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Chimeric m2/m3 Muscarinic Receptors: Role of Carboxyl Terminal Receptor Domains in Selectivity of Ligand Binding and Coupling to Phosphoinositide Hydrolysis

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SUMMARY

The cloning and expression of five mammalian muscarinic receptor genes (m1–m5) have shown that the individual receptor subtypes differ in their functional and ligand-binding properties. To study the role of the carboxyl terminal receptor domains in this pharmacological diversity, we constructed chimeric m2/m3 receptors in which a region comprising part of transmembrane domain VI, the third extracellular loop, transmembrane region VII, and the cytoplasmic tail (collectively referred to as C-terminal domains) was exchanged between the human m2 and the rat m3 receptor. The ability of the cloned receptors to mediate stimulation of phosphoinositide hydrolysis and to bind subtype-selective muscarinic ligands was studied after their transient expression in COS-7 cells. Whereas wild-type m3 strongly stimulated phosphoinositide breakdown, wild-type m2 gave only a poor response. Exchange of the C-terminal domains between m2 and m3 had no significant effect on the magnitude of these responses. In *N*-[^{3}H]methylscopolamine competition binding studies, the muscarinic antagonists AF-DX 116 and methoctramine showed 11- and 23-fold higher affinities, respectively, for m2 than for m3, whereas hexahydro-silad-ifenidol (HHSiD) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) displayed the reverse selectivity profile, having approximately 10-fold higher affinities for m3. In comparison with wild-type m3, the mutant m3 receptor containing the C-terminal domains of m2 displayed 2.5- and 8-fold higher affinities for AF-DX 116 and methoctramine but 7- and 3-fold lower affinities for HHSiD and 4-DAMP, respectively. The mutant m2 receptor with the C-terminal domains of m3 showed 2–3-fold lower affinities for AF-DX 116 and methoctramine but 2–3-fold higher affinities for HHSiD and 4-DAMP, as compared with wild-type m2. These data suggest that the C-terminal domains of the muscarinic receptors are not involved in conferring selectivity of coupling to phosphoinositide hydrolysis but contain major structural determinants of antagonist binding selectivity.

Muscarinic receptors share a high degree of structural homology with all other G protein-coupled hormone and neurotransmitter receptors (1). All of these receptors are thought to be composed of seven hydrophobic membrane-spanning domains (I–VII) connected by alternating cytoplasmic and extracellular loops, a glycosylated extracellular N-terminal sequence, and an intracellular C-terminal tail. Whereas the ligand binding site of these receptors appears to be formed by the hydrophobic transmembrane domains, various intracellular regions are thought to be involved in the coupling of the activated receptors to distinct G proteins and signal transduction pathways (for a review, see Ref. 2).

Molecular cloning studies have revealed the existence of five mammalian muscarinic receptors (m1–m5) (3–7), which can be subdivided into two distinct functional classes. Whereas m1, m3, and m5 preferentially couple to stimulation of PI metabolism (7–11), m2 and m4 strongly inhibit adenylate cyclase (11, 12) but only weakly stimulate PI breakdown (11, 13). Studies involving the use of chimeric muscarinic receptors suggest that the putative third cytoplasmic loop is sufficient to determine the coupling selectivity of the individual receptor subtypes (13–15). We have recently shown, for example, that exchange of the third cytoplasmic loop between m2 and m3 caused a reversal in the ability of the resulting hybrid receptors to couple to specific second messenger pathways (15). However, the maximum responses to stimulation of these chimeric receptors were smaller than the corresponding wild-type responses. We have, therefore, speculated that other intracellular domains, besides the third cytoplasmic loop, may be involved in conferring optimum coupling efficiency (15).

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; AF-DX 116, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzodiazepine-6-one; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; HHSiD, hexahydro-sila-difenidol; NMS, N-methylscopolamine; Gpp(NH)p, 5′-guanylyl imidodiphosphate; PI, phosphoinositide; IP_1, inositol monophosphate; kb, kilobases; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

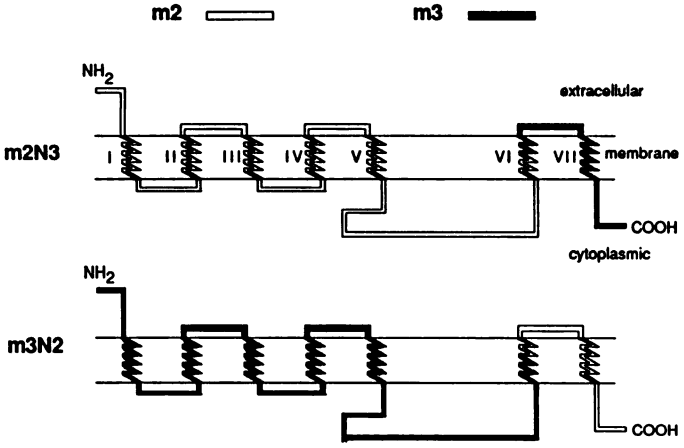

Fig. 1. Structure of the chimeric muscarinc receptors m2N3 and m3N2, composed of human m2 (*open bars*) and rat m3 (*black bars*) sequences. The seven transmembrane regions are numbered I–VII, from the NH_2 terminus (extracellular) to the COOH terminus (intracellular). The fact that m3 differs from m2 in the lengths of the N-terminal portion preceding transmembrane segment I (+44 amino acids), the third cytoplasmic loop (+59 amino acids), and the C-terminal sequence following segment VII (+20 amino acids) is ignored. In total, the human m2 and the rat m3 receptors are composed of 466 and 589 amino acids, respectively (6). The two chimeric receptors are composed as follows (amino acid numbers are given in parentheses): m2N3, m2 (1–400) and m3 (504–589); m3N2, m3 (1–503) and m2 (401–466).

Whereas the first two intracellular loops are relatively well conserved among all five muscarinic receptors, considerable sequence divergence is found in the C-terminal receptor portion following transmembrane region VII (3–7). Moreover, this region is considerably longer in m1, m3, and m5 (34–43 amino acids) than in m2 and m4 (23 amino acids). To study the potential functional importance of this receptor domain, we have constructed cDNAs encoding the chimeric receptors m2N3 and m3N2 (N refers to the *NheI* site used to create these receptors), in which the last 66 amino acids of human m2 and the last 86 amino acids of rat m3 were exchanged between these two receptors (Figs. 1 and 2). Following transient expression of these receptors in COS-7 cells, their ability to mediate agonist-induced PI hydrolysis was examined.

Besides their functional profiles, m1–m5 also differ in their agonist- (13, 16, 17) and antagonist-binding properties (5, 18, 19). Studies with chimeric α_2/β_2- (20) and β_1/β_2-adrenergic receptors (21) have shown that transmembrane domains VI and VII contain major structural determinants of antagonist binding specificity. To investigate whether this is also true for muscarinic receptors, the ligand-binding properties of m2N3 and m3N2 were examined by using several subtype-selective muscarinic antagonists.

Experimental Procedures

Materials. Tissue culture reagents were from GIBCO Laboratories. COS-7 cells were from the American Type Culture Collection. [^{3}H]NMS (71 Ci/mmol) and *myo*-[^{3}H]inositiol (23 Ci/mmol) were purchased from New England Nuclear and American Radiolabeled Chemicals Inc., respectively. Methoctramine and 4-DAMP were obtained from Research Biochemicals Inc., AF-DX 116 and HHSiD were kindly donated by Thomae (Biberach, FRG) and Dr. G. Lambrecht (University of Frankfurt, FRG), respectively. All other reagents were from Sigma unless otherwise noted.

Construction of expression plasmids and transfections. An *NheI* site homologous to the one at amino acids 496–498 in transmembrane region VI of the rat m3 receptor was created at amino acids 393–395 in the human m2 expression clone previously described (6). This mutation was introduced by oligonucleotide-directed mutagenesis (22) without changing the amino acid sequence. To obtain plasmid m2N3, the 1.5-kb *SalI*-*SacI* and the 0.33-kb *SacI*-*NheI* fragments from the mutated m2 clone were ligated with the 4.7-kb *NheI*-*SalI* fragment from the rat m3 expression plasmid (6). Plasmid m3N2 was created by ligating the 4.1-kb *NheI*-*SalI* fragment from the mutated m2 clone with the 2.1-kb *NheI*-*SalI* fragment from the m3 expression plasmid. The identity of the constructs was confirmed by sequencing (23) of the regions derived from the synthetic oligodeoxyribonucleotides and by restriction endonuclease analysis.

For transfections, COS-7 cells were seeded into 10-cm plates at a density of 7.5×10^5 cells/ plate. Twenty-four hours later, cells were transfected with 20 μg of plasmid DNA by calcium phosphate precipitation (24).

Binding studies. Cells were harvested 72 hr after transfections, and membrane preparations were obtained as described previously (19). Binding buffer consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM $MgCl_2$. Assays were conducted in 1-ml total volume. Final membrane protein concentrations (in μg/ml) were: m2, 4; m3, 4; m2N3, 3; and m3N2, 6. In the [^{3}H]NMS saturation experiments, six or seven different concentrations of the radioligand (12.5 to 800 pM) were used. In the [^{3}H]NMS displacement studies, 10 different concentrations of the unlabeled inhibitors were employed. The [^{3}H]NMS concentration used in the inhibition experiments was 200 pM. Specific binding was defined as the difference in [^{3}H]NMS binding in the absence and presence of 1 μM atropine. Incubations were carried out at 22° for 3 hr. Binding was terminated by filtration, using a Brandel cell harvester, onto Whatman GF/C filters. Membranes were washed three times with ice-cold binding buffer, transferred to 10 ml of scintillation fluid (NEN Aquasol), and counted in an LKB Beta counter.

Data from direct binding experiments were fitted to the equation $a = (B_{max}\, x^n/K_D)/(1 + x^n/K_D)$ to derive the Hill number n and to $a = (B_{max}\, x/K_D)/(1 + x/K_D)$ to obtain the dissociation constant K_D and the binding capacity B_{max} (a = [^{3}H]NMS specifically bound and x = [^{3}H]NMS concentration). Data from inhibition experiments were fitted to the equation percentage of [^{3}H]NMS bound = $100 - [100\, x^n/k/(1 + x^n/k)]$ to obtain the Hill number n and to percentage of [^{3}H]NMS bound = $100 - [100\, x/IC_{50}/(1 + x/IC_{50})]$ to derive the IC_{50} value (x = concentration of the unlabeled inhibitor). IC_{50} values were converted to K_i values according to the method of Cheng and Prusoff (25). Data

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      ----VI----                  --------VII--------          i4
m2  401--APYNVMVLINTFCAPCIPNTVWTIGYWLCYINSTINPACYALCNATFKKTFKHLLMCHYKNIGATR 466
m3  504--TPYNIMVLVNTFCDSCIPKTYWNLGYWLCYINSTVNPVCYALCNKTFRTTFKTLLLCQCDKRKRRKQQYQQRQSVIFHKRVPEQAL 589
         *** *** ****  *** * *  ********** ** ****** **  *** ** *
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Fig. 2. comparison of the amino acid sequences of the C-terminal domains of the human m2 and the rat m3 receptor. The sequences shown were exchanged between the two receptors to give chimeric receptors m2N3 and m3N2 (Fig. 1). Sequences were taken from Refs. 6 and 7. *Numbers* in front and at the end of the sequences, amino acid positions. Transmembrane domains (*VI*, *VII*) and the cytoplasmic tail (*i4*) are indicated above the sequences. *, amino acid identities.

TABLE 1

Ligand-binding properties of chimeric muscarinic receptors transiently expressed in COS-7 cells

Affinity constants (K_D) for the radioligand [^{3}H]NMS, inhibition constants (K_i), and IC_{50} values were determined as described in Experimental Procedures. Hill coefficients are given in parentheses. B_{max} values indicate maximum number of binding sites/mg of membrane protein. Data are presented as means ± standard errors of three independent experiments, each performed in duplicate.

Receptor	Direct binding studies		[^{3}H]NMS competition experiments					
			Carbachol, IC_{50}					
	B_{max}	[^{3}H]NMS K_D	Control	+Gpp(NH)p (100 μM)	AF-DX 116, K_i	Methoctramine, K_i	HHSiD, K_i	4-DAMP, K_i
	fmol/mg	pM	μM		nM	nM	nM	nM
m2	1270 ± 190	79 ± 9 (0.91 ± 0.11)	1.2 ± 0.1 (0.68 ± 0.03)*	1.3 ± 0.2 (0.62 ± 0.04)*	80 ± 10 (0.89 ± 0.12)	9.5 ± 1.8 (1.09 ± 0.19)	56 ± 5 (1.04 ± 0.03)	3.9 ± 0.1 (0.99 ± 0.02)
m3	1200 ± 60	28 ± 1 (1.04 ± 0.13)	35 ± 3 (0.76 ± 0.02)*	36 ± 4 (0.75 ± 0.03)*	917 ± 64 (1.00 ± 0.03)	217 ± 1 (1.05 ± 0.05)	4.4 ± 0.3 (1.09 ± 0.04)	0.50 ± 0.02 (0.99 ± 0.04)
m2N3	2170 ± 490	41 ± 5 (1.00 ± 0.06)	17 ± 2 (0.77 ± 0.03)*	15 ± 5 (0.78 ± 0.03)*	174 ± 12 (0.92 ± 0.02)	27 ± 5 (1.08 ± 0.02)	22 ± 3 (1.02 ± 0.05)	2.2 ± 0.1 (0.95 ± 0.03)
m3N2	710 ± 60	95 ± 3 (1.01 ± 0.07)	42 ± 5 (0.74 ± 0.05)*	45 ± 7 (0.77 ± 0.07)*	360 ± 32 (0.95 ± 0.02)	26 ± 11 (1.01 ± 0.07)	29 ± 5 (0.98 ± 0.04)	1.5 ± 0.1 (1.05 ± 0.03)

* Hill number significantly different from unity ($p < 0.05$ in an unpaired, one-tailed Student's t test).

were analyzed by a nonlinear least squares curve-fitting procedure, using the program DATAPLOT run on a VAX II computer.

Protein concentrations were determined according to the method of Bradford (26), using a Bio-Rad protein assay kit.

Measurement of PI hydrolysis. Twenty-four hours after transfection, COS-7 cells were incubated with 3 μ Ci/ml *myo*-[^{3}H]inositol for 48 hr. The cells were then washed twice with phosphate-buffered saline and lifted from the dish bottoms with 2 ml of divalent cation-free Dulbecco's modified Eagle's medium containing 1 mM EDTA. After centrifugation, cells were resuspended in 8 ml of Dulbecco's modified Eagle's medium containing 25 mM HEPES and 10 mM LiCl and were incubated at room temperature for 15 min. Aliquots (0.5 ml) of the cell suspension were then transferred into 13 × 100-mm glass tubes and, following the addition of different concentrations of carbachol, incubated for 1 hr at 37°. The reaction was then stopped by the addition of 0.5 ml of 15% (w/v) ice-cold trichloroacetic acid, followed by 30-min incubation on ice. The trichloroacetic acid was extracted with water-saturated diethyl ether (3 × 4 ml), and levels of IP_1 were determined by anion exchange chromatography (27) and an LKB liquid scintillation counter.

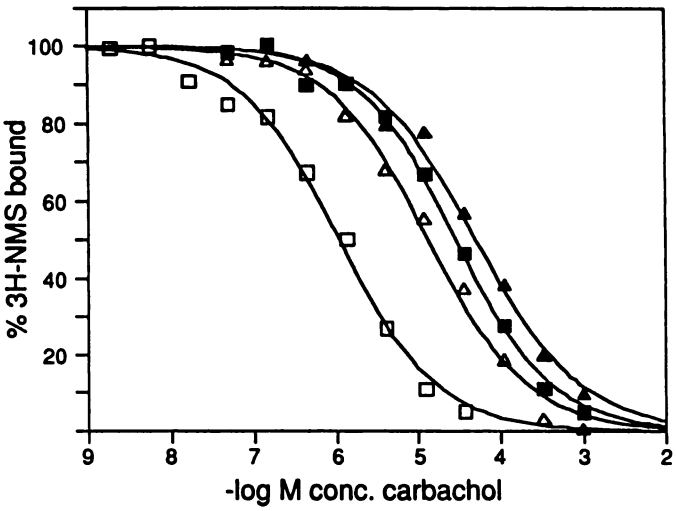

Fig. 3. Displacement of specific [^{3}H]NMS binding to m2 (□), m3 (■), m2N3 (△), and m3N2 (▲) by carbachol in COS-7 cells. The [^{3}H]NMS concentration used was 200 pM. Binding assays were performed as described in Experimental Procedures. Each curve is representative of three independent experiments, each done in duplicate. Gpp(NH)p (100 μM) had no significant effect on the carbachol binding parameters (see Table 1). Curves were generated by computer fit, assuming a variable Hill number (for binding parameters, see Table 1).

Results

Binding studies. The muscarinic antagonist [^{3}H]NMS bound specifically to membranes prepared from COS-7 cells that had been transfected with the various wild-type and chimeric muscarinic receptor genes (Table 1). No specific [^{3}H]NMS binding could be detected in nontransfected cells. As observed previously (13, 15), m3 bound [^{3}H]NMS with approximately 3-fold higher affinity than did m2. The [^{3}H]NMS affinities for m2N3 and m3N2 approximated those for m3 and m2, respectively.

The agonist carbachol inhibited [^{3}H]NMS binding to m2 with approximately 30-fold higher potency, compared with m3 (Fig. 3, Table 1). The carbachol IC_{50} values obtained for m2N3 and m3N2 were 14- and 35-fold higher, respectively, than the corresponding m2 value. Gpp(NH)p (100 μ M) had no significant effect on the carbachol binding parameters (Table 1).

All four of the tested muscarinic antagonists displaced [^{3}H]NMS binding to the different receptors with Hill coefficients that were close to unity (Table 1). AF-DX 116 and methoctramine displayed a considerably higher affinity (11-and 23-fold, respectively) for m2 than for m3. HHSiD and 4-DAMP showed the reverse selectivity profile, exhibiting a 13- and 8-fold higher affinity, respectively, for m3 than for m2 (Table 1, Fig. 4). AF-DX 116 and methoctramine displayed 2.5- and 8-fold higher affinities, respectively, for m3N2 than for m3 but approximately 2–3-fold lower affinities for m2N3 than for m2. HHSiD and 4-DAMP showed the reverse affinity pattern; both antagonists exhibited 2–3-fold higher affinities for m2N3 than for m2 but 7- and 3-fold lower affinities, respectively, for m3N2 than for m3.

Measurement of PI hydrolysis. The ability of m2, m3, m2N3, and m3N2 to mediate stimulation of PI hydrolysis upon incubation with carbachol was determined by studying increases in intracellular IP_1 levels. Whereas nontransfected COS-7 cells did not give a significant PI response (data not shown), carbachol-induced increases in IP_1 production were observed with cells expressing the different wild-type and chimeric muscarinic receptors (Fig. 5). As previously observed (11, 13, 15), the PI response following m3 stimulation was consid-

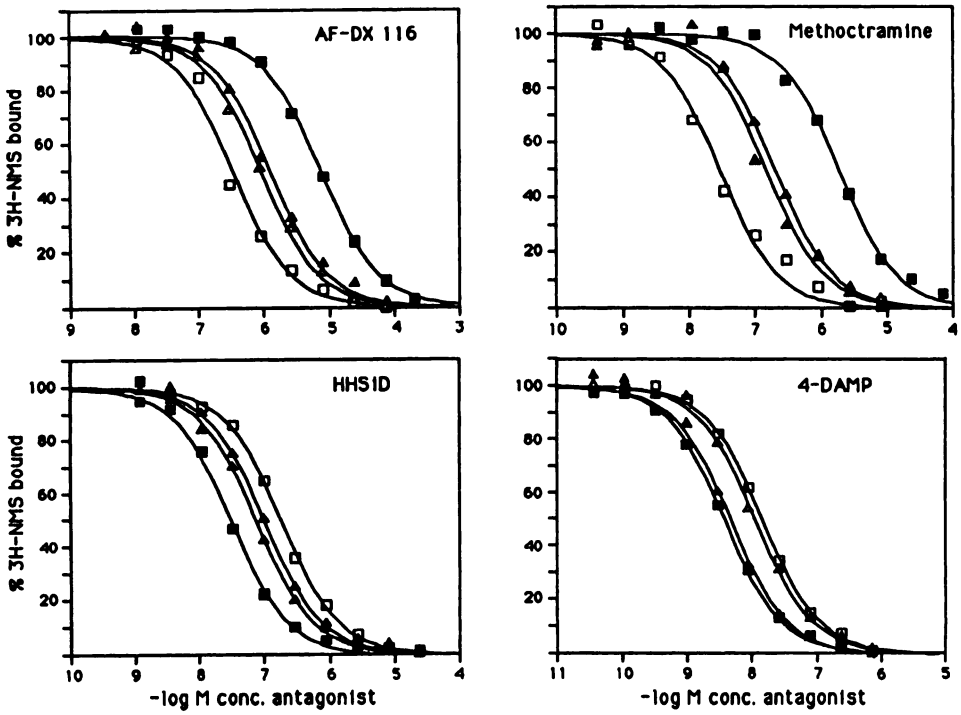

Fig. 4. Displacement of specific [^{3}H]NMS binding to m2 (□), m3 (■), m2N3 (△), and m3N2 (▲) by several selective muscarinic antagonists in COS-7 cells. The [^{3}H]NMS concentration used was 200 pM. Binding studies were carried out as described in Experimental Procedures. Each curve is representative of three independent experiments, each carried out in duplicate. Curves were generated by computer fit, according to a one binding site model.

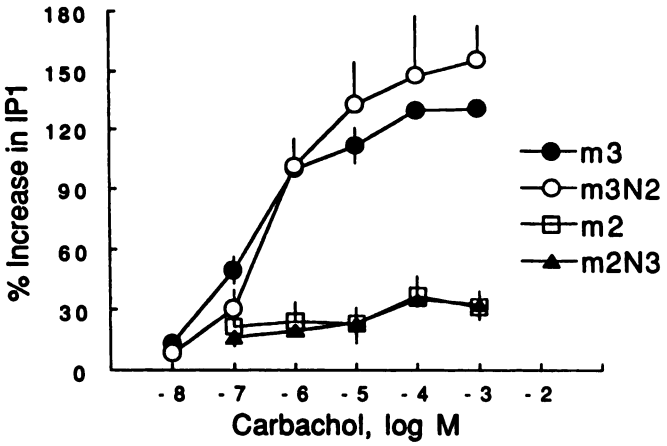

Fig. 5. Carbachol-induced stimulation of PI hydrolysis in COS-7 cells transiently expressing m2, m3, and m2/m3 hybrid receptors. Transfected cells were incubated with increasing concentrations of carbachol for 1 hr at 37°, and the production of IP_1 was determined as described in Experimental Procedures. The responses are shown as percentage of increase in IP_1 above basal levels measured in the absence of carbachol. Basal IP_1 levels were (in cpm/sample): m2, 805 ± 67; m3, 782 ± 71; m2N3, 923 ± 109; m3N2, 734 ± 45. Data are presented as means ± standard errors of two or three independent experiments, each performed in duplicate.

erably more pronounced than that mediated by m2 (maximum increases in IP_1 above basal levels, 131 ± 3 and 37 ± 9%, respectively). The chimeric receptors m2N3 and m3N2 stimulated IP_1 production to similar maximum levels (35 ± 4 and 156 ± 18%, respectively) and with dose-response curves virtually identical to those of wild-type m2 and m3, respectively (Fig. 5). All responses could be completely blocked by 10 μM atropine.

Discussion

Previous studies have shown that sequences within the third cytoplasmic loop are responsible for the functional selectivity of the individual muscarinic receptor subtypes (13–15). However, based on the reduced activity of chimeric muscarinic receptors in which this intracellular loop had been exchanged between m2 and m3, we have hypothesized that additional receptor domains may be required for full coupling efficiency (15). To study the potential functional role of the C-terminal receptor regions, we have created two chimeric muscarinic receptors, m2N3 and m3N2, in which the C-terminal domains have been exchanged between m2 and m3, and studied their ability to mediate stimulation of PI hydrolysis.

As previously reported (11, 13, 15), we found that m3 strongly stimulated PI hydrolysis, whereas m2 only weakly coupled to this second messenger response. Exchange of the C-terminal sequences between the two receptors had no significant effect on the magnitude of the wild-type responses. This finding suggests that the receptor region comprising the C-terminal portion of transmembrane region VI, the third extracellular loop, transmembrane domain VII, and the cytoplasmic tail is not involved in determining the coupling efficiency of muscarinic receptors to PI turnover. In agreement with our results, it has been demonstrated in *Xenopus* oocytes that the current responses mediated by m1 and m2 remained unaffected by exchange of a similar region between these two receptors (14). These data suggest that the C-terminal muscarinic receptor domains are not involved in conferring effector coupling selectivity.

In contrast to our findings, it has been reported that replacement of the N-terminal portion of the cytoplasmic tail of the α_1-adrenergic receptor by the corresponding β_2 sequence resulted in a considerable impairment of the ability of the α_1 receptor to activate PI metabolism (28). Similar results have been obtained with chimeric α_2/β_2-adrenergic receptors regarding stimulation of adenylyl cyclase (29). One possible explanation for the discrepancy between these and our findings may be that structure-function relationships are different for mus-

carinic and adrenergic receptors. Alternatively, it is also conceivable that the relatively small substitutions made in the case of the chimeric adrenergic receptors may have led to unfavorable conformational effects, resulting in a reduced efficiency of receptor-G protein coupling.

Whereas intracellular receptor domains are thought to be involved in the functional coupling, the ligand binding site of G protein-linked receptors appears to be formed by amino acids located within the transmembrane domains. It has been demonstrated, for example, that aspartic acid residues within transmembrane region III are essential for ligand binding to muscarinic (30, 31) and adrenergic receptors (32). Furthermore, biochemical studies have shown that the visual pigment rhodopsin covalently binds its endogenous ligand, retinal, to a lysine residue in transmembrane domain VII (33). Studies with chimeric α_2/β_2 (20) and β_1/β_2 receptors (21) suggest that transmembrane regions VI and VII are critically involved in determining the binding specificity of subtype-selective antagonists.

To study the potential role of the C-terminal muscarinic receptor domains in the selective binding of muscarinic ligands, the binding properties of m2N3 and m3N2 have been evaluated by using the agonist carbachol and various subtype-selective muscarinic antagonists. In agreement with previous findings (13, 15), we observed that carbachol bound to m2 with considerably higher affinity than to m3. Both chimeric receptors, however, displayed relatively low affinities for carbachol, making the data difficult to interpret. In fact, we have recently shown that the subtype-selective binding of carbachol is mainly dependent on sequences within the third cytoplasmic loop (15). Because all four receptors studied were able to mediate a functional response, the insensitivity of all carbachol binding parameters to the presence of Gpp(NH)p is somewhat unexpected. At present, no clear explanation for this rather commonly reported phenomenon can be offered.

In agreement with their reported selectivities, the muscarinic antagonists AF-DX 116 (34) and methoctramine (35) showed considerably higher affinities for m2 than for m3, whereas HHSiD (36) and 4-DAMP (37) displayed the reverse selectivity profile. m2N3 bound AF-DX 116 and methoctramine with significantly lower affinities than did m2, whereas the affinities of HHSiD and 4-DAMP for this chimeric receptor were increased (in comparison with m2). Analogously, m3N2 bound AF-DX 116 and methoctramine with substantially higher affinities than did m3 but showed significantly lower affinities (compared with m3) for HHSiD and 4-DAMP. In agreement with this pattern, the [^{3}H]NMS binding affinities for m2N3 and m3N2 were similar to those for m3 and m2, respectively. However, in all cases, with the exception of [^{3}H]NMS binding to m3N2, the antagonist affinities for m2N3 and m3N2 approached but never equaled those for m3 and m2, respectively. Given the highly consistent pattern of reciprocal changes in affinities for the chimeric receptors for the m2- versus the m3-selective antagonists, these data suggest that the subtype-selective binding of muscarinic antagonists is critically, although not exclusively, dependent on the C-terminal receptor domains. However, as is the case for all studies involving mutagenesis, one cannot completely exclude the possibility that the observed changes in binding characteristics may be due to artifactual alterations in receptor structure that could not be predicted.

As far as the exchanged transmembrane segments are concerned, m2 and m3 differ in only six amino acids (m2–m3); Ala^{401}-Thr (VI), Val^{405}-Ile (VI), Ile^{409}-Val (VI), Ile^{424}-Leu (VII), Ile^{435}-Val (VII), and Ala^{438}-Val (VII). Assuming that the hydrophilic receptor regions, as shown for the β_2 receptor (38), are not involved in antagonist binding, future experiments have to show which of these six residues is (are) specifically involved in conferring antagonist binding selectivity.

This study further underscores the value of chimeric receptors in elucidation of the structural basis of receptor function and ligand binding specificity. Given the great therapeutic potential of subtype-selective muscarinic drugs (39), such studies should provide the basis for a more rational approach towards the development of novel therapeutic agents.

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