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Homo- or Hetero-Dimerization of Muscarinic Receptor Subtypes is Not Mediated by Direct Protein-Protein Interaction Through Intracellular and Extracellular Regions

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The oligomerization of G-proteincoupled receptors (GPCRs) has been shown to occur by various mechanisms, such as via disulfide covalent linkages, noncovalent (ionic, hydrophobic) interactions of the N-terminal, and/or transmembrane and/or intracellular domains. Interactions between GPCRs could involve an association between identical proteins (homomers) or non-identical proteins (heteromers), or between two monomers (to form dimers) or multiple monomers (to form oligomers). It is believed that muscarinic receptors may also be arranged into dimeric or oigomeric complexes, but no systematic experimental evidence exists concerning the direct physical interaction between receptor proteins as its mechanism. We undertook this study to determine whether muscarinic receptors form homomers or a heteromers by direct protein-protein interaction within the same or within different subtypes using a yeast two-hybrid system. Intracellular loops (i1, i2 and i3) and the C-terminal cytoplasmic tails (C) of human muscarinic (Hm) receptor subtypes, Hm1, Hm2 and Hm3, were cloned into the vectors (pB42AD and pLexA) of a two-hybrid system and examined for heteromeric or homodimeric interactions between the cytoplasmic domains. No physical interaction was observed between the intracellular domains of any of the Hm/Hm receptor sets tested. The results of our study suggest that the Hm1, Hm2 and Hm3 receptors do not form dimers or oligomers by interacting directly through either the hydrophilic intracellular domains or the C-terminal tail domains. To further investigate extracellular domain interactions, the N-terminus (N) and extracellular loops (o1 and o2) were also cloned into the two-hybrid vectors. Interactions of Hm2N with Hm2N, Hm2o1, Hm2o2, Hm3N, Hm3o1 or Hm3o2 were examined. The N-terminal domain of Hm2 was found to have no direct interaction with any extracellular domain. From our results, we excluded the possibility of a direct interaction between the muscarinic receptor subtypes (Hm1, Hm2 and Hm3) as a mechanism for homo- or hetero-meric dimerization/oligomerization. On the other hand, it remains a possibility that interaction may occur indirectly or require proper conformation or subunit formation or hydrophobic region involvement.

Key words: Muscarinic receptors subtypes, Oligomerization, Protein-protein interaction, Two-hybrid system

INTRODUCTION

A number of cell surface receptors mediate their actions *via* dimerization that alters the ligand-binding, and signaling properties of the receptors. G protein coupled receptors (GPCRs) mediate a variety of signaling processes, such as neurotransmission, hormonal response, olfaction, and light transduction. Traditional molecular models that describe the interaction of GPCRs with their G proteins predict that GPCRs function as monomers and couple to G protein in a 1:1 ratio. However, several recent studies have reported interactions between GPCRs, and it is apparent that these interactions could involve an association between identical proteins (homomers) or non-identical proteins (heteromers) to form either dimers or oligomers. Indirect evidences for existence of GPCR dimers has been provided by a number of studies, upon issue such as, complex binding in ligand-binding analysis, photoaffinity labeling with radioiodinated ligands, cross-linking, target size analysis and

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immunoprecipitation using antibodies to endogenous receptors (for reviews, see Bouvier, 2001; Gomes *et al.*, 2001). In recent years, differential epitope tagging of GPCR cDNA followed by selective immunoprecipitation of the dimer has been used to directly confirm the ability of GPCRs to dimerize in heterologous cells (Hebert *et al.*, 1996 1998; Cvejic and Devi, 1997; Jordan and Devi, 1999 Fukushima *et al.*, 1997; Wregget and Wells, 1995; Abe *et al.*, 1999; Zeng and Wess, 1999). The existence of GPCR dimers in living cells has been demonstrated more recently using biophysical techniques such as fluorescence resonance energy transfer and bioluminescence resonance energy transfer (Overton and Blumer, 2000; Cornea *et al.*, 2001 Angers *et al.*, 2000; McVey *et al.*, 2001; Rocheville *et al.* 2000; Roess *et al.*, 2000).

GPCR dimerizations have shown to occur by various mechanisms, including disulfide covalent linkage and covalent interactions with the N-terminal, transmembrane (TM) or intracellular domains. In the case of the β2 adrenergic receptor, dimerization may depend on hydrophobic contacts involving residues located on TM VI (Hebert *et al.*, 1996). On the other hand, homodimerization of the δ-opioid receptor has been shown to be dependent on the integrity of the C-terminal "tail" (Cvejic and Devi, 1997). Likewise, the formation of the $GABA_BR1$/ $GABA_BR2$ receptor heterodimers appears to involve direct C-terminal (coiled-coil) interaction (Jones *et al.*, 1998; Kaupman *et al.*, 1998; Kuner *et al.*, 1999; White *et al.*, 1998). In contrast, the metabotropic glutamate receptor 5 (Romano *et al.*, 1996) and the calcium-sensing receptor (Bai *et al.*, 1998; Pace *et al.*, 1999; Ward *et al.*, 1998) appear to form disulfide-linked dimers associated with their large amino termini.

Muscarinic receptors have been suggested to be arranged in dimeric or oligomeric complexes by radioligand binding studies (Potter *et al.*, 1991; Hirschberg and Schimerlik, 1994; Wreggett and Wells, 1995; Chidiac *et al.*, 1997). The complex agonist binding properties of muscarinic receptors expressed in rabbit heart and rat brain stem were consistent with the presence of two agonist binding sites located on dimeric receptor molecules (Potter *et al.*, 1991). Likewise, computer simulations of the binding properties of an agonist, [^{3}H]oxotremorine-M, at m2 muscarinic receptors expressed in cultured cells or porcine atria were consistent with the existence of receptor dimers and/or monomers (Hirschberg and Schimerlik, 1994). Similar findings were obtained in studies using purified muscarinic receptors (Wreggett and Wells, 1995), suggesting that the complex binding properties of agonists are due to different classes of G proteins. A report of the coexpression of α2/ m3 and m3/α2 hybrid receptors, which resulted in near wild-type binding and activity raised the possibility of a muscarinic receptor heterodimer (Maggio *et al.*, 1993a). Zeng and Wess (1999) modified rat m3 muscarinic receptor by deleting the central portion of the i3 loop and investigated molecular aspects of its assembly and dimerization/ oligomerization, by immunoprecipitation by using the differential epitope tagging of the receptor cDNA. They reported that the dimerization/oligomerization of the modified m3 muscarinic receptor appears to be receptor subtype-selective and to involve disulfide-cross linking of the receptor monomers. Although muscarinic receptor oligomerization has been speculated from the studies mentioned above, direct systematic experimental evidences concerning the nature of the dimerization mechanism are largely lacking.

In the present study, we examined using yeast two-hybrid system whether muscarinic receptors form a homomer or a heteromer by direct protein-protein interaction of intracellular and exptracellular domains within the same or different subtypes. Each cytoplasmic loop and C-terminal cytoplasmic tail of the human muscarinic (Hm) receptor subtypes, Hm1, Hm2 and Hm3, were cloned into the vectors of a two hybrid system and direct protein-protein interactions between cytoplasmic domains were examined. In addition, interactions within extracellular loops and N-terminal regions were also tested for their involvement in muscarinic receptor dimerization.

MATERIALS AND METHODS

Construction of muscarinic receptor fragment constructs

DNAs encoding the intracellular and extracellular domains of Hm1, Hm2 and Hm3 receptors were amplified by polymerase chain reaction (PCR) using template cDNAs and primers containing *EcoRI* and *XhoI* restriction site for cloning into yeast two-hybrid system vectors (Table I). PCR primers 1-34 were used to construct the DNA fragments encoding the intracellular domain (i1, i2, i3, C-termini) and the extracellular domain (N-termini, o1, o2) of Hm1, Hm2 and Hm3. Primers 1 and 2 were used for Hm1i1 that contains amino acids Lys51 to Asn61. Primers 3 and 4 were used for Hm1i2 containing amino acids Asp122 to Arg141, primers 5 and 6 for Hm1i3 containing amino acids Arg210 to Thr296, and primers 7 and 8 for Hm1C containing amino acid Asn422 to Cys460. The primers 9 and 10 were used for Hm2i1 containing Lys 49 to Asn 59, primers 11 and 12 for Hm2i2 containing Asp120 to Lys138, primers 13 and 14 for Hm2i3 containing His 208 to Thr388, and primers 15 and 16 for Hm2C containing Cys443 and Arg466. Primers 17 and 18 were used to construct Hm3i2 containing Asp165 to Arg184, primers 19 and 20 for Hm3i3 containing Arg253 to Thr492, and primers 21 and 22 for Hm3C containing Asn548 to Leu590. The primers 23 and 24 were used for Hm2N containing amino acids Met1 to Glu22, primers 25 and 26

Table I. Sequences of oligonucleotides (5'→3') for cloning of the intracellular and extracellular domains of human muscarinic receptor

Primers	Forward	Backward
Intracellular domains		
M1i1	G*GAATTC*AAGGTCAAC	CCG*CTCGAG*TTATTGACTGT
M1i2	G*GAATTC*GACCGCTACTTC	CCG*CTCGAG*CCGGCGGGGTGT
M1i3	G*GAATTC*CGCATCTACCGG	CCG*CTCGAG*GGTCCGAGCC
M1c	G*GAATTC*TGCAACAAAG	CCG*CTCGAG*TCAGCATTGGCG
M2i1	G*GAATTC*AAAGTCAACCGC	CCG*CTCGAG*ATTGTTGACGGT
M2i2	G*GAATTC*GACAGGTACTTC	CCG*CTCGAG*TTTTGTGGTCCG
M2i3	G*GAATTC*CACATATCCCGA	CCG*CTCGAG*TGTCCTGGTGAC
M2c	G*GAATTC*TGCAATGCCACC	CCG*CTCGAG*TTACCTTGTAGC
M3i2	G*GAATTC*GACAGATACTTT	CCG*CTCGAG*GCTCTCTTTGT
M3i3	G*GAATTC*AGGATCTATAAG	CCG*CTCGAG*GGTCTGGGC
M3c	G*GAATTC*AACAAAACATTC	CCG*CTCGAG*CTACAAGGCCTG
Extracellular domains		
M2N	G*GAATTC*ATGAATAACTCA	CCG*CTCGAG*TTCAAATGTCTT
M2o1	G*GAATTC*ACCCTCTACACT	CCG*CTCGAG*GTCACACACCACA
M2o2	G*GAATTC*CAGTTCATTGTA	CCG*CTCGAG*AGCATTGGAAAA
M3N	G*GAATTC*ATGACCTTGCAC	CCG*CTCGAG*TTGCCAGACGGT
M3o1	G*GAATTC*ACGACCTACATC	CCG*CTCGAG*GTCACAGGCCAA
M3o2	G*GAATTC*CAATACTTTGTT	CCG*CTCGAG*GGGCTCACTGAG

a: GAATTC; *Eco*RI restriction site
b: CTCGAG; *Xho*I restriction site
Human muscarinic receptor (Hm) was designated as M.

for Hm2o1 containing amino acids Thr81 to Asp, and primers 27 and 28 for Hm2o2 containing amino acids Gln163 to Ala184. The primers 29 and 30 were used for Hm3N containing amino acid Met1 to Val42, primers 31 and 32 for Hm3o1 containing Thr126 to Asp142, and primers 33 and 34 for Hm3o2 containing Gln208 to Pro229. PCR reactions utilized Pfu DNA polymerase (Stratagene, La Joli, CA, USA) using the following conditions: 5-min precycle of denaturation at 95°C followed by 30 cycles of, denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The PCR products were digested with *EcoR* and *XhoI* enzymes (TaKaRa, Otsu, Shiga, Japan) and cloned into the corresponding sites of yeast two-hybrid system vectors, pLexA and pB42AD (Clontech, Palo Alto, CA, USA). The resulting LexA and pB42AD constructs encoded fusion protein containing the DNA binding domain of the bacterial protein ($LexA_{BD}$) under the control of the *adh*1 promoter and containing the B42 acid patch transcriptional activation domain ($B42_{AD}$) under the control of the *gal*1 promoter.

Yeast two-hybrid assays: Yeast transformation and interaction trap

The yeast two-hybrid system was utilized in this study to determine direct protein-protein interaction between muscarinic receptor subtypes. The yeast reporter strain used was EGY48/pSH18-34. The yeast strain EGY48 (MATα, ura3-52, his3, trp1, leu2::pLEU2-LexAop6) carries the LexAop-Leu2 reporter, and the plasmid pSH18-34 contains the LexAop-LacZ reporter. The pLexA- and pB42AD-muscarinic receptor fragment fusion constructs were introduced into EGY48/pSH18-34 by using the lithium acetate transformation method (Gietz *et al.*, 1992) for interaction trap selections. The suspension of co-transformed cells were spread on glucose-Ura$^-$His$^-$Trp$^-$ plates and a single colony was selected, which was streaked onto a glucose-Ura$^-$His$^-$Trp$^-$ plate and incubated at 30°C for two or three days. Transformed yeast cells for interaction trap were plated onto media Leu$^-$Ura$^-$His$^-$Trp$^-$ and grown at 30 for 2-4 days. No growth on Leu$^-$ plates containing glucose but growth on Leu$^-$ plates containing galactose indicated an interaction between the DNA binding domain hybrid protein and the transcriptional activation domain hybrid protein. Protein-protein interactions were further confirmed by plating the transformed yeast cells onto media containing 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-gal). White growth on X-gal plates containing glucose, but blue growth on X-gal plates containing galactose indicated an interaction between the DNA binding domain hybrid protein and the transcriptional activation domain hybrid protein.

Electrophoresis and immunoblot analysis

The expressions of the fusion proteins of appropriate

sizes were confirmed by Western blotting. Yeast cells in selective media (glucose-$Ura^-His^-Trp^-$ media for the detection of pLexA fusion constructs and glucose- or galactose-$Ura^-His^-Trp^-$ media for the detection of pB42AD fusion constructs) were grown to OD_{600} 0.8-1.0 and centrifuged at 3,000 × g for 5 min. Protein extracts of yeast cells were prepared in 200 μL of yeast lysis buffer (50 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 0.5% SDS, 0.5 mM PMSF, 5 μg/μL aprotinin, 1 μg/μL leupeptin, 1 mM benzamidine) and 200 μL of glass beads. The protein extracts were then SDS-polyacrylamide gel electrophoresed and electro-transferred to polyvinylidene fluoride (PVDF) membranes. To detect the LexA fusion proteins from the pLexA constructs and activation domain fusion proteins from pB42AD constructs, NC membranes were incubated with LexA antibody and anti-HA antibody 12CA5, respectively. The protein bands were detected by chemiluminescence (ECL Amersham, Buckinghamshire, UK) visualized using LAS 1000 (Fuji Co., Tokyo, Japan).

RESULTS

Production of fusion proteins

The intracellular domains of the Hm1, Hm2 or the Hm3 receptors (Fig. 1) were fused both to the DNA binding domain of the bacterial protein $LexA_{BD}$ in pLexA and to the acidic domain of the B42 transcriptional activating factor ($B42_{AD}$) in pB42AD. The production of fusion proteins in the correct reading frame with the expected sizes was confirmed by immunoblot analyses. Anti-LexA antibody detected bands, which corresponded to the masses of Hm1i1-, Hm1i2-, Hm1i3-, Hm1c-, Hm2i1-, Hm2i2-, Hm2i3-, Hm2C-, Hm3i2-, Hm3i3-, and Hm3C-$LexA_{BD}$ fusion proteins (26 kDa, 28 kDa, 45 kDa, 27 kDa, 26 kDa, 27 kDa, 45 kDa, 24 kDa, 26 kDa, 49 kDa and 31 kDa, respectively) (Fig. 2A). Anti-HA antibody detected bands of 21 kDa, 24 kDa, 38 kDa, 24 kDa, 21 kDa, 25 kDa, 40 kDa, 22 kDa, 22 kDa 46 kDa and 21 kDa, that correspond to Hm1i1-, Hm1i2-, Hm1i3-, Hm1C-, Hm2i1-, Hm2i2-, Hm2i3-, Hm2C-, Hm3i2-, Hm3i3-, and Hm3C-$B42_{AD}$ fusion proteins, respectively. Since the expression of the activator gene is controlled by the *gal*1 promoter, the fusion proteins of Hm-$B42_{AD}$ were detected with HA antibody in galactose-containing (+) media, but not in glucose-containing (-) media (Fig. 2B). Extracellular domain interactions were also examined in the same way as the intracellular interactions. Extracellular domains of Hm2 and Hm3 were also fused to $LexA_{BD}$ in pLexA and $B42_{AD}$ in pB42AD. We also confirmed that the fusion proteins were produced in the correct reading frame. Bands of 28 kDa, 26 kDa, 26 kDa, 27 kDa 27 kDa and 27 kDa proteins, which corresponded to Hm2N-, Hm2o1-, Hm2o2-, Hm3N-, Hm3o1- and Hm3o2-$LexA_{BD}$ fusion proteins were detected by using anti-LexA antibody (Fig. 2C). HA antibody detected bands of 24 kDa, 21 kDa, 21 kDa, 23 kDa, 22 kDa, and 24 kDa proteins, which also correspond to Hm2N-, Hm2o1-, Hm2o2-, Hm3N-, Hm3o1-, and Hm3o2-$B42_{AD}$ fusion proteins. The detection of fusion proteins with the anti-HA antibody was galactose-dependent as is shown by Fig. 2D. The detected sizes agreed well with the predicted sizes of the fusion proteins.

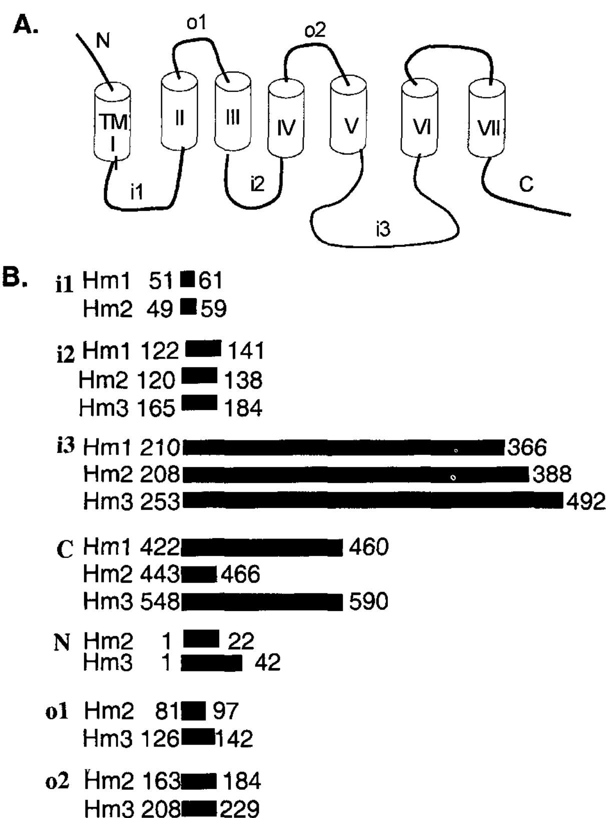

Fig. 1. Intra- and extra-cellular domains of Hm1, Hm2 and Hm3 receptors used in the yeast two-hybrid assay. C-terminal tail, each intracellular loop (i1, i2, i3), N-terminus, extracellular o1 or o2 loop of Hm1, Hm2 or Hm3 receptors were amplified by PCR. Residues numbered by beginning with the N-terminal methionine as a residue 1.

Screening for the intracellular and extracellular regions involved in the homomeric or heteromeric interactions between muscarinic receptor subtypes

Under protein-protein interaction, transformed yeast cells are grown on galactose-$Leu^-Ura^-His^-Trp^-$ media, and blue colonies on galactose-$Ura^-His^-Trp^-$ containing X-gal. Endogenous DNA-binding and transcriptional activating capabilities were examined before testing target protein interactions. EGY48/pSH18-34/pLexA/pB42AD was used as a negative control and EGY48/pSH 18-34/pLexA/α-loop of Na^+, K^+-ATPase/pB42AD/cofilin construct, which had already been confirmed for their interaction (Yoon and Lee, 1998) was used as a positive control (Table II, lanes I & II in Fig. 3). When most of the hybrid constructs were coexpressed

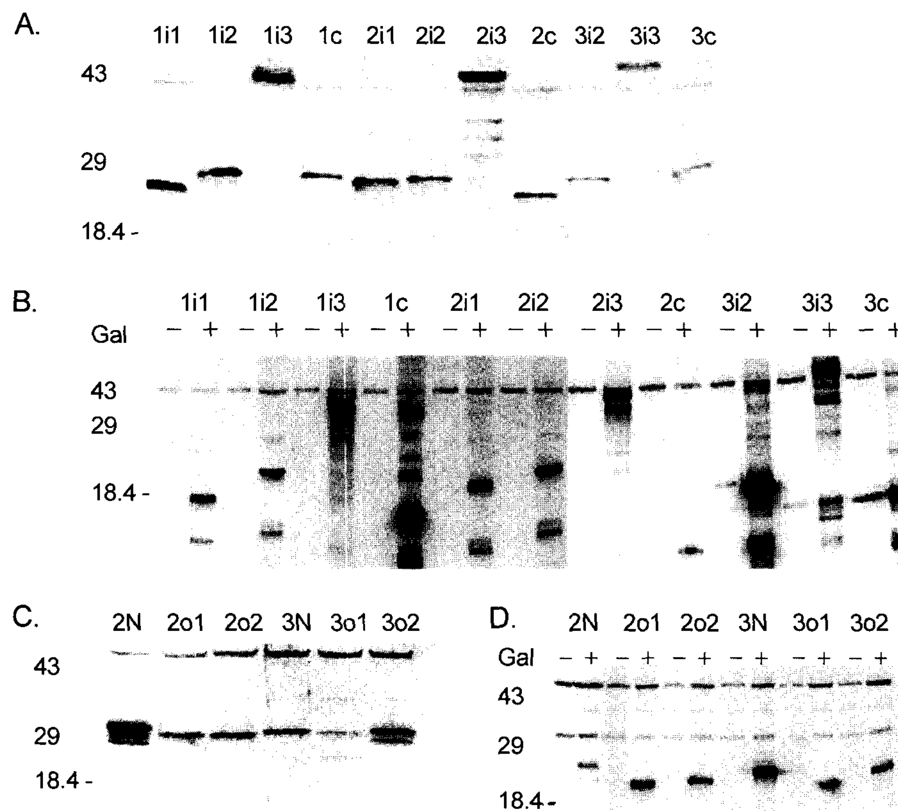

Fig. 2. Expression of fusion proteins confirmed by Immunoblot analyses. Yeast cells EGY48/pSH18-34 were transformed with indicated plasmids containing intracellular (A, B) and extracellular domains (C, D) of Hm1, Hm2 or Hm3 plasmids in glucose (glu; –) or galactose (gal; +) $Ura^{-}Trp^{-}His^{-}$ media. Protein lysates prepared from those yeast cells were SDS-polyacrylamide gel electrophoresed. The rabbit polyclonal LexA antibody was used for the detection of LexA binding domain-Hm domain fusion proteins (A, C) and anti-HA monoclonal antibody 12CA5 for the detection of B42 transcriptional activation domain-Hm domain fusion proteins (B, D). The western analyses confirmed the production of fusion protein in the correct reading frame with expected sizes.

Table II. Two hybrid assays for endogenous DNA-binding and activating capabilities

Constructs		Media			
DNA binding domain	Transcriptional activation domain	Glu U-H-T-L-	Gal U-H-T-L-	Glu U-H-T-X-gal	Gal U-H-T-X-gal
Control					
PLexA	pB42AD	–	–	White	White
pLexA/Na^{+} pump	pB42AD/cofilin	–	+	White	Blue
Intracellular domains					
PLexA	pB42AD/ M1i1,M1i2,M1i3,M1c,M2i1,M2i2,M2i3,M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/ M1i1,M1i2,M1i3,M1c,M2i1,M2i2,M2i3,M2c, M3i2,M3i3,M3c	pB42AD	–	–	White	White
Extracellular domains					
PLexA	pB42AD/M2N,M2o1,M2o2,M3N,M3o1,M3o2	–	–	White	White
pLexA/M2N	pB42AD	–	–	White	White
PLexA/M2o1,M2o2,M3N,M3o1,M3o2	pB42AD	+	+	Blue	Blue

Human muscarinic receptor (Hm) was designated as M.

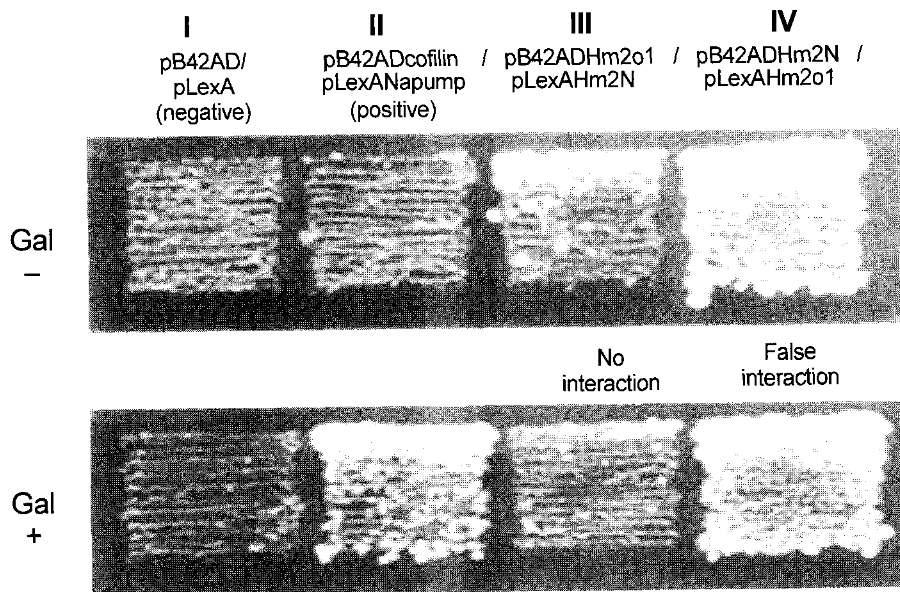

Fig. 3. Leu2 expression of EGY48/pSH18-34 yeast cells transformed with the indicated plasmids. The trasformants were streaked onto glucose-Ura-His-Trp-Leu- (Glu+) & galactose-Ura-His-Trp-Leu- (Gal+) plates to test for Leu2 gene expression. The gal-ependent growth of lane II (positive control) indicates a protein-protein interaction, whereas lanes I and III show no interactions. The nonspecific growth of lane IV might be due to the activation function of the Hm2o1 itself.

with corresponding empty vector, transcriptions of the reporter gene were not activated, which ensures no false binding or transcriptional activation activity of the tested domains themselves. The constructs pLexA/Hm2o1, Hm2o2, Hm3N, Hm3o1 and Hm3o2 were excluded from the two-hybrid assay system because each construct enhanced the growth of yeast cells on galactose-Leu$^-$Ura$^-$His$^-$Trp$^-$ media, indicating that these domains in themselves can activate transcription (Table II, lane IV in Fig. 3).

The constructs were cotransformed in pairs to search for the intracellular domains involved in heterotypic or homotypic interaction. As shown in Table III, no growth was observed on galactose-Leu$^-$Ura$^-$His$^-$Trp$^-$ medium in yeast cells expressing pairs of each intracellular loop region (i1, i2 or i3) or the C-terminal tail of Hm1, Hm2 and Hm3. Blue colonies on galactose-Ura$^-$His$^-$Trp$^-$ containing

Table II. Examination of homotypic or heterotypic interactions between intracellular domain of Hm1, Hm2 & Hm3 subtypes and between extracellular domain of Hm2 & Hm3 subtypes by using an yeast two-hybrid assay

Constructs		Media			
DNA binding domain	Transcriptional activation domain	Glu U-H-T-L-	Gal U-H-T-L-	Glu U-H-T-X-gal	Gal U-H-T-X-gal
Intracellular domains					
pLexA/M1i1	pB42AD/M1i1,M1i2,M1i3,M1c,M2i1,M2i2,M2i3,M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/M1i2	pB42AD/M1i2,M1i3,M1c,M2i1,M2i2,M2i3,M2c, M3i2,M3i3,M3c	–	–	White	White
pLexA/M1i3	pB42AD/M1i3,M1c,M2i1,M2i2,M2i3,M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/M1c	pB42AD/M1c,M2i1,M2i2,M2i3,M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/M2i1	pB42AD/M2i1,M2i2,M2i3, M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/M2i2	pB42AD/M2i2,M2i3,M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/M2i3	pB42AD/M2i3,M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/M2c	pB42AD/M2c,M3i2,M3i3,M3c	–	–	White	White
pLexA/M3i2	pB42AD/M3i2,M3i3,M3c	–	–	White	White
pLexA/M3i2	pB42AD/M3i3,M3c	–	–	White	White
Extracellular domains					
pLexA/M2N	pB42AD/M2N,M2o1,M2o2,M3N,M3o1,M3o2	–	–	White	White

X-gal medium from yeast cells expressing the intracellular domain combinations tested were also not detected, while blue colonies were grown from the positive control cells. These results indicate that intracellular domains of Hm1, Hm2 and Hm3 do not interact with any identical or non-identical intracellular domain of the muscarinic receptor subtypes to form dimeric or oligomeric arrays. To further test the involvement of the interactions between the extracellular regions in muscarinic receptor dimerization, the pLexA/Hm2N construct was examined for its interaction with pB42AD/Hm2N, Hm2o1, Hm2o2, Hm3N, Hm3o1 or Hm3o2. No growth of yeast cells expressing the extracellular domains of Hm2 and Hm3 was detected on galactose-$Leu^-Ura^-His^-Trp^-$ medium (Table III, lane III in Fig. 3), which indicates that the Hm2 N-terminal did not interact with any of extracellular domains of Hm2 or Hm3 muscarinic receptor. Growth of no blue colonies, but growth of white colonies was observed, which ensures no interactions between the extracellular domains of muscarinic receptor subtypes tested.

DISCUSSION

GPCR-protein assemblies are regarded as dynamic complexes that involve interactions, such as those between, receptors, receptors and G protein, and receptors and other proteins, all of which contribute to the finely tuned processes of downstream cellular signaling. A number of GPCRs contain a sequence motif that is known to direct protein-protein interactions, and therefore, these species have the theoretical capacity to interact with a wide range of the proteins. Using experimental approaches, such as yeast two-hybrid analysis, a considerable number of such interactions have been reported. Yeast two-hybrid assay is a technique well suited to the monitoring of the interactions that occur between protein motifs defined by relatively short linear peptide sequences within proteins. The technique is not appropriate for the analysis of full-length polypeptides, such as GPCRs, which contain transmembrane helices. This is because the technique requires the transcriptional regulation that is induced in the nucleus of yeast by such protein-protein interactions. However, internal regions of GPCRs, such as the third intracellular (i3) loop and the C-terminal tail, can be isolated and used as 'bait' in yeast two-hybrid protein interaction screening. Moreover, there is evidence that external regions of some GPCRs might be amenable to yeast two-hybrid screening for interacting proteins, especially the N-terminal tail (Milligan and White, 2001). In terms of the intracellular portion of GPCRs, both the C-terminal tail and the i3 loop have been focused upon during examination of protein-protein interactions owing to their appropriate size. Recently, the formation of $GABA_BR1$/ $GABA_BR2$ receptor heterodimers was shown to involve the short domain in the C-terminal cytoplasmic tail, by using a yeast two-hybrid system (Jones *et al.*, 1998; Kapupman *et al.*, 1998; Kuner *et al.*, 1999). The present study also tested the possibility of the involvement of the C-terminal tail region in the dimerization/oligomerization of muscarinic receptors. However, direct interactions between C-terminal regions of the muscarinic receptor subtypes, Hm1, Hm2 and Hm3, were not observed.

Several studies have reported that i3 loop of GPCRs could also be involved in direct protein-protein interactions between signaling molecules or cytoskeletal anchoring polypeptides (Li, 2000). However, no report is available upon the protein-protein interactions between receptors through i3 and other intracellular loops. In the present study, we examined whether intracellular domains are involved in the dimerization of muscarinic receptors. Using yeast two-hybrid assays, no detectable interaction between the i3 loop of muscarinic receptors or of any other intracellular hydrophilic domain was observed. These results indicate that muscarinic receptors do not interact directly through intracellular loops. Our results may support the idea that the domain swapped dimer Goudlson's hypothesis (Gouldson *et al.*, 1998, 2000) or contact dimer between the interfaces of hydrophobic helices is likely to be responsible for interactions of muscarinic receptors. Gouldson *et al.* (1998, 2000) proposed domain swapped dimer models for multi-domain proteins, and they hypothesized that some TM helices of GPCR constitute the first domain while the rest of helices constitute the second domain in monomer and these two domains are connected by hinge loops. If two monomers merely touch each other, a contact dimer can form, but if the hinge loop opens out, the two domains can exchange to form a domain swapped dimer. Domain swapped dimers are less common than contact dimers, but have the major advantage that interactions between the domains, already present in the monomers, can be reused to reform the dimers, thus domain swapping is an efficient way of forming dimerization interfaces. The length of the hinge loop is important in this process for GPCRs and Gouldson et al. noted that the i3 loop, the hinge loop connecting two domains, is frequently the longest loop in GPCRs. Moreover, Maggio *et al.* (1996) strengthened this hypothesis, by showing that shortening the i3 hinge loop resulted in the loss of the receptor's ability to form dimers. They initially created short chimeric α2 adrenergic/m3 muscarinic receptors, in which 196 amino acids were deleted from the i3 loop (α2/m3-short and m3/α2-short). Although cotransfection with α2/m3 and m3/α2 resulted in specific binding, the coexpression of the two short constructs (α2/m3-short and m3/α2-short) together or in combination with α2/m3 and m3/α2, respectively, did not result in any

detectable binding activity. Based on our results, which suggest no direct protein-protein interaction between the i3, loops of identical or non-identical muscarinic receptor subtypes, the role of the i3 loop in the dimerization of muscarinic receptor could also be explained in terms of a domain swapping model.

How many types and what the physiological roles of dimerization/oligomerization are among the muscarinic receptor subtypes are not fully understood. However, it can be speculated that heteromeric interaction between receptor subtypes as well as homodimeric interactions between identical subtypes could be formed and these may have distinct functions. For example, interaction between different receptor subtypes could stimulate different G proteins. When wild type m2 or m3 muscarinic receptors were expressed together with gene fragments originating from m3 or m2 receptors, respectively, antagonist binding showed the presence of two populations of binding sites; one representing wild-type m2 or m3 receptors, the other the heterodimeric m2/m3 receptor (Maggio *et al*., 1993b). These findings demonstrated that m2 and m3 muscarinic receptor subtypes coupled to Gq/11 and Gi/o proteins, respectively, can interact with each other and form a new pharmacological heterodimeric receptor. In support of this view, binding studies and gel electrophoresis experiments using purified m2 muscarinic receptor from porcine atria demonstrated a cooperative interaction. (Wreggett and Wells, 1995). However, such data does not provide direct evidence as to whether m2/m3 heterodimerization may be accomplished through direct protein-protein interaction. Various attempts to reveal both the existence of dimerization and the mechanism of interaction are required. The muscarinic receptor sets tested using yeast two-hybrid assays in the present study failed to show any physical interaction between the intracellular loops of m2 and m3 muscarinic receptors. The N-terminal of m2 also showed no direct interaction with any extracellular domain of m2 and m3. A study by Zeng and Wess (1999) suggested that the formation of disulfide-linked m3 muscarinic receptor dimer appeared to be receptor subtype-specific because coimmunoprecipitation revealed that the m3 receptor is unlikely to interact with m1 or m2. However, different types of heterodimerization of muscarinic receptors may exist, and this issue has not been fully elucidated. Interactions between closely related receptor members with similar second messenger systems, such as m1 and m3 receptors, both coupled to Gq/11 proteins and between related members with different second messenger systems, such as m1 and m2 receptors, coupled to Gq/11 and Gi/o proteins, respectively, are possible. Dimerization between muscarinic receptors and distantly related GPCR members may also exist. The functional implications resulting from these different types of dimerizations would present an interesting study. Results from the present study using a yeast two-hybrid system indicate that homodimerization and heterodimerization among the three muscarinic receptor subtypes do not occur, at least, via direct physical interaction.

In conclusion, our results exclude the possibility of direct interaction between the three muscarinic receptor subtypes, Hm1, Hm2 and Hm3, as a mechanism for homomeric or heteromeric dimerization/oligomerization. These results raise the possibility that the interactions required to dimerize muscarinic receptors might occur indirectly or that they require conformation adjustment, subunit formation or hydrophobic region involvement. Other mechanisms of muscarinic receptor dimerization and the role of transmembrane regions in dimerization and of their functions are items that require further study.

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