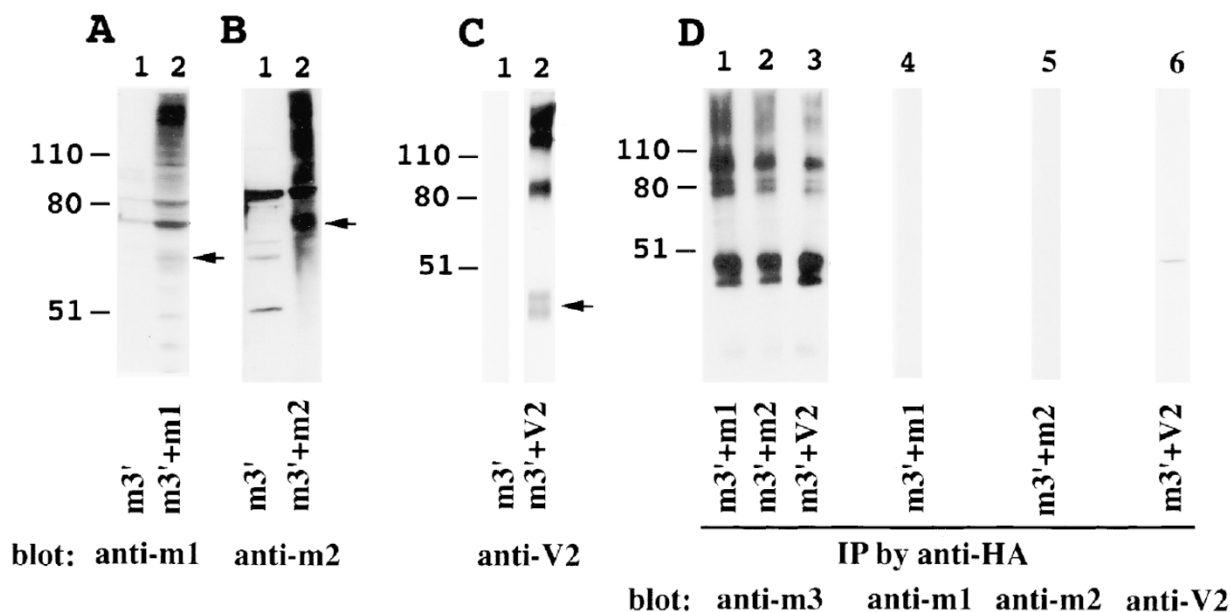


Figure 7. Coimmunoprecipitation studies examining the receptor subtype specificity of M₃' receptor dimer formation. COS-7 cells were transfected/cotransfected with the following receptor constructs: M₃' (see Figure 1), V₂ (human V₂ vasopressin receptor), m1 (human M₁ muscarinic receptor), and m2 (human M₂ muscarinic receptor). The generation of anti-m1 and anti-m2 rabbit antisera has been described by Zeng and Wess (1999). (A–C) Selectivity of anti-m1, anti-m2, and anti-V₂ antibodies used for immunoblotting studies. Membrane extracts (20 μg/lane) prepared from COS-7 cells transfected/cotransfected with the indicated receptor constructs were subjected to 12% SDS-PAGE and Western blotting using the indicated antibodies (non-reducing conditions). The 80-kDa band seen in panel B, lane 1, was also observed using cell lysates prepared from vector-transfected cells (data not shown). (D) Coimmunoprecipitation studies. Membrane extracts prepared from COS-7 cotransfected with the indicated receptor constructs were subjected to immunoprecipitation (IP) by the anti-HA antibody (Zeng and Wess 1999). Note that only the M₃' receptor contained an HA epitope tag. Immunoprecipitated receptors proteins were separated via 12% SDS-PAGE and detected via immunoblotting using the indicated antibodies (non-reducing conditions). Protein molecular mass standards (in kDa) are indicated. Taken from Zeng and Wess (1999).

M₃ Receptor Dimers/Oligomers Are Expressed on the Cell Surface

To examine whether M₃' receptor dimers were actually present on the cell surface, intact M₃'-transfected COS-7 cells were labeled with the anti-HA monoclonal antibody (Zeng and Wess 1999). This antibody is directed against the extracellular N-terminus of the M₃' receptor protein and does not penetrate the plasma membrane barrier (Schöneberg et al. 1995). Antibody-bound cell surface receptors were separated from intracellular receptors via immunoprecipitation using protein A-Sepharose 4B beads. The two resulting receptor fractions were then analyzed via non-reducing SDS-PAGE and immunoblotting using the anti-m₃ antibody. As shown in Figure 8, M₃' receptor aggregates were found both intracellularly as well as on the cell surface. This observation excludes the possibility that the higher molecular mass M₃' receptor forms are misfolded receptor aggregates that are retained intracellularly.

M₃ Receptor Dimers/Oligomers Retain Ligand-binding Activity

To study whether M₃' receptor dimers/multimers are able to bind muscarinic ligands, we incubated membrane extracts prepared from M₃' receptor-expressing COS-7 cells with an ABT-agarose affinity gel (ABT [3-(2'-

aminobenzhydryloxy)-tropane] is a muscarinic antagonist that binds to muscarinic receptors with nanomolar affinity; Haga and Haga 1985). Subsequently, specifically bound M₃' receptor species were eluted from the affinity gel with a buffer containing 100 μ M atropine and subjected to SDS-PAGE and Western blotting analysis (non-reducing conditions) (Zeng and Wess 1999). Figure 9 shows that the atropine eluate yielded the same pattern of high molecular mass M₃' receptor species as was found with control samples that had not been subjected to ABT-agarose affinity chromatography. This observation strongly suggests that M₃' receptor dimers/multimers retain ligand-binding activity.

M₃ Receptor Dimer/Oligomer Levels Are Not Regulated by Agonist Stimulation

To evaluate whether M₃' receptor dimer formation was agonist-sensitive, M₃' receptor-expressing COS-7 cells were incubated with increasing concentrations (0.01–1 mM) of the muscarinic agonist, carbachol (Zeng and Wess 1999). Subsequently, membrane extracts were prepared and subjected to SDS-PAGE and Western blotting analysis (non-reducing conditions). These studies showed that agonist-induced receptor stimulation had no significant effect on the M₃' receptor monomer/dimer ratio (Zeng and Wess 1999).

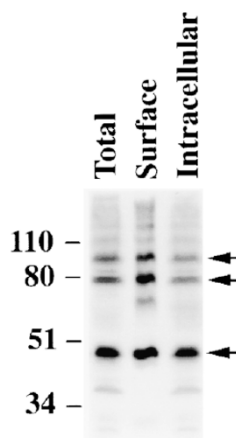


Figure 8. High molecular mass M₃' receptor aggregates are present on the cell surface. Following transient expression of the M₃' construct in COS-7 cells, cell surface M₃' receptors were labeled by incubation of intact cells with the anti-HA antibody (Zeng and Wess 1999). Antibody-bound (surface) receptors were separated from receptors that did not bind antibody (intracellular receptors) by the use of protein A-Sepharose 4B beads. The two fractions were subjected to 12% SDS-PAGE and Western blot analysis using the anti-m₃ antibody (non-reducing conditions). Lane 1 ("total" receptors): Membrane extract prepared from M₃'-expressing cells that had not been subjected to protein A-Sepharose 4B treatment. Protein molecular mass standards (in kDa) are indicated. Taken from Zeng and Wess (1999).

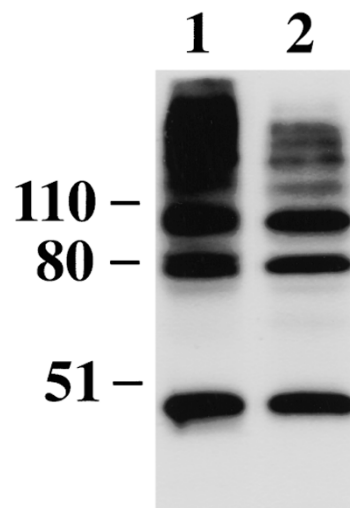


Figure 9. Binding of high molecular mass M₃' receptor species to ABT-agarose affinity gels. Membrane lysates were prepared from M₃' receptor-transfected COS-7 cells and subjected to ABT-agarose affinity chromatography as described (Zeng and Wess 1999). Receptors bound to the affinity gel were eluted with a buffer containing 100 μ M atropine. Eluates (lane 2) were subjected to 12% SDS-PAGE and immunoblotting using the anti-m₃ antibody (non-reducing conditions). Lane 1: Control sample that had not been subjected to ABT-agarose affinity chromatography. Protein molecular mass standards (in kDa) are indicated. Taken from Zeng and Wess (1999).

As observed with the M3' muscarinic receptor, dimerization of the calcium-sensing receptor expressed in HEK293 cells was also found to be agonist-insensitive (Bai et al. 1998). On the other hand, evidence has been presented that agonist stimulation of β_2 -adrenergic receptor-transfected COS-7 cells stabilized the dimeric state of this receptor subtype (Hebert et al. 1996). Similarly, AbdAlla et al. (1999) showed that dimerization/oligomerization of the B₂ bradykinin receptor was dependent on agonist stimulation. In contrast, agonist incubation of δ -opioid receptor-expressing CHO cells was found to lead to a reduction in the formation of receptor dimers (Cvejic and Devi 1997). It remains to be seen whether these discrepant results are due to differences in experimental conditions or whether agonist-dependent regulation of receptor dimerization varies from receptor to receptor.

Cysteine Residues Involved in M3' Receptor Crosslinking

As shown in Figure 3, the M3' receptor contains 10 native Cys residues, four of which are located on the extracellular receptor surface (Cys140, Cys220, Cys516, and Cys519). Since disulfide bridges primarily occur on the extracellular surface of integral membrane proteins, we speculated that one or more of these four extracellular Cys residues might participate in the formation of disulfide-linked M3' receptor dimers. To test this hypothesis, these Cys residues were systematically targeted by site-directed mutagenesis (Figure 3).

As expected, biochemical analysis of a series of Cys>Ala/Ser mutant M3' receptors showed that disulfide-crosslinking was dependent on the presence of extracellular Cys residues (Figure 10). A mutant receptor in which Cys140 and Cys220 were simultaneously converted to alanine almost completely lacked the ability to form disulfide-linked receptor dimers (Figure 10, lane 5). Subsequent analysis of mutant M3' receptors containing either the C140A or the C220A single point mutations indicated that both mutant receptors were impaired in their ability to form disulfide-linked receptor aggregates (Zeng and Wess 1999), indicating that Cys140 and Cys220 play key roles in the covalent association of M3' receptor monomers.

Interestingly, Cys140 and Cys220 are conserved among almost all GPCRs of the rhodopsin family (Watson and Arkininstall 1994; Strader et al. 1994). Considerable evidence suggests that these two conserved Cys residues are engaged in an intramolecular disulfide bond, thus covalently linking the first and second extracellular loops (see, for example, Curtis et al. 1989; Kurtenbach et al. 1990). As outlined above, our findings indicate that the two conserved extracellular Cys residues can also participate in the formation of intermolecular disulfide bonds. One possibility is that the forma-

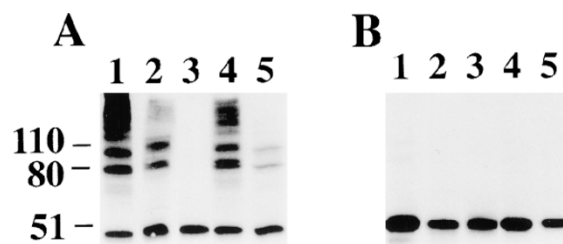


Figure 10. Cys140 and Cys220 play key roles in the formation of disulfide-linked M3' receptor dimers. COS-7 cells were transfected with mutated M3' receptor constructs containing the following amino acid substitutions (also see Figure 3): M3' (lane 1), M3'(C111S, C532A, C542A, C546S, C560S, C562S) (lane 2), M3'(C140A, C220A, C516A, C519A) (lane 3), M3'(C516A, C519A) (lane 4), and M3'(C140A, C220A) (lane 5). Membrane extracts were prepared from transfected COS-7 cells and subjected to 12% SDS-PAGE and Western blotting analysis using the anti-m3 antibody (A), non-reducing conditions; (B), reducing conditions. Protein molecular mass standards (in kDa) are indicated. Taken from Zeng and Wess (1999).

tion of intermolecular disulfide bonds involves disulfide bond exchange reactions between individual receptor monomers. In such a model, the formation of non-covalent receptor aggregates is predicted to precede intermolecular disulfide-crosslinking.

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 in PI assays (>10,000-fold), as compared with the M3' receptor (Zeng et al. 1999). It remains to be explored whether these functional deficits are due to the absence of intramolecular or intermolecular disulfide bonds.

Previous work has demonstrated that the mGluRs (Romano et al. 1996; Robbins et al. 1999) and the calcium-sensing receptor (Ward et al. 1998; Bai et al. 1998) (together with the GABA_B receptors, these receptors form a unique GPCR subfamily; Wess 1998) are also able to form disulfide-linked receptor dimers. These studies also showed that the disulfide-linked dimeric receptor species are expressed on the cell surface (Romano et al. 1996; Bai et al. 1998). Several investigators reported that disulfide-crosslinking of mGluRs (Romano et al. 1996; Okamoto et al. 1998; Robbins et al. 1999) and calcium-sensing receptor monomers (Goldsmith et al. 1999; Pace et al. 1999; Ray et al. 1999) involves cysteine residues located within the large extracellular N-terminal domain that is characteristic for this class of receptors.

In an additional set of experiments, we also addressed the question whether the loss of disulfide-crosslinking prevented non-covalent association of M3' receptor monomers. Specifically, we generated two versions of the M3'(C140A, C220A) mutant receptor that carried either a C-terminal rhodopsin or V2 tag. Coim-

munoprecipitation studies showed that the anti-Rh antibody was able to coprecipitate the V2-tagged receptor species (Figure 11). As expected, no higher molecular mass disulfide-crosslinked species were observed when SDS-PAGE/Western blot analysis was carried out under non-reducing conditions (Figure 11).

FUTURE OUTLOOK

An important question that remains to be addressed is to what extent muscarinic receptor dimers/multimers differ functionally from the monomeric receptor species. The identification and functional analysis of mutant receptors that fail to oligomerize as well as the use of reagents that can prevent the formation of muscarinic receptor multimers should shed light on this question. Several recent studies strongly suggest that GPCR homodimerization is in fact of functional relevance. For example, Hebert et al. (1996) demonstrated that the extent of β_2 -adrenergic receptor dimerization correlated well with receptor-mediated G-protein activation. Cvejic and Devi (1997) showed that a dimerization-defective mutant δ -opioid receptor lacked the ability to undergo agonist-dependent internalization. AbdAlla et al.

(1999) found that a dimerization-impaired mutant B_2 bradykinin receptor was not phosphorylated, did not desensitize, and was not down-regulated upon receptor stimulation with bradykinin. More detailed analysis of other GPCRs, including the muscarinic receptors, will show whether or not attenuation of receptor-mediated signaling is a general phenomenon associated with GPCR oligomerization.

Future work should also help elucidate the molecular mechanisms underlying the non-covalent association of muscarinic receptor (or other GPCR) monomers. Biochemical studies with the β_2 -adrenergic receptor, for example, have demonstrated that dimerization of this receptor subtype may depend on hydrophobic contacts involving residues located on TM VI (Hebert et al. 1996). On the other hand, homodimerization of the δ -opioid receptor was shown to be dependent on the integrity of the C-terminal "tail" (Cvejic and Devi 1997). Likewise, the formation of GABA_BR1/ GABA_BR2 receptor heterodimers appears to involve amino acids within the C-terminal receptor regions (White et al. 1998; Kuner et al. 1999). Finally, biochemical analysis of a B_2 bradykinin receptor truncation mutant has implicated the N-terminal extracellular domain in receptor dimerization, further highlighting the structural diversity by which GPCR oligomerization can be achieved.

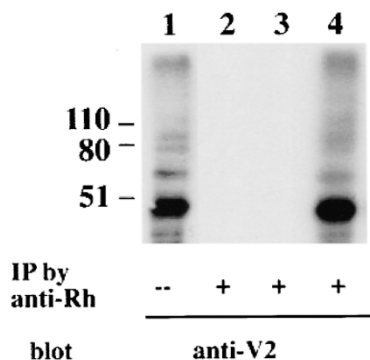


Figure 11. Coimmunoprecipitation studies with the M3'(C140A, C220A) receptor construct carrying different C-terminal epitope tags. C-terminally tagged versions of the M3'(C140A, C220A) mutant receptor [M3'(C140A, C220A)-V2 and M3'(C140A, C220A)-Rh] were generated as described in the legend to Figure 6. Lane 1: Membrane extract prepared from cotransfected COS-7 cells (M3'(C140A, C220A)-V2 plus M3'(C140A, C220A)-Rh) that had not been subjected to immunoprecipitation (IP). Samples run in lanes 2–4 were subjected to IP using the anti-Rh antibody, followed by 12% SDS-PAGE and Western blotting analysis using the anti-V2 antibody (non-reducing conditions). Lanes 2 (M3'(C140A, C220A)-V2 expressed alone) and 3 (M3'(C140A, C220A)-Rh expressed alone) confirm the specificity of the anti-V2 and anti-Rh antibodies. Lane 4 (cotransfection of M3'(C140A, C220A)-V2 with M3'(C140A, C220A)-Rh) indicates that the M3'(C140A, C220A) receptor retains the ability to form non-covalently linked, SDS-sensitive aggregates. Taken from Zeng and Wess (1999).

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