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Reconstitution of functional muscarinic receptors by co-expression of amino- and carboxyl-terminal receptor fragments

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Truncated m2 and m3 muscarinic receptors (referred to as m2- and m3-trunc), containing transmembrane domains I–V and the N-terminal portion of the third cytoplasmic loop, were co-expressed in COS-7 cells with the corresponding C-terminal receptor fragments (referred to as m2- and m3-tail; containing transmembrane domains VI and VII). Expression of any of these four polypeptides alone did not result in any detectable [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) binding activity. However, specific [^{3}H]NMS binding sites were observed after co-expression of m2-trunc with m2-tail and m3-trunc with m3-tail. These sites displayed ligand binding properties similar to those of the two wild-type receptors. The 'reconstituted' m3-trunc/m3-tail receptor was also able to stimulate agonist-dependent phosphatidyl inositol hydrolysis in a fashion similar to the wild-type m3 receptor, whereas all other polypeptide combinations were inactive. These data suggest that muscarinic receptors are assembled in a fashion analogous to two-subunit receptors.

G protein-coupled receptor; Muscarinic receptor; Phosphatidyl inositol hydrolysis; Protein folding; Truncated receptor

1. INTRODUCTION

The muscarinic acetylcholine receptors (m1–m5) are members of the family of plasma membrane receptors that transduce their intracellular signals via coupling to guanine nucleotide-binding regulatory proteins (G proteins) [1–3]. Like all other G protein-coupled receptors, the muscarinic receptors are predicted to be composed of seven hydrophobic (α-helical) membrane-spanning domains connected by alternating cytoplasmic and extracellular loops, a glycosylated extracellular N-terminal domain and an intracellular C-terminal region. The seven transmembrane domains (TM I–VII) are predicted to enclose a highly conserved cavity in which the binding of muscarinic ligands is thought to occur [3–7]. In contrast, the selectivity of G protein coupling displayed by the individual receptor subtypes appears to be primarily dependent on the membrane-proximal portions of the third cytoplasmic loop (i3) [8–12].

Little is known about the molecular mechanisms controlling the folding and assembly of muscarinic and other G protein-linked receptors. Interestingly, biochemical studies have shown that bacteriorhodopsin (a structurally related transmembrane protein which functions as a light-driven proton pump) consists of several 'autonomous folding domains' which, following their independent insertion into the lipid bilayer, can interact with each other to form a functional transmembrane protein (for reviews, see [13,14]).

To test the hypothesis that the biosynthetic assembly of muscarinic receptors is based on a similar mechanism, we prepared a series of fragmented m2 and m3 muscarinic receptor genes encoding the polypeptides shown in Fig. 1. m2-trunc and m3-trunc (containing TM I–V) represent muscarinic receptors which have been truncated within the i3 domain, whereas m2-tail and m3-tail consist of the C-terminal half of i3 and the remaining C-terminal receptor sequences (including TM VI and VII). The different polypeptides were expressed in COS-7 cells, either alone or in various combinations, and studied for their ability to bind muscarinic ligands and to stimulate phosphatidyl inositol (PI) hydrolysis.

2. MATERIALS AND METHODS

2.1. *Construction of expression plasmids encoding fragmented muscarinic receptors*

Hm2pcD and Rm3pcD, two mammalian expression vectors containing the entire coding sequences of the human m2 and the rat m3 muscarinic receptors [15], respectively, were used to construct various fragmented muscarinic receptor genes coding for m2-trunc, m3-trunc, m2-tail, and m3-tail (Fig. 1). Expression plasmids coding for these four polypeptides (pcDm2-trunc, pcDm3-trunc, pcDm2-tail, and pcDm3-tail) were created as described in Fig. 2.

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Abbreviations: NMS, *N*-methylscopolamine chloride; G protein, guanine nucleotide-binding protein; i3, third cytoplasmic loop; PI, phosphatidyl inositol; TM I–VII, the seven transmembrane domains of G protein-coupled receptors; IP_1, inositol monophosphate; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide.

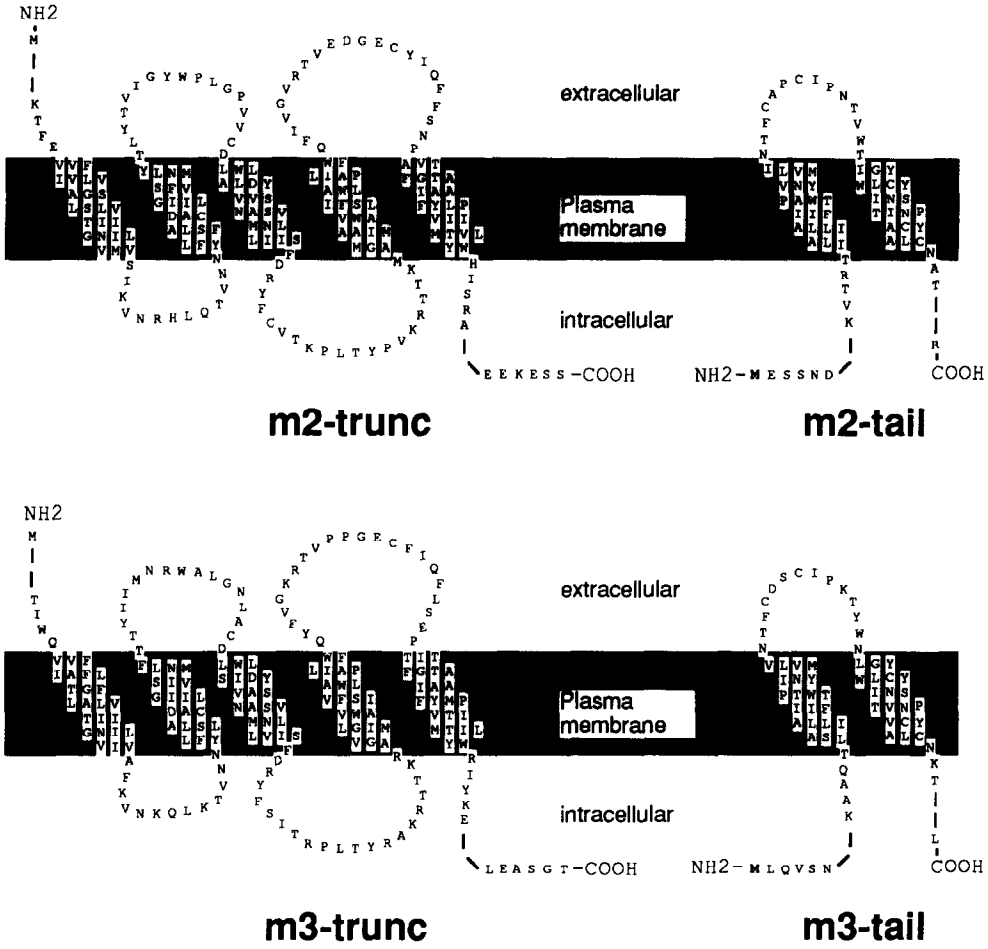

Fig. 1. Amino acid sequence and predicted transmembrane disposition of fragmented human m2 and rat m3 muscarinic receptors. Expression plasmids coding for the various polypeptides were prepared as described in section 2. The different receptor fragments are composed as follows (amino acid numbers of the wild-type m2 or m3 receptor sequences are given in parentheses): m2-trunc (1–283), m2-tail (281–466), m3-trunc (1–272), and m3-tail (388–589). Only the membrane-proximal portions of the N- and C-termini of the various polypeptides are shown (dashed lines indicate receptor sequences not shown in this scheme). The intracellular C-termini of m2- and m3-trunc consist of 56 and 21 amino acids, respectively. The intracellular N-termini of m2- and m3-tail are composed of 109 and 105 amino acids, respectively. The methionine codons at the N-termini of m2- and m3-tail (shown in bold) have been added to provide a proper translation start site.

Table I

Antagonist binding properties of muscarinic receptor fragments co-expressed in COS-7 cells

Receptor (complex)	B_{max} (fmol/mg)	[^{3}H]NMS		4-DAMP	
		K_D (pM)	n_H	K_i (nM)	n_H
m2 (wild-type)	1,215 ± 189	62 ± 7	0.99 ± 0.18	3.3 ± 0.3	1.02 ± 0.04
m2-trunc/m2-tail	475 ± 16	79 ± 10	0.92 ± 0.09	3.7 ± 0.2	0.98 ± 0.04
m2-trunc/m3-tail	389 ± 25	34 ± 2	1.09 ± 0.12	1.0 ± 0.05	0.96 ± 0.04
m3 (wild-type)	1,141 ± 51	29 ± 2	1.05 ± 0.10	0.40 ± 0.03	0.99 ± 0.05
m3-trunc/m3-tail	114 ± 17	23 ± 2	1.00 ± 0.11	0.40 ± 0.02	0.99 ± 0.05
m3-trunc/m2-tail	no specific [^{3}H]NMS binding detectable				

[^{3}H]NMS affinity constants (K_D) were determined in direct binding assays. Inhibition constants (K_i) for 4-DAMP were obtained in competition binding experiments as described in section 2 (n_H = Hill coefficient). B_{max} values represent maximal number of binding sites, expressed as fmol/mg of membrane protein. Data are presented as means ± S.E.M. of two or three independent experiments, each performed in duplicate.

Plasmid	Fragments used for ligation	Linker sequence
pcDm2-trunc	Sst I - Sst I (5.3 kb)	(Self-ligation creates an in-frame stop codon after Ser283)
pcDm3-trunc	Bstx I - Nhe I (6.1 kb)	Stop 5' CTGGCTGGCCTACAGGCTCTGGGACATGAG 3' 3' TCTCGACCGACCGGATGTCCGAGACCCTGTACTCGATC 5' Bstx I Nhe I
pcDm2-tail	Sst I - Hind III (4.5 kb) Hind III - Pst I (0.5 kb)	Met 5' GATTAGAGAACGCAAAATGGAGAGCT 3' 3' ACGTCTAATCTCTTGCGTTTTACCTC 5' Pst I Sst I
pcDm3-tail	Pst I - Cfr 10 (4.1 kb) Cfr 10 - BamH I (1.4 kb)	Met 5' GATCCCTATGTCAGAGAGTCACAATGCTGCA 3' 3' GGATACAGTCTCTCAGTGTTACG 5' BamH I Pst I

Fig. 2. Construction of expression plasmids encoding muscarinic receptor fragments. Hm2pcD and Rm3pcD, two mammalian expression plasmids containing the entire coding sequences of the human m2 and the rat m3 muscarinic receptors [15], respectively, were used to construct various fragmented muscarinic receptor genes. DNA linkers were prepared on an Applied Biosystems 3280B DNA Synthesizer. Single-stranded oligonucleotides were purified by polyacrylamide gel electrophoresis, annealed (resulting in the creation of cohesive ends), and then ligated with the indicated restriction fragments prepared from either Hm2pcD (pcDm2-tail) or Rm3pcD (pcDm3-trunc and pcDm3-tail) (see section 2).

2.1.1. pcDm2-trunc

A 5.3 kb *Sst*I-fragment was removed from Hm2pcD and subjected to self-ligation, resulting in the creation of a stop codon after amino acid codon 283 of the human m2 sequence.

2.1.2. pcDm3-trunc

A 0.70 kb *Bst*XI–*Nhe*I fragment was removed from Rm3pcD and replaced with a synthetic linker sequence containing an in-frame stop codon after amino acid codon 272 of the rat m3 sequence.

2.1.3. pcDm2-tail and pcDm3-tail

To construct pcDm2-tail, a 0.90 kb *Pst*I–*Sst*I restriction fragment was removed from Hm2pcD and replaced with a synthetic DNA linker sequence containing an in-frame translation initiation codon (ATG) and a short stretch of adjacent 5′ untranslated sequence present in Hm2pcD. The resulting plasmid encodes the C-terminal 185 amino acids (amino acids 281–466) of the human m2 receptor. For the creation of pcDm3-tail, a 1.3 kb *Bam*HI–*Pst*I restriction fragment was cut out from Rm3pcD and replaced with a synthetic DNA linker sequence containing an in-frame translation initiation codon (ATG) and a short stretch of adjacent 5′ untranslated sequence of Rm3pcD. The resulting construct codes for the C-terminal 202 amino acids (amino acids 388–589) of the rat m3 muscarinic receptor.

The identity of all constructs was confirmed by dideoxy sequencing [16] of the regions derived from the synthetic oligonucleotides and by restriction endonuclease analysis.

2.2. *Construction of expression plasmids containing two transcriptional units*

To allow the efficient co-expression of the various muscarinic receptor fragments, plasmids with two transcriptional units were constructed. The *Sal*I–*Tth*111I fragments containing the SV40 early region promoter, the m2-tail or m3-tail coding sequence, and the segment carrying the SV40 late region polyadenylation signal were removed from pcDm2-tail or pcDm3-tail, respectively, blunt-ended, and cloned into the blunt-ended *Sal*I site present in pcDm2-trunc or pcDm3-trunc. Restriction endonuclease analysis verified that both transcriptional units were present in the same orientation. All possible pcDtrunc/tail combinations were constructed: pcDm2-trunc/m2-tail, pcDm2-trunc/m3-tail, pcDm3-trunc/m3-tail, and pcDm3-trunc/m2-tail. The structural features of the pcD vector system used to construct the various expression plasmids have been described by Okayama and Berg [17].

2.3. *Transfections and membrane preparation*

Unless otherwise indicated, COS-7 cells were transfected with 20 μg of plasmid DNA in 100-mm plates by using a calcium phosphate precipitation method [18]. Cells were harvested 72 h after transfections, and membrane homogenates were prepared as described previously [19]. Protein concentrations were determined according to the method of Bradford [20] using a Bio-Rad protein assay kit.

2.4. *Ligands*

[^{3}H]*N*-Methylscopolamine ([^{3}H]NMS; 78.9 Ci/mmol) was purchased from Dupont-New England Nuclear. 4-Diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) was obtained from Research Biochemicals Inc. Acetylcholine, carbachol and atropine were from Sigma.

2.5. *Ligand binding studies*

Radioligand binding studies were carried out with membrane homogenates essentially as described [19]. In the competition binding experiments, a [^{3}H]NMS concentration of 200 pM was used. Nonspecific binding was determined in the presence of 1 μM atropine. Data were analyzed by a non-linear least-squares curve fitting procedure as described [19].

2.6. *PI assays*

Transfected COS-7 cells were incubated with 3 μCi/ml *myo*-[^{3}H]inositol (23 Ci/mmol, American Radiolabeled Chemicals Inc.) for 48 h. Carbachol-induced increases in intracellular inositol monophosphate (IP_1) levels were determined as described [21].

3. RESULTS

For co-expression experiments, expression plasmids containing two transcriptional units (see section 2) were used. The ligand binding and functional properties of the expressed polypeptides (polypeptide complexes; Fig. 1) were determined and compared with those of the wild-type m2 and m3 muscarinic receptors.

3.1. *Antagonist binding studies*

None of the four muscarinic receptor fragments (Fig. 1) showed detectable [^{3}H]NMS binding activity when expressed alone in COS-7 cells. In contrast, a considerable number of specific [^{3}H]NMS binding sites was observed after co-expression of m2-trunc with m2-tail (m2- trunc/m2-tail) and of m3-trunc with m3-tail (m3-trunc/m3-tail) (Table I). The calculated [^{3}H]NMS K_D values were similar to those found for the wild-type m2 and m3 receptors, respectively (Table I). [^{3}H]NMS binding activity (K_D = 34 ± 2 pM) was also found in cells co-expressing the chimeric polypeptide combination m2-trunc/m3-tail (Table I). Interestingly, cells transfected with the chimeric m3-trunc/m2-tail construct displayed no specific [^{3}H]NMS binding. Co-transfection of m3- trunc/m2-tail plasmid DNA (pcDm3-trunc/m2-tail) with an expression plasmid coding for either m2-trunc (pcDm2-trunc) or m3-tail (pcDm3-tail) resulted in a 'recovery' of muscarinic binding sites (data not shown), indicating that pcDm3-trunc/m2-tail in fact directs the expression of both encoded polypeptides.

The subtype-selective muscarinic antagonist, 4-DAMP, bound to the m2-trunc/m2-tail and m3-trunc/m3-tail receptor complexes with affinities that were not significantly different from those found for the wild-type m2 and m3 receptors, respectively (Table I). The chimeric m2-trunc/m3-tail receptor complex bound 4-DAMP with a K_i value that was intermediate between those determined for the two wild-type receptors (Table I).

3.2. *Agonist binding studies*

The 'reconstituted' m2-trunc/m2-tail and m3-trunc/m3-tail receptor complexes exhibited agonist binding properties similar to those obtained for the wild-type m2 and m3 receptors, respectively, and thus retained the same spectrum of agonist binding selectivity displayed by the wild-type receptors (Table II). The chimeric m2-trunc/m3-tail receptor complex displayed agonist binding affinities that were intermediate between those found for the two wild-type receptors.

3.3. *Stimulation of PI hydrolysis*

The ability of the various muscarinic receptor fragments and fragment combinations to mediate carbachol-induced stimulation of PI hydrolysis was determined. Carbachol stimulation of non-transfected COS-7 cells or of cells transfected with the various muscarinic receptor fragments alone did not result in any detectable PI activity. Consistent with their known functional selectivity [8,9,22], the wild-type m3 receptor mediated a pronounced stimulation of PI hydrolysis, whereas the m2 receptor was inactive (Table III, Fig. 3). The m3-trunc/m3-tail receptor complex stimulated PI hydrolysis in a fashion similar to the wild-type m3 receptor (Table III, Fig. 3). Like the wild-type m2 receptor, the 'reconstituted' m2-trunc/m2-tail and the 'chimeric' receptor complexes were unable to mediate a significant increase in inositol phosphate levels upon carbachol stimulation.

4. DISCUSSION

We have shown that expression of m2 or m3 muscarinic receptors as two separate polypeptides (one containing TM I–V, and the other, TM VI and VII; see Fig. 1) results in the 'reconstitution' of functional receptor complexes. The reconstituted receptors displayed ligand-binding properties similar to those of the two wild-type receptors. These data suggest that the two parts of the muscarinic receptors that are covalently linked by the i3 loop in the native receptors have the capability to fold independently of each other, being able to adopt a conformation in the lipid bilayer that allows them to recognize each other and form a stable receptor complex.

Furthermore, functional studies showed that the m3-trunc/m3-tail receptor complex mediated a stimulation of PI hydrolysis in a fashion similar to the wild-type m3

Table II

Agonist binding properties of muscarinic receptor fragments co-expressed in COS-7 cells

Receptor (complex)	Acetylcholine		Carbachol	
	IC_{50} (μM)	n_H	IC_{50} (μM)	n_H
m2 (wild-type)	0.87 ± 0.17	0.63 ± 0.04	2.14 ± 0.6	0.49 ± 0.06
m2-trunc/m2-tail	0.31 ± 0.10	0.49 ± 0.03	0.65 ± 0.2	0.49 ± 0.02
m2-trunc/m3-tail	3.62 ± 0.32	0.81 ± 0.03	22.0 ± 1.9	0.77 ± 0.03
m3 (wild-type)	12.3 ± 1.3	0.76 ± 0.02	59.3 ± 8.7	0.71 ± 0.03
m3-trunc/m3-tail	8.3 ± 1.9	0.58 ± 0.04	47.7 ± 4.8	0.81 ± 0.05

IC_{50} values were obtained in competition binding experiments using [^{3}H]NMS (200 pM) as a radioligand. Hill coefficients (n_H) were significantly smaller than 1 ($P < 0.05$). Data are presented as means ± S.E.M. of two or three independent experiments, each performed in duplicate.

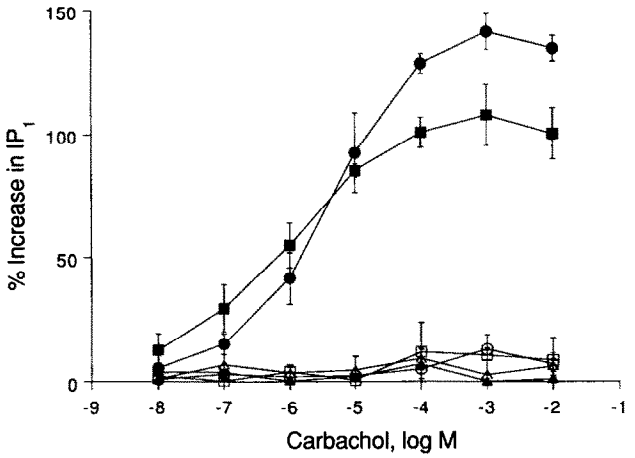

Fig. 3. Stimulation of PI hydrolysis by m2 and m3 muscarinic receptor fragments co-expressed in COS-7 cells. The following symbols are used: wild-type m2 (▲), m2-trunc/m2-tail (○), m2-trunc/m3-tail (□), wild-type m3 (●), m3-trunc/m3-tail (■), and m3-trunc/m2-tail (△). Transfected COS-7 cells were incubated with increasing concentrations of carbachol for 1 h at 37°C. Increases in intracellular IP_1 levels were determined by anion-exchange chromatography as described [21]. Responses are expressed as percentage increase of IP_1 above basal levels determined in the absence of carbachol. The basal IP_1 levels observed for the various 'trunc/tail' receptor complexes were not significantly different from those found for the wild-type m2 and m3 receptors. Data are presented as means ± S.E.M. of two or three independent experiments.

receptor. This finding indicates that a covalent connection between TM V and VI (i3) is not required for efficient G protein activation. Similar to the wild-type m2 receptor, carbachol stimulation of the m2-trunc/m2-tail receptor complex did not result in a significant PI response, indicating that the reconstituted polypeptide complexes retained the functional selectivity of the wild-type receptor from which they were derived. Furthermore, the chimeric m2-trunc/m3-tail receptor was also unable to mediate stimulation of PI hydrolysis. This finding is consistent with the notion that the N-terminal portion of the i3 domain (contained in m2-trunc) is the primary structural determinant of how efficiently the individual muscarinic receptors are coupled to PI hydrolysis [8,9].

The chimeric m2-trunc/m3-tail receptor complex was characterized by ligand binding affinities that were intermediate between those found for the wild-type m2 and m3 receptors. This finding is consistent with the notion that the C-terminal muscarinic receptor domains (including TM VI and VII) contain major structural determinants of ligand binding selectivity [21,23]. The lack of detectable [^{3}H]NMS binding activity observed after transfection with pcDm3-trunc/m2-tail may be due to conformational incompatibilities between the N-terminal m3 and the C-terminal m2 receptor sequences which do not allow the formation of functional receptor complexes.

Our data support and considerably expand previous findings obtained with truncated β2-adrenergic receptors [24]. In this case, coinjection into *Xenopus* oocytes of mRNAs coding for two fragmented β2-adrenergic receptors ('split' within the i3 loop) resulted in the appearance of specific adrenergic binding sites. However, in contrast to our findings with the m3-trunc/m3-tail receptor complex, which proved to be functionally highly active, the reconstituted β2-adrenergic receptor was found to be severely impaired in its ability to mediate adenylyl cyclase activation [24]. The reason for this discrepancy remains unclear at present, particularly since no EC_{50} or B_{max} values were given in this earlier study.

Our findings, together with previous mutagenesis studies, suggest that, as a general rule, G protein-coupled receptors consist of at least two independent folding domains (one containing TM I–V, and the other, TM V and VI). Interestingly, studies on the microassembly of the structurally related integral membrane protein, bacteriorhodopsin, have shown that bacteriorhodopsin can be functionally reassembled in lipid vesicles starting from various denatured proteolytic fragments (for a review, see [13]). In addition, two natural

Table III

Stimulation of PI hydrolysis by muscarinic receptor fragments co-expressed in COS-7 cells

Receptor (complex)	Amount of transfected DNA (μg)	B_{max} (fmol/mg)	Maximum increase in IP_1 levels above basal (%)	Carbachol EC_{50} (μM)
m2 (wild-type)	4	83 ± 12	7 ± 5	–
m2-trunc/m2-tail	20	393 ± 28	13 ± 1	–
m2-trunc/m3-tail	20	291 ± 13	12 ± 11	–
m3 (wild-type)	4	108 ± 15	141 ± 7	6.1 ± 2.5
m3-trunc/m3-tail	20	70 ± 18	107 ± 12	0.96 ± 0.32
m3-trunc/m2-tail	20	–	9 ± 4	–

EC_{50} values for carbachol-induced increases in IP_1 levels were determined graphically from plots of log carbachol concentration vs. percentage response. B_{max} values were determined in [^{3}H]NMS saturation binding assays. The amount of transfected wild-type m2 and m3 plasmid DNA was reduced to 4 μg/100 mm dish to obtain B_{max} values similar to those found for the various 'trunc/tail' receptor complexes. Basal IP_1 levels, determined in the absence of carbachol, were not significantly different for the wild-type m2 and m3 receptors and the various 'tail/trunc' receptor complexes. Data are presented as means ± S.E.M. of two or three independent experiments, each performed in duplicate.

cases have been described (cytochrome b_6/subunit IV and nicotinamide nucleotide transhydrogenase) in which a single polypeptide in one type of membrane or organism appears to be split into two subunits in another [13]. Taken together, these findings are consistent with the idea that integral membrane proteins are composed of two or more independent folding domains that are inserted into the lipid bilayer as independent units and then assembled to form a functional transmembrane protein [14]. Based on the high structural homology found among all G protein-coupled receptors, the findings described here should be of general importance for this entire class of integral membrane proteins.

REFERENCES

[1] Nathanson, N.M. (1987) Annu. Rev. Neurosci. 10, 195–236.
[2] Bonner, T.I. (1989) Trends Neurosci. 12, 148–151.
[3] Hulme, E.C., Birdsall, N.J.M. and Buckley, N.J. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 633–673.
[4] Fraser, C.-M., Wang, C.-D., Robinson, D.A., Gocayne, J.D. and Venter, J.C. (1989) Mol. Pharmacol. 36, 840–847.
[5] Hulme, E.C., Kurtenbach, E. and Curtis, C.A.M. (1991) Biochem. Soc. Trans. 19, 133–138.
[6] Wess, J., Gdula, D. and Brann, M.R. (1991) EMBO J. 10, 3729–3734.
[7] Wess, J., Maggio, R., Palmer, J.R. and Vogel, Z. (1992) J. Biol. Chem. 267, 19313–19319.
[8] Wess, J., Brann, M.R. and Bonner, T.I. (1989) FEBS Lett. 258, 133–136.
[9] Wess, J., Bonner, T.I., Dörje, F. and Brann, M.R. (1990) Mol. Pharmacol. 38, 517–523.
[10] Lechleiter, J., Hellmiss, R., Duerson, K., Ennulat, D., David, N, Clapham, D. and Peralta, E. (1990) EMBO J. 9, 4381–4390.
[11] Shapiro, R.A. and Nathanson, N.M. (1989) Biochemistry 28, 8946–8950.
[12] Okamoto, T. and Nishimoto, I. (1992) J. Biol. Chem. 267, 8342–8346.
[13] Popot, J.-L. and de Vitry, C. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 369–403.
[14] Popot, J.-L. and Engelman, D.M. (1990) Biochemistry 29, 4031–4037.
[15] Bonner, T.I., Buckley, N.J., Young, A.C. and Brann, M.R. (1987) Science 237, 527–532.
[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[17] Okayama, H. and Berg, P.A. (1983) Mol. Cell. Biol. 3, 280–289.
[18] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752.
[19] Dörje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E. and Brann, M.R. (1991) J. Pharmacol. Exp. Ther. 256, 727–733.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Wess, J., Bonner, T.I. and Brann, M.R. (1990) Mol. Pharmacol. 38, 872–877.
[22] Peralta, E.G., Ashkenazi, A., Winslow, J.W., Ramachandran, J and Capon, D.J. (1988) Nature 334, 434–437.
[23] Wess, J., Gdula, D. and Brann, M.R. (1992) Mol. Pharmacol 41, 369–374.
[24] Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G. and Lefkowitz, R.J. (1988) Science 240, 1310–1316.