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Intramolecular Interactions in Muscarinic Acetylcholine Receptors Studied with Chimeric m2/m5 Receptors

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SUMMARY

Current models of the three-dimensional structures of muscarinic acetylcholine receptors and other G protein-coupled receptors are based primarily on high-resolution electron diffraction data obtained with bacteriorhodopsin, the molecular structure of which is characterized by the presence of seven α-helical transmembrane domains (TM I-VII). However, bacteriorhodopsin does not couple to G proteins and its primary sequence lacks a series of amino acids that are conserved among virtually all G protein-coupled receptors. Therefore, it remains to be shown experimentally whether the molecular structures of these functionally different proteins are in fact identical. To address this question, we have analyzed the pharmacological properties of a series of hybrid human m2/m5 muscarinic receptors. Initially, we identified several chimeric constructs that, upon transient expression in COS-7 cells, were unable to bind significant amounts of the muscarinic antagonists *N*-[^{3}H]methylscopolamine and [^{3}H]quinuclidinyl benzilate. A common structural feature of these constructs was the presence of m2 receptor sequence in TM VII and of m5 receptor sequence in TM I. The ligand-binding activity of these "pharmacologically inactive" hybrid receptors could be restored by replacing TM I (consisting of m5 receptor sequence) with the corresponding m2 receptor domain. These data provide the first direct experimental evidence that the molecular architecture of muscarinic receptors (and, most likely, that of other G protein-coupled receptors) resembles that of bacteriorhodopsin, in that the seven TM helices are arranged in a ring-like fashion such that TM I lies directly adjacent to TM VII.

Muscarinic acetylcholine receptors are members of the superfamily of plasma membrane receptors that transduce their intracellular signals via coupling to G proteins (1–3). The primary sequences of muscarinic receptors and other G protein-coupled receptors contain seven stretches of primarily hydrophobic amino acids, which are predicted to correspond to seven α-helical TM domains (TM I-VII). Current models of the three-dimensional structures of G protein-coupled receptors (4–6) are based primarily on high-resolution electron diffraction data for bacteriorhodopsin (7), an integral membrane protein obtained from *Halobacterium halobium*. Bacteriorhodopsin, however, is not coupled to G proteins (it functions as a light-driven proton pump) and shares virtually no sequence homology with G protein-coupled receptors. It therefore remains uncertain whether the three-dimensional structures of these proteins are in fact identical.

A recently published projection map of rhodopsin (a G protein-coupled photoreceptor), obtained by electron crystallography of two-dimensional crystals (8), suggests that the structure of rhodopsin is similar to that of bacteriorhodopsin, in that it contains a bundle of seven TM helices. However, a comparison of the projection structures of the two proteins revealed clear differences in the overall arrangement of the various TM domains. These differences could not be defined in further structural detail because of the low resolution of the rhodopsin projection map, precluding the assignment of particular TM helices to the peaks in the projection structure.

In this study, we have chosen a mutagenesis approach involving the creation of a series of chimeric m2/m5 muscarinic receptors to elucidate the three-dimensional structure of muscarinic acetylcholine receptors. Initially, we identified several hybrid m2/m5 receptors that, upon transient expression in COS-7 cells, were unable to bind significant amounts of the muscarinic antagonists [^{3}H]NMS or [^{3}H]QNB, apparently due to improper folding of the receptor proteins. The molecular basis underlying this phenomenon was studied by systematic structural modifications of these pharmacologically inactive mutant receptors, resulting in a "rescue" of receptor function. Our data provide the first direct experimental evidence that the three-dimensional structure of muscarinic receptors is similar to that of bacteriorhodopsin, in that the seven TM domains are arranged in a ring-like fashion such that TM I lies directly adjacent to TM VII. Moreover, the data are consistent with a recent mutagenesis study that used chimeric α_2/β_2-adrenergic receptors and demonstrated that a specific amino acid in TM VII of the adrenergic receptors can interact with the amino-terminal TM helices (9).

ABBREVIATIONS: TM, transmembrane; TM I-VII, transmembrane domains I-VII; NMS, *N*-methylscopolamine chloride; QNB, quinuclidinyl benzilate; kb, kilobase(s).

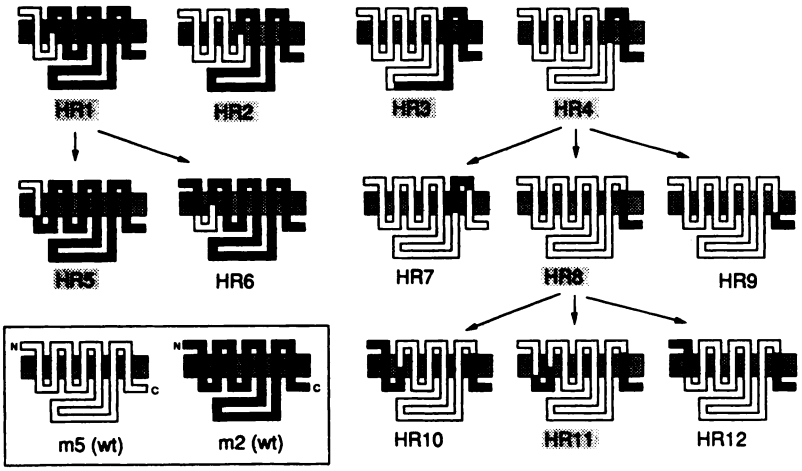

Fig. 1. Structure of hybrid m2/m5 muscarinic receptors (HR1-HR12). The amino terminus (*N*) of the receptor proteins is predicted to be located extracellularly, whereas the carboxyl terminus (*C*) is thought to reside on the cytoplasmic side of the plasma membrane (*gray area*). The fact that the m2 receptor differs from the m5 receptor in the lengths of the extracellular amino-terminal segment (−7 amino acids), the third cytoplasmic loop (−48 amino acids), and the cytoplasmic carboxyl-terminal tail (−11 amino acids) is ignored. Transfection of COS-7 cells with the *highlighted* constructs (HR1-HR5, HR8, and HR11) did not result in an appreciable number of specific [^{3}H]NMS or [^{3}H]QNB binding sites. However, as shown by Northern blot analysis, all of these constructs gave mRNA levels that were similar to those found with the wild-type (*wt*) m2 receptor.

TABLE 1

Ligand-binding properties of chimeric m2/m5 muscarinic receptors

Affinity constants for the radioligands [^{3}H]NMS and [^{3}H]QNB were determined in direct binding assays using membrane homogenates prepared from transfected COS-7 cells. All [^{3}H]NMS and [^{3}H]QNB saturation binding curves were characterized by Hill coefficients (n_H) close to unity. Binding parameters for the agonist carbachol were assessed in competition binding assays as described in Experimental Procedures, using [^{3}H]NMS (200 pM) as a radioligand. Transfection of COS-7 cells with the chimeric constructs HR1-HR5, HR8, and HR11 (Fig. 1) did not result in the appearance of an appreciable number of specific [^{3}H]NMS or [^{3}H]QNB binding sites. Data are presented as means ± standard errors of two to five independent experiments, each performed in duplicate.

Receptor	[^{3}H]NMS binding		[^{3}H]QNB binding		Carbachol binding	
	K_d	B_{max}	K_d	B_{max}	IC_{50}	n_H
	pM	fmol/mg	pM	fmol/mg	μM	
m2 (wild-type)	64 ± 14	1280 ± 240	75 ± 11	1320 ± 290	5.1 ± 1.6	0.80 ± 0.04
m5 (wild-type)	64 ± 10	1050 ± 180	72 ± 10	1230 ± 210	10.8 ± 2.7	0.80 ± 0.10
HR6	65 ± 11	670 ± 130	74 ± 8	930 ± 190	2.3 ± 1.2	0.65 ± 0.05
HR7	88 ± 7	910 ± 240	87 ± 14	945 ± 170	4.8 ± 0.6	0.74 ± 0.04
HR9	60 ± 10	440 ± 60	55 ± 9	690 ± 110	12.9 ± 3.4	0.89 ± 0.20
HR10	102 ± 33	1040 ± 150	86 ± 15	1350 ± 250	6.7 ± 1.5	0.71 ± 0.06
HR12	71 ± 10	440 ± 50	78 ± 12	550 ± 90	15.3 ± 5.5	0.64 ± 0.12

Experimental Procedures

Construction of chimeric muscarinic receptor genes. Hybrid human m2/m5 muscarinic receptor genes were created by standard polymerase chain reaction techniques (10). The plasmids Hm2pcD (11) and Hm5pcDp1 (12) served as templates for the construction of the various mutant receptors. In total, the human m2 and m5 receptors are composed of 466 and 532 amino acids, respectively (11, 12). The individual hybrid m2/m5 muscarinic receptors are composed as follows (numbers refer to amino acid positions in the human m2 and m5 receptor sequences): HR1, m5 1–77/m2 71–466; HR2, m5 1–162/m2 156–466; HR3, m5 1–337/m2 301–466; HR4, m5 1–445/m2 391–466; HR5, m5 1–53/m2 47–466; HR6, m2 1–46/m5 54–77/m2 71–466; HR7, m5 1–445/m2 391–421/m5 477–532; HR8, m5 1–477/m2 423–466; HR9, m5 1–497/m2 444–466; HR10, m2 1–70/m5 78–477/m2 423–466; HR11, m5 1–53/m2 47–70/m5 78–477/m2 423–466; HR12, m2 1–46/m5 54–477/m2 423–466. The identity of the desired mutations and the correctness of all polymerase chain reaction-derived coding sequences were verified by dideoxy sequencing of the mutant plasmids (13).

Transfections and binding assays. COS-7 cells were seeded into 100-mm plates at a density of 1×10^6 cells/plate. About 24 hr later, cells were transfected with 20 μg of plasmid DNA by calcium phosphate precipitation, as described (14). Cells were harvested approximately 72 hr after transfections, for the preparation of membrane homogenates (15) to be used in radioligand binding assays.

Radioligand binding studies were carried out essentially as described (15). In brief, binding buffer consisted of 25 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$. In saturation binding assays, six different concentrations (25–800 pM) of the radioligands [^{3}H]NMS (78.9 Ci/mmol; DuPont-New England Nuclear) and [^{3}H]QNB (43.0 Ci/mmol; DuPont-New England Nuclear) were used. Carbachol competition binding studies were carried out using 200 pM [^{3}H]NMS. Nonspecific binding was determined in the presence of 1 μM atropine. Incubations were carried out for 3 hr at 22°. Binding data were analyzed by using a nonlinear least squares curve-fitting procedure as described (15).

Protein concentrations were determined according to the method of Bradford (16), using a Bio-Rad protein assay kit.

Northern analysis. Total RNA was isolated from transfected COS-7 cells by using a guanidinium isothiocyanate procedure (17). RNA was electrophoresed (10 μg/lane) on a 1% denaturing agarose gel and blotted onto a Nytran nylon membrane. Blots were hybridized with a random-primed ^{32}P-labeled 0.43-kb *Dra*III-*Kpn*I Hm2pcD restriction fragment, containing the coding region for TM VII and the cytoplasmic "tail" of the human m2 receptor and approximately 0.29 kb of 3′ untranslated sequence. Blots were washed at 56° in 0.1× standard saline citrate containing 1% sodium dodecyl sulfate (3 × 15 min), dried, and exposed to X-ray film for 8 hr at −70°.

Results and Discussion

This study was designed based on the initial observation that a series of four chimeric m2/m5 muscarinic receptors (HR1-HR4) (Fig. 1) were unable to bind significant amounts of the radioligands [^{3}H]NMS and [^{3}H]QNB when transiently expressed in COS-7 cells. Only at high radioligand concentrations (1.6–2 nM) could a very low number of specific muscarinic binding sites (B_{max} < 20 fmol/mg of membrane protein) be detected. In contrast, the wild-type m2 and m5 muscarinic receptors were expressed at very high levels (B_{max} ~ 1 pmol/

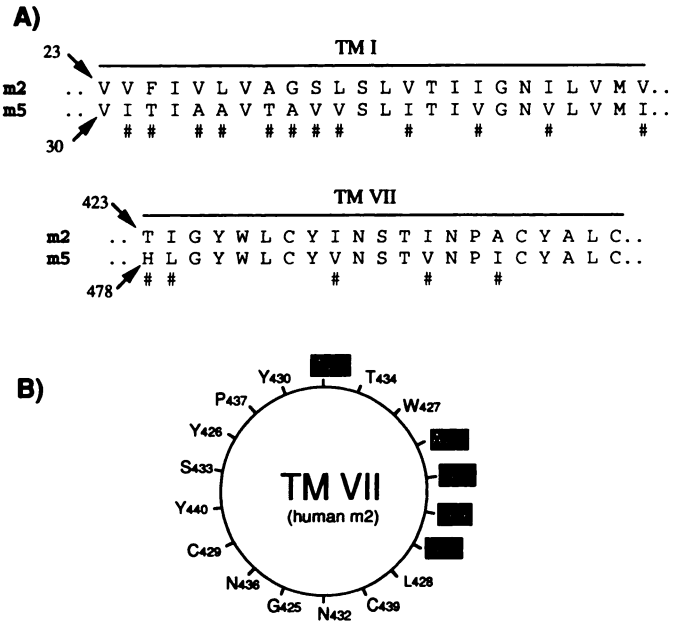

Fig. 2. Amino acid sequences of TM I and VII of the human m2 and m5 muscarinic receptors. A, Comparison of m2 and m5 muscarinic receptor sequences (TM I and VII). *Numbers at the beginning of the sequences*, amino acid positions within the human m2 and m5 muscarinic receptors (11, 12). #, Positions at which the m2 receptor sequence differs from the m5 receptor sequence. B, Helical wheel model of TM VII of the human m2 muscarinic receptor. The direction of view is normal to the extracellular surface of the plasma membrane. Only the first 16 amino acids of TM VII (m2 receptor) are shown (the remaining TM VII sequence is identical to that of the m5 receptor). *Amino acids highlighted in black* differ from the corresponding residues present in the m5 receptor.

mg of membrane protein) and bound [^{3}H]NMS and [^{3}H]QNB with K_d values ranging from 64 to 75 pM (Table 1). Northern analysis of total RNA isolated from transfected COS-7 cells (using a random-primed ^{32}P-labeled 0.43-kb *Dra*III-*Kpn*I Hm2pcD restriction fragment as a probe) indicated that mRNA levels were similar for HR1-HR4 and the wild-type m2 receptor (data not shown). Therefore, the relative lack of ligand binding seen after transfection of COS-7 cells with constructs HR1-HR4 is not due to decreased mRNA levels but rather appears to depend on the structural properties of the encoded receptor proteins.

A common structural feature of constructs HR1-HR4 is that their carboxyl-terminal portions (including most of TM VI and TM VII) consist of m2 receptor sequence, whereas their amino-terminal domains are derived from the m5 receptor. It is therefore likely that a conformational incompatibility exists between the carboxyl-terminal m2 and amino-terminal m5 receptor sequences, resulting in improperly folded receptor proteins that are unable to bind muscarinic ligands. An alternative explanation for the relative lack of ligand binding seen with HR1-HR4 is that the encoded chimeric proteins are not stably integrated into plasma membrane because of aberrant intracellular trafficking or reduced protein stability. Because of the lack of suitable antibodies, we were unable to distinguish between these different possibilities. However, independently of the precise molecular mechanisms underlying this phenomenon, it seems reasonable to assume that the inability of HR1-HR4 to yield functional membrane receptors is based on an improper protein fold caused by the incompatibility of adjacent m2 and m5 receptor sequences.

To more precisely locate the m2 receptor region responsible for the "functional inactivation" of HR1-HR4, three additional chimeric m2/m5 receptors (HR7, HR8, and HR9) were created (Fig. 1) and pharmacologically characterized in radioligand binding studies. HR7 and HR9 were expressed at high levels (as determined in [^{3}H]NMS and [^{3}H]QNB saturation binding studies) and were able to bind muscarinic ligands with affinities similar to those found with the two wild-type receptors (Table 1). This finding indicates that the presence of m2 receptor sequence in TM VI, the third extracellular loop, and the cytoplasmic tail is not responsible for the improper folding of HR1-HR4. In contrast to HR7 and HR9, transfection of COS-7 cells with HR8 did not result in the appearance of a significant number of muscarinic binding sites. The inability of construct HR8 (as well as of HR1-HR4) to produce functional muscarinic receptors thus appears to depend on the presence of m2 receptor sequence in TM VII.

In an attempt to restore the pharmacological activity of HR8 by replacing amino-terminal m5 receptor domains with the corresponding m2 receptor sequences, three additional hybrid receptors (HR10, HR11, and HR12) (Fig. 1) were created and pharmacologically analyzed. No specific muscarinic binding sites were found with HR11, indicating that the presence of m5 receptor sequence in the first intracellular loop and the amino-terminal portion of TM II is not responsible for the lack of pharmacological activity seen with HR8 (and HR1-HR4). This notion is further supported by the observation that HR6 (an m2 receptor in which the first intracellular loop and the amino-terminal portion of TM II were replaced with m5 receptor sequence) (Fig. 1) displayed ligand-binding properties similar to those of the wild-type receptors (Table 1).

In contrast to the lack of ligand-binding activity seen with construct HR11, structural modifications of HR8 that included the substitution of TM I (m5 receptor sequence) with m2 receptor sequence resulted in receptors (HR10 and HR12) that displayed pharmacological properties similar to those of the wild-type receptors (Table 1). The lack of pharmacological activity seen with HR5 (an m2 receptor in which the amino-terminal segment including TM I was replaced with homologous m5 receptor sequence) (Fig. 1) and the ability of construct HR6 to yield functional muscarinic receptors are in agreement with this observation. Taken together, all experimental results are consistent with the notion that TM I lies in direct proximity to TM VII and that specific molecular interactions between these two TM domains are required for the proper folding of muscarinic receptors. Our findings are in good agreement with a recent theoretical study of the probable arrangement of the seven TM helices present in all G protein-coupled receptors, which was based on a detailed comparison of the amino acid sequences of a large number of heptahelical receptors (18).

Whereas the primary sequences of the m2 and m5 muscarinic receptors differ considerably in TM I, the TM VII sequences of the two receptors differ in only five amino acids (Fig. 2A). It should be of interest to determine (e.g., by site-directed mutagenesis) which of these five residues is (are) specifically responsible for the inability of TM VII of the m2 receptor to properly interact with TM I of the m5 receptor. Because it is reasonable to assume that such residues are located directly at the interface between TM I and TM VII, these studies should provide direct experimental evidence regarding how TM I and TM VII are oriented relative to each other.

To identify intramolecular interactions in the adrenergic receptors, Suryanarayana *et al.* (9) recently described a mutagenesis approach to restore function to mutationally inactivated adrenergic receptors. Using chimeric α_2/β_2-adrenergic receptors, they could demonstrate the interaction of a specific amino acid in TM VII with TM I, or with TM I together with TM II. Interestingly, TM I alone did not complement the TM VII mutation, whereas TM I together with TM II did so. In addition, immunocytochemical evidence that the nonfunctional receptor proteins were translated and retained in an intracellular compartment was presented (9).

In conclusion, our data provide the first direct experimental evidence that muscarinic acetylcholine receptors show a molecular architecture similar to that of bacteriorhodopsin (7), in that TM I lies directly adjacent to TM VII. Based on the high degree of structural homology that muscarinic receptors share with other G protein-coupled receptors, it is therefore highly likely that all of these proteins have similar three-dimensional structures. The findings described here provide a rational basis for future molecular modeling studies, which should help define the molecular architecture of G protein-coupled receptors in greater structural detail.

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