Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3103–3107, April 1993
Neurobiology

Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors

(receptor dimerization/site-directed mutagenesis/chimeric receptors/truncated receptors/phosphatidylinositol hydrolysis)

ROBERTO MAGGIO, ZVI VOGEL, AND JÜRGEN WESS*

Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, Building 36, Room 3D-02, Bethesda, MD 20892

Communicated by Marshall Nirenberg, December 30, 1992

ABSTRACT **We have tested the hypothesis that guanine-nucleotide-binding-protein-coupled receptors may be able to interact with each other at a molecular level. To address this question, we have initially created two chimeric receptors, α_2/m3 and m3/α_2, in which the C-terminal receptor portions (containing transmembrane domains VI and VII) were exchanged between the α_{2C}-adrenergic and the m3 muscarinic receptor. Transfection of COS-7 cells with either of the two chimeric constructs alone did not result in any detectable binding activity for the muscarinic ligand *N*-[^{3}H]methylscopolamine or the adrenergic ligand [^{3}H]rauwolscine. However, cotransfection with α_2/m3 and m3/α_2 resulted in the appearance of specific binding sites (30–35 fmol/mg of membrane protein) for both radioligands. These sites displayed ligand binding properties similar to those of the two wild-type receptors. Furthermore, COS-7 cells cotransfected with α_2/m3 and m3/α_2 were able to mediate a pronounced stimulation of phosphatidylinositol hydrolysis upon stimulation with the muscarinic agonist carbachol ($E_{max} \approx$ 40–50% of wild-type m3). A mutant m3 receptor (containing 16 amino acids of m2 receptor sequence at the N terminus of the third cytoplasmic loop) that was capable of binding muscarinic ligands but was virtually unable to stimulate phosphatidylinositol hydrolysis was also used in various cotransfection experiments. Coexpression of this chimeric receptor with other functionally impaired mutant muscarinic receptors (e.g., with an m3 receptor containing a Pro → Ala point mutation in transmembrane region VII) resulted in a considerable stimulation of phosphatidylinositol breakdown after carbachol treatment ($E_{max} \approx$ 40–50% of wild-type m3). Thus, these data suggest that guanine-nucleotide-binding-protein-coupled receptors can interact with each other at a molecular level. One may speculate that the formation of receptor dimers involving the intermolecular exchange of N- and C-terminal receptor domains (containing transmembrane domains I–V and VI and VII, respectively) may underlie this phenomenon.**

Muscarinic acetylcholine receptors (m1–m5) are integral membrane proteins that, upon agonist stimulation, interact with guanine nucleotide binding proteins (G proteins) to induce a variety of intracellular responses (1, 2). Like all other G-protein-coupled receptors, the muscarinic receptors are predicted to be composed of seven hydrophobic transmembrane domains (TMD I–VII), connected by alternating intra- and extracellular loops. Whereas several of the transmembrane regions have been shown to be involved in ligand binding (2–4), the specificity of G-protein coupling is primarily determined by the membrane-proximal portions of the third cytoplasmic loop (i3; refs. 5–8).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

We have shown (9) that muscarinic receptors behave structurally in a fashion analogous to two-subunit receptors. When truncated m2 or m3 receptors (containing TMD I–V) were coexpressed in COS-7 cells with gene fragments coding for the corresponding C-terminal receptor portions (containing TMD VI and VII), functional muscarinic receptors with ligand binding properties similar to those of the two wild-type receptors were obtained. Similar findings have also been described for "split" β_2-adrenergic receptors coexpressed in *Xenopus* oocytes (10). Based on these results, we have speculated that the association of N- (containing TMD I–V) and C-terminal receptor domains (containing TMD VI and VII) may occur not only intra- but also intermolecularly, thus providing a molecular basis for receptor dimerization.

To test the hypothesis that G-protein-coupled receptors are in fact able to interact with each other at a molecular level, we initially created two chimeric receptor molecules, α_2/m3 and m3/α_2 (Fig. 1), in which the C-terminal receptor domains (including TMD VI and VII) were exchanged between the α_{2C}-adrenergic (in the following referred to as α_2) and the m3 muscarinic receptor. The two mutant receptors were expressed in COS-7 cells, either alone or in combination, and studied for their ability to bind muscarinic and adrenergic ligands and to stimulate phosphatidylinositol (PI) hydrolysis. Furthermore, coexpression studies were performed with various mutant m3 muscarinic receptors that functionally were severely impaired or completely inactive (Fig. 1). The results of these studies suggest that muscarinic receptors can functionally interact with each other at the protein level.

MATERIALS AND METHODS

Preparation of Mutant Receptor Constructs. Rm3pcD (12) and Rα_2pRc/CMV (ref. 13; kindly provided by H. Chin, National Institutes of Health, Bethesda, MD), two mammalian expression vectors containing the entire coding sequence of the rat m3 muscarinic and the rat α_{2C}-adrenergic receptor, respectively, were used to construct α_2/m3 and m3/α_2 (Fig. 1). Expression plasmids coding for these two chimeric receptors (pcDα_2/m3 and pcDm3/α_2) were created as follows.

pcDα_2/m3. A 0.67-kb *Hin*fI–*Acc* I fragment (containing the coding sequence for TMD I–V of the α_2-adrenergic receptor) was removed from Rα_2pRc/CMV and ligated, via a synthetic DNA linker sequence (encoding the last amino acid of TMD V of the α_2-adrenergic receptor and the first 10 amino acids of the i3 loop of the m3 muscarinic receptor), with the 5.7-kb *Ppu*MI–*Bst*XI restriction fragment from Rm3pcD. The resulting construct encoded a chimeric receptor (α_2/m3) con-

Abbreviations: G protein, guanine nucleotide binding protein; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; i3, the third cytoplasmic loop of G-protein-coupled receptors; PI, phosphatidylinositol; IP_1, inositol monophosphate; NMS, *N*-methylscopolamine; TMD, transmembrane domain of G-protein-coupled receptors.
*To whom reprint requests should be addressed.

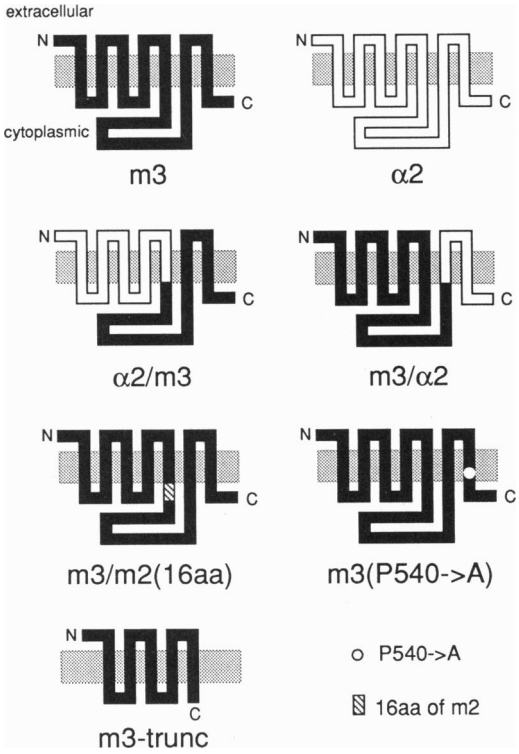

FIG. 1. Structure of chimeric α_2-adrenergic/m3 muscarinic receptors (α_2/m3 and m3/α_2) and various other mutant m3 receptors. The N terminus is predicted to be located extracellularly, whereas the C terminus is thought to reside on the cytoplasmic side of the plasma membrane (shaded area). In m3/m2(16aa), the first 16 amino acids of the i3 loop of the m3 receptor have been replaced with the corresponding segment of the m2 muscarinic receptor (5, 6). In m3(P540→A), Pro-540 (located in TMD VII) has been replaced with Ala (11). m3-trunc represents an m3 receptor that has been truncated at the beginning of the i3 loop (containing only the first 21 amino acids of i3).

sisting of the first 187 amino acids of the α_2-adrenergic receptor and the last 338 amino acids of the m3 muscarinic receptor (Fig. 1).

pcDm3/α_2. An *Nhe* I site was created at the C terminus of the i3 loop in Rm3pcD by a PCR procedure, leaving unchanged the amino acid sequence of the i3 loop. A 0.95-kb *Dra* III–*Nhe* I restriction fragment was removed from this construct and replaced with a 0.23-kb PCR fragment encoding the C-terminal domains of the α_2-adrenergic receptor (including TMD VI and VII). The encoded chimeric receptor (m3/α_2) consisted of the first 491 amino acids of the m3 muscarinic receptor and the last 75 amino acids of the α_2-adrenergic receptor (Fig. 1).

pcDm3-trunc. A 0.70-kb *Bst*XI–*Nhe* I restriction fragment was removed from Rm3pcD and replaced with a synthetic linker containing an in-frame stop codon after amino acid codon 272 of the rat m3 receptor sequence (Fig. 1). The encoded truncated receptor (m3-trunc) contained only the first 21 amino acids of the i3 loop.

pcDm2-trunc. A 5.3-kb *Sst* I restriction fragment was removed from Hm2pcD (an expression plasmid coding for the human m2 muscarinic receptor, see ref. 12) and subjected to self-ligation, resulting in the creation of a stop codon after amino acid codon 283 (located in the middle of the i3 loop) of the m2 receptor sequence.

The construction of expression plasmids coding for m3/m2(16aa) (a receptor in which the first 16 amino acids of the i3 loop of the rat m3 receptor have been replaced with the corresponding segment of the human m2 receptor) and m3(P540→A) (an m3 receptor in which Pro-540 was replaced with Ala) has been described (5, 11). The identity of all mutations and the correctness of all PCR-derived coding sequences were confirmed by dideoxynucleotide sequencing of the mutant plasmids (14).

Transfections and Binding Assays. COS-7 cells were transfected in 100-mm plates with a total amount of 20 μg of plasmid DNA by calcium phosphate precipitation as described (15). Cells were harvested ≈72 hr after transfection, and radioligand binding studies were carried out with membrane homogenates as described (16). In competition binding experiments, the muscarinic antagonist *N*-[^{3}H]methylscopolamine([^{3}H]NMS; 200 pM) and the adrenergic antagonist [^{3}H]rauwolscine (2 nM) were used. Nonspecific binding was determined in the presence of 1 μM atropine (for muscarinic ligands) or 100 μM noradrenaline (for adrenergic ligands).

PI Assays. Transfected COS-7 cells were incubated with *myo*-[^{3}H]inositol (3 μCi/ml; 23 Ci/mmol; 1 Ci = 37 GBq; American Radiolabeled Chemicals, St. Louis) for 48 hr. Carbachol-induced increases in intracellular inositol monophosphate (IP_1) levels were determined as described (17).

Ligands. [^{3}H]NMS (78.9 Ci/mmol) and [^{3}H]rauwolscine (77.9 Ci/mmol) were purchased from DuPont/NEN. 4-Diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) was obtained from Research Biochemicals (Natick, MA). All other ligands were from Sigma.

RESULTS

Ligand Binding Properties of Chimeric α_2-Adrenergic/m3 Muscarinic Receptors Expressed in COS-7 Cells. Two chimeric α_2-adrenergic/m3 muscarinic receptors, α_2/m3 and m3/α_2 (Fig. 1), were expressed in COS-7 cells, either alone or in combination, and tested for their ability to bind muscarinic and adrenergic ligands. Untransfected COS-7 cells or cells transfected with either of the two chimeric constructs alone did not display any specific binding for the muscarinic antagonist [^{3}H]NMS or the adrenergic antagonist [^{3}H]rauwolscine. In contrast, cotransfection of COS-7 cells with α_2/m3 and m3/α_2 resulted in the appearance of specific binding sites for both radioligands. Except for an ≈5-fold decrease in their affinity for acetylcholine (Table 1), these sites displayed ligand binding properties similar to those of the two wild-type receptors (Table 1 and Fig. 2). The maximum number of binding sites (B_{max}) detected with either of the two radioligands amounted to 30–35 fmol/mg of protein. In comparison, transfection of COS-7 cells with plasmid DNA (20 μg) coding for either of the two wild-type receptors resulted in B_{max} levels of ≈1 pmol/mg of protein.

Stimulation of PI Hydrolysis After Coexpression of Chimeric α_2-Adrenergic/m3 Muscarinic Receptors. The ability of α_2/m3 and m3/α_2 to mediate stimulation of PI hydrolysis was also examined. Carbachol stimulation of nontransfected COS-7 cells or of cells transfected with either of the two chimeric constructs alone did not result in any significant activation of PI metabolism (Fig. 3*A* and Table 2). On the other hand, carbachol treatment of cells cotransfected with α_2/m3 and m3/α_2 resulted in a pronounced increase in intracellular IP_1 levels [83 ± 15% (mean ± SEM) above basal], although this effect did not reach the magnitude of the wild-type response (191 ± 29%; Fig. 3*A* and Table 2). The carbachol EC_{50} determined for this response (4.3 ± 2.9 μM) was only ≈3-fold higher than that found with the wild-type m3 receptor (1.5 ± 0.4 μM) (Table 2).

Stimulation of PI Hydrolysis After Coexpression of Functionally Impaired Mutant m3 Receptors. Additional coexpression experiments were performed with mutant m3 muscarinic receptors that were unable or severely impaired in their ability to mediate stimulation of PI hydrolysis. m3/m2(16aa), an m3 receptor in which the N-terminal portion of the i3 loop has been replaced with the corresponding m2 receptor sequence (Fig. 1), has been shown to bind muscarinic ligands

Table 1. Ligand binding properties of chimeric α_2-adrenergic/m3 muscarinic receptors coexpressed in COS-7 cells

	Muscarinic ligand				Adrenergic ligand	
Receptor	[^{3}H]NMS K_d, pM	4-DAMP K_i, nM	Acetylcholine IC_{50}, μM	Carbachol IC_{50}, μM	[^{3}H]Rauwolscine K_d, nM	Yohimbine K_i, nM
m3 wild type	23 ± 3 (0.91 ± 0.06)	2.9 ± 0.1 (0.89 ± 0.02)	8.8 ± 1.2 (0.75 ± 0.04)	52 ± 7 (0.77 ± 0.07)	—	
α_2 wild type	—				1.8 ± 0.2 (0.94 ± 0.10)	4.1 ± 0.3 (1.02 ± 0.06)
α_2/m3 + m3/α_2	18 ± 2 (1.03 ± 0.12)	3.0 ± 0.3 (0.87 ± 0.07)	47 ± 6 (0.81 ± 0.07)	65 ± 12 (0.64 ± 0.04)	1.9 ± 0.5 (1.08 ± 0.13)	4.2 ± 0.6 (0.96 ± 0.13)

No specific [^{3}H]NMS or [^{3}H]rauwolscine binding sites could be detected after transfection of COS-7 cells with α_2/m3 or m3/α_2 alone. Affinity constants (K_d) for [^{3}H]NMS and [^{3}H]rauwolscine were determined in direct binding assays. Inhibition constants (K_i) and IC_{50} values were obtained in competition binding experiments as described (16). Hill coefficients are given in parentheses. Data are presented as means ± SEM of two or three experiments, each carried out in duplicate.

with wild-type affinities (5, 6) but is virtually unable to mediate stimulation of PI hydrolysis (refs. 5 and 6; Fig. 3*B*). m3-Trunc (Fig. 1) represents a truncated m3 receptor that was unable to bind muscarinic ligands or to mediate a functional response (Fig. 3*B* and Table 2). However, when m3/m2(16aa) and m3-trunc were coexpressed in COS-7 cells, a considerable increase in intracellular IP_1 levels (84 ± 15% above basal) was observed after carbachol stimulation.

In another set of experiments, m3/m2(16aa) was coexpressed with a mutant m3 receptor (m3[P540→A]; Fig. 1) containing a point mutation in TMD VII. m3(P540→A) has been shown to bind muscarinic agonists and antagonists with high affinity but is functionally severely impaired (ref. 11; Fig. 3*C*). Interestingly, m3/m2(16aa) and m3(P540→A) were able to induce a significant stimulation of PI breakdown (89 ± 4%) when the two mutant receptors were coexpressed in COS-7 cells (Fig. 3*C* and Table 2).

Coexpression of a Truncated m2 Receptor With α_2/m3. To exclude the possibility that, in the coexpression experiments

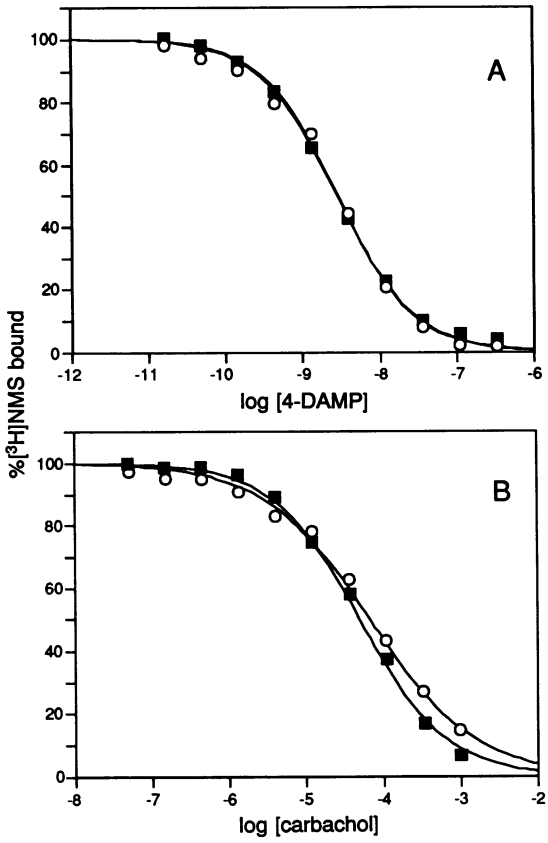

FIG. 2. Ligand binding properties of chimeric α_2-adrenergic/m3 muscarinic receptors (α_2/m3 and m3/α_2) coexpressed in COS-7 cells. The muscarinic antagonist 4-DAMP (*A*) and the muscarinic agonist carbachol (*B*) were tested for their ability to compete for the binding of the radioligand [^{3}H]NMS (200 pM), by using membranes prepared from transfected COS-7 cells. ■, Wild-type m3; ○, α_2/m3 coexpressed with m3/α_2. Curves were generated by computer fit as described (16). Actual binding parameters are given in Table 1.

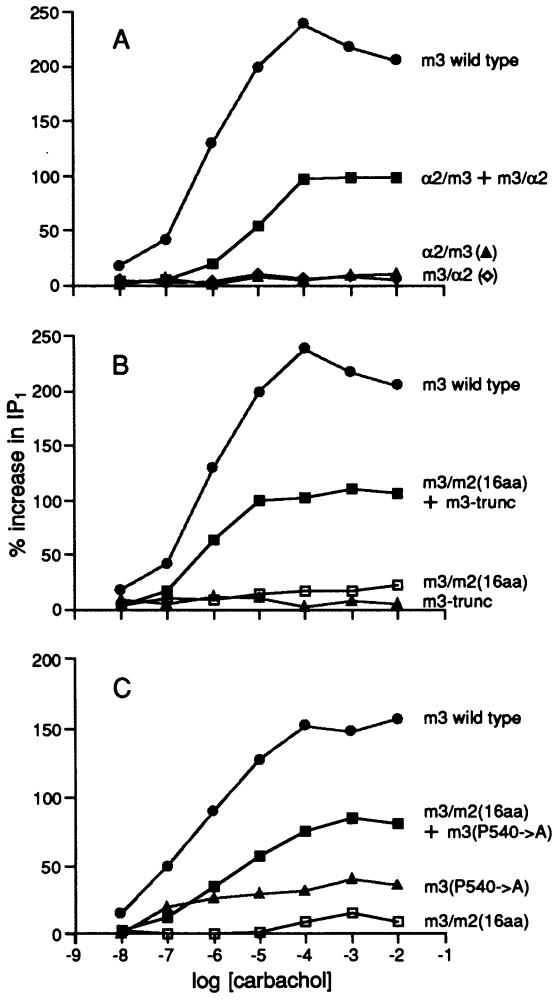

FIG. 3. Carbachol-induced PI hydrolysis after coexpression of chimeric α_2-adrenergic/m3 muscarinic receptors (*A*) and various other mutant m3 receptors (*B* and *C*). Transfected COS-7 cells were incubated with increasing concentrations of carbachol for 1 hr at 37°C, and the resultant increases in intracellular IP_1 levels were determined as described (17). Responses are expressed as percent increase in IP_1 above basal levels determined in the absence of carbachol. Basal IP_1 levels were similar in all experiments. Each curve is representative of two to four experiments, each carried out in duplicate.

Table 2. Carbachol-induced stimulation of PI hydrolysis after coexpression of chimeric α_2-adrenergic/m3 muscarinic receptors and other mutant m3 receptors in COS-7 cells

Receptor	Amount of transfected DNA, μg	B_{max}, fmol/mg	PI hydrolysis: Maximum increase in IP_1 levels above basal, %	PI hydrolysis: Carbachol EC_{50}, μM
m3 wild type	2	225 ± 32	191 ± 29	1.5 ± 0.4
m3/α_2	20	—	NS	—
α_2/m3	20	—	NS	—
α_2/m3 + m3/α_2	10 + 10	32 ± 4	83 ± 15	4.3 ± 2.9
m3/m2(16aa)	20	203 ± 13	15 ± 3	ND
m3(P540→A)	20	42 ± 8	44 ± 8	0.37 ± 0.17
m3/m2(16aa) + m3(P540→A)	10 + 10	147 ± 24	89 ± 4	3.7 ± 0.8
m3-trunc	20	—	NS	—
m3/m2(16aa) + m3-trunc	10 + 10	107 ± 2	84 ± 15	0.16 ± 0.03

B_{max} values (binding sites per mg of membrane protein) were determined in [^{3}H]NMS saturation binding experiments. The amount of transfected wild-type m3 plasmid DNA was reduced to 2 μg to obtain B_{max} values similar to those found for the various mutant receptors. Basal IP_1 levels, determined in the absence of carbachol, were not significantly different for wild-type m3 and the various mutant receptors (expressed either alone or in different combinations). Data are presented as means ± SEM of two to four independent experiments. NS, no statistically significant stimulation of PI hydrolysis; ND, not determinable with sufficient accuracy.

described above, homologous recombination events may have led to the recreation of wild-type receptor plasmid DNA, the following experiment was designed: An m2 muscarinic receptor was truncated in the middle of the i3 loop (m2-trunc) and was coexpressed in COS-7 cells with α_2/m3. Transfection of COS-7 cells with either m2-trunc or α_2/m3 alone did not result in any detectable [^{3}H]NMS binding activity, whereas cotransfection with these two mutant constructs gave a considerable number of specific [^{3}H]NMS binding sites (≈50 fmol/mg of protein). Since m2-trunc shares virtually no sequence homology with the m3 receptor sequence present in α_2/m3 (18), the appearance of functional muscarinic receptors in the various cotransfection experiments appears to be due to protein–protein interactions rather than to homologous recombination events.

DISCUSSION

To test the hypothesis that G-protein-coupled receptors are able to interact with each other at a molecular level, two chimeric receptors, α_2/m3 and m3/α_2 (Fig. 1), in which a region including TMD VI and VII was exchanged between the α_2-adrenergic and the m3 muscarinic receptor, were created. Expression in COS-7 cells of either of the two chimeric proteins alone did not result in functional receptors. However, coexpression of α_2/m3 and m3/α_2 resulted in a significant number of specific binding sites for the muscarinic ligand [^{3}H]NMS and the adrenergic ligand [^{3}H]rauwolscine. In general, these sites displayed a pharmacology similar to that of the two wild-type receptors. In addition, carbachol stimulation of COS-7 cells coexpressing α_2/m3 and m3/α_2 resulted in a considerable stimulation of PI hydrolysis (E_{max} ≈ 40–50% of wild-type m3). These data suggest that the two chimeric proteins must be able to physically interact with each other to create functional receptors.

Various observations, in addition to the significant number of detectable receptor sites, argue against the possibility that homologous recombination events (resulting in the creation of wild-type plasmid DNA), rather than intermolecular interactions at the protein level, are responsible for the appearance of functional receptors after coexpression of α_2/m3 and m3/α_2. (*i*) Coexpression of a truncated m2 receptor, m2-trunc (containing TMD I–V and the N-terminal half of the i3 loop), with α_2/m3 resulted in a considerable number of [^{3}H]NMS binding sites (≈50 fmol/mg of protein), whereas none of the two mutant proteins was able to bind muscarinic ligands when expressed alone. Since m2-trunc shares virtually no sequence homology with the m3 receptor sequence present in α_2/m3 (18), protein–protein interactions, rather than homologous recombination events, appear to be responsible for this phenomenon. (*ii*) The muscarinic binding sites detected after cotransfection of COS-7 cells with α_2/m3 and m3/α_2 showed an ≈5-fold lower affinity for acetylcholine than the wild-type receptor, thus clearly arguing against the "accidental" creation of wild-type m3 receptor due to homologous recombination events.

Coexpression experiments involving fragmented muscarinic (9) or adrenergic (10) receptors provide a possible clue as to the molecular basis underlying the functional interaction between α_2/m3 and m3/α_2. These studies have shown that muscarinic and β_2-adrenergic receptors behave in a fashion analogous to two-subunit receptors (Fig. 4). For example, coexpression in COS-7 cells of a truncated m3 receptor (containing TMD I–V) with the corresponding C-terminal receptor fragment (containing TMD VI and VII) resulted in the "reconstitution" of functional muscarinic receptors that were able to stimulate PI hydrolysis to a maximum extent similar to that of the wild-type receptor (9). One may therefore speculate that, in the coexpression experiments described above, the C-terminal portion of α_2/m3 is able to interact with the N-terminal portion of m3/α_2 to create

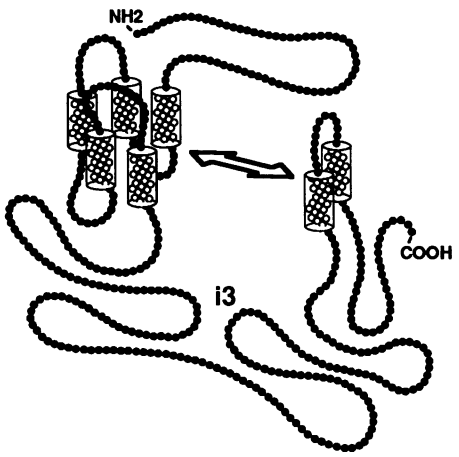

FIG. 4. Model depicting the hypothetical subunit character of muscarinic receptors based on coexpression studies with fragmented m2 and m3 receptors (9). The seven cylinders represent TMD I–VII, whereas the solid circles mark extramembranous receptor sequences. The third cytoplasmic loop (i3) is thought to act as a linker sequence between two structural receptor subunits (one containing TMD I–V and the other containing TMD VI and VII).

functional muscarinic receptors. An analogous mechanism may explain the appearance of α_2-adrenergic binding sites observed after coexpression of α_2/m3 and m3/α_2.

Further evidence supporting the concept of intermolecular cross-talk between G-protein-coupled receptors was derived from cotransfection experiments involving the use of two additional mutant m3 muscarinic receptors, m3/m2(16aa) and m3(P540→A) (for structures, see Fig. 1). In contrast to α_2/m3 and m3/α_2, these two mutant receptors, when expressed alone, were able to bind muscarinic agonists and antagonists with high affinity (5, 6, 11). However, both mutant proteins were found to be severely impaired in their ability to activate PI metabolism (5, 6, 11). Coexpression of m3/m2(16aa) and m3(P540→A) in COS-7 cells, followed by carbachol stimulation, resulted in a significant stimulation of PI hydrolysis ($E_{max} \approx$ 40–50% of wild-type m3). This finding further supports the concept that muscarinic receptors are able to interact with each other at the receptor level, possibly in a fashion analogous to that described above for the interaction between α_2/m3 and m3/α_2.

Consistent with this proposal, photoaffinity labeling studies (followed by gel electrophoresis) have shown that rat heart and brain muscarinic receptors can exist in dimeric forms (19, 20). A similar conclusion has been reached based on a systematic analysis of the agonist binding properties of muscarinic receptors in various rat tissues (21, 22). Interestingly, it has been proposed that serotonin 5-HT_2 and 5-HT_{1A} receptors, based on the presence of leucine-zipper sequences in individual transmembrane segments, may also be capable of dimer formation (23).

In conclusion, we have demonstrated that muscarinic receptors are able to functionally interact with each other at a molecular level. Moreover, we have proposed a model in which receptor dimerization is dependent on the intermolecular exchange of N- and C-terminal receptor domains (containing TMD I–V and VI and VII, respectively). Based on the high structural homology found among all G-protein-coupled receptors, our findings should be of general importance for the entire receptor superfamily.

We thank Prerana Sangani for skillful technical assistance.

1. Nathanson, N. M. (1987) *Annu. Rev. Neurosci.* **10,** 195–236.
2. Hulme, E. C., Birdsall, N. J. M. & Buckley, N. J. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 633–673.
3. Hulme, E. C., Kurtenbach, E. & Curtis, C. A. M. (1991) *Biochem. Soc. Trans.* **19,** 133–138.
4. Wess, J., Gdula, D. & Brann, M. R. (1991) *EMBO J.* **10,** 3729–3734.
5. Wess, J., Brann, M. R. & Bonner, T. I. (1989) *FEBS Lett.* **258,** 133–136.
6. Wess, J., Bonner, T. I., Dörje, F. & Brann, M. R. (1990) *Mol. Pharmacol.* **38,** 517–523.
7. Lechleiter, J., Hellmiss, R., Duerson, K., Ennulat, D., David, N., Clapham, D. & Peralta, E. (1990) *EMBO J.* **9,** 4381–4390.
8. Okamoto, T. & Nishimoto, I. (1992) *J. Biol. Chem.* **267,** 8342–8346.
9. Maggio, R., Vogel, Z. & Wess, J. (1993) *FEBS Lett.* **319,** 195–200.
10. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Science* **240,** 1310–1316.
11. Wess, J., Nanavati, S., Vogel, Z. & Maggio, R. (1993) *EMBO J.* **12,** 331–338.
12. Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. (1987) *Science* **237,** 527–532.
13. Voigt, M. M., McCune, S. K., Kanterman, R. Y. & Felder, C. C. (1991) *FEBS Lett.* **278,** 45–50.
14. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
15. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
16. Dörje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E. & Brann, M. R. (1991) *J. Pharmacol. Exp. Ther.* **256,** 727–733.
17. Wess, J., Bonner, T. I. & Brann, M. R. (1990) *Mol. Pharmacol.* **38,** 872–877.
18. Bonner, T. I. (1989) *Trends NeuroSci.* **12,** 148–151.
19. Avissar, S., Moscona-Amir, E. & Sokolovsky, M. (1982) *FEBS Lett.* **150,** 343–346.
20. Avissar, S., Amitai, G. & Sokolovsky, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 156–159.
21. Potter, L. T., Ferrendelli, C. A. & Hanchett, H. E. (1988) *Cell. Mol. Neurobiol.* **8,** 181–191.
22. Potter, L. T., Ballesteros, L. A., Bichajian, L. H., Ferrendelli, C. A., Fisher, A., Hanchett, H. E. & Zhang, R. (1991) *Mol. Pharmacol.* **39,** 211–221.
23. Hartig, P., Kao, H.-T., Macchi, M., Adham, N., Zgombick, J., Weinshank, R. & Branchek, T. (1990) *Neuropsychopharmacology* **3,** 335–347.