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Identification of a small intracellular region of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover

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Molecular cloning studies have demonstrated the existence of five different muscarinic receptors (m1–m5). While m1, m3 and m5 strongly couple to stimulation of phosphoinositide (PI) hydrolysis, m2 and m4 are more efficiently linked to inhibition of adenylate cyclase. The sequences of m1–m5 have a short segment at the N-terminal portion of the putative third cytoplasmic loop (i3) which is highly conserved among m1, m3 and m5, but different from the sequence which is well conserved among m2 and m4. To study the role of this region in conferring coupling selectivity, we constructed cDNAs encoding chimeric m2/m3 receptors. Transient expression of these receptor hybrids in COS-7 cells showed that a 17 amino acid segment at the N-terminal portion of i3 is a major determinant of how efficiently the different muscarinic receptors are coupled to PI hydrolysis.

Muscarinic receptor subtype; Chimeric receptor; Phosphoinositide hydrolysis; COS-7 cell; Effector coupling; cDNA expression

1. INTRODUCTION

Molecular cloning studies have established the existence of five molecularly distinct muscarinic receptors (m1–m5) [1–4]. Accumulating evidence suggests that the individual receptor subtypes have the capacity to preferentially activate or inhibit specific cellular signal transduction pathways [5–11]. Expression studies in mammalian cells have shown that m1, m3 and m5 are efficiently coupled to phosphoinositide (PI) breakdown [6,7,10,11] but do not inhibit adenylate cyclase [11]. In contrast, m2 and m4 are not or only poorly linked to PI hydrolysis but strongly inhibit adenylate cyclase activity [11].

A recent electrophysiological study on m1/m2 receptor chimeras suggests that the putative third cytoplasmic loop (i3; fig.2) plays a crucial role in the selective coupling of muscarinic receptors to their intracellular effector systems [13]. Sequence analysis reveals that a 16–17 amino acid segment at the N-terminal portion of i3 is highly conserved among m1, m3 and m5, but different from the sequence which is well conserved in m2 and m4 (fig.1). In order to study the potential role of this domain in conferring coupling selectivity, we constructed cDNAs encoding chimeric m2/m3 receptors in which either this small fragment or the whole i3 loop have been exchanged between the human m2 and the rat m3 receptor (fig.2). The ability of these receptor chimeras to stimulate PI hydrolysis was studied in transfected COS-7 cells transiently expressing the clonal receptors.

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2. MATERIALS AND METHODS

2.1. *Constructs*

Two restriction sites were introduced into the human m2 expression clone described in [3] to facilitate the creation of chimeric receptors. An Nhe I site homologous to the one in transmembrane region VI of the rat m3 receptor [3] was introduced by oligonucleotide-directed mutagenesis [14] without changing the amino acid sequence. A Hpa I site was created at amino acids 225–226 in the i3 region of m2 which changed amino acid 226 from Ala to Asn. These sites were used in conjunction with a BspH I site found in transmembrane region V of both m2 (amino acids 201–203) and m3 (amino acids 246–248) and a Stu I site at amino acids 268–269 in the i3 segment of m3. To change the whole i3 domain (and causing a Ile–Val change in transmembrane

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          --V---          i3

          **  ** **o o*o *o*o o oo     * *
R=H m4    IMTVLYIHISLASRSRVHKHRPEGPKEKKAKTLAFLK
  R m2    IMTVLYWHISRASKSRIKKEKKEPVANQDPVSPSLVQ
  H m2    IMTVLYWHISRASKSRIKKDKKEPVANQDPVSPSLVQ

R=H m3    IMTILYWRIYKETEKRTKELAGLQASGTEAEAENFVH
  R m5    VMTILYCRIYRETEKRTKDLADLQGSDSVAEAKKREP
  H m5    VMTILYCRIYRETEKRTKDLADLQGSDSVTKAEKRKP
R=H m1    VMCTLYWRIYRETENRARELAALQGSETPGKGGGSSS
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Fig.1. Comparison of the primary sequences of m1–m5 (H = human, R = rat) at the amino terminus of the third cytoplasmic loop (i3). V indicates the C-terminal portion of the putative fifth transmembrane spanning segment. Asterisks represent positions at which all muscarinic receptors are either identical or only conservative substitutions occur. Circles indicate residues which are conserved only within the two functional classes of receptors (even vs odd). The underlined sequences have been exchanged between the human m2 and the rat m3 receptor.

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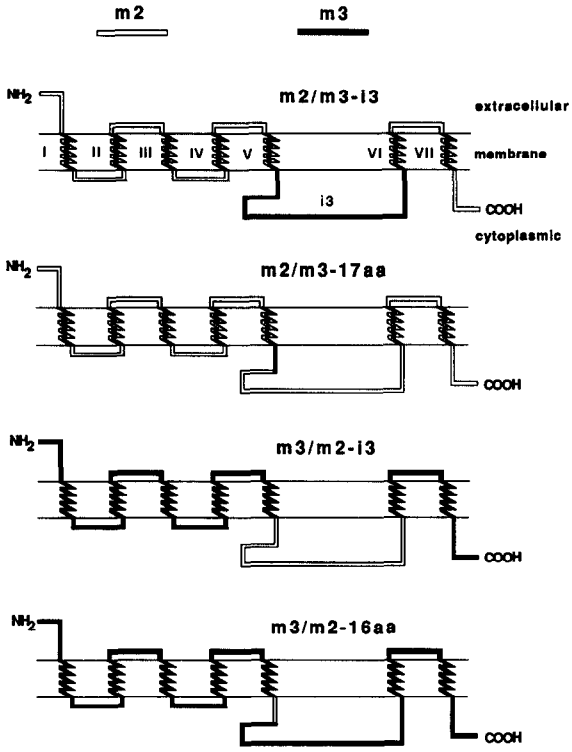

Fig.2. Structure of m2/m3 receptor chimeras. The putative transmembrane segments are numbered I–VII from the NH_2-terminus (extracellular) to the COOH-terminus (intracellular). The putative third cytoplasmic loop is named i3. Portions shown in black represent m3 sequences, while open lines indicate m2 sequences.

region V), the BspH I–Nhe I fragments from the two receptors were exchanged. To change the first 16–17 amino acids of the i3 region, double-stranded oligodeoxyribonucleotides of the appropriate sequence were prepared with an automatic DNA synthesizer (Applied Biosystems) to substitute for the BspH I–Hpa I fragment of m2 or the BspH I–Stu I fragment of m3. The identity of the constructs was confirmed by sequencing [15] the regions derived from the synthetic oligodeoxyribonucleotides and by restriction endonuclease analysis.

2.2. *Transfections and binding assays*

COS-7 cells (~70% confluent) were transfected in 10 cm plates with 20 μg plasmid DNA by calcium phosphate precipitation [16]. The plasmid DNAs used for transfections consisted of the muscarinic constructs inserted into the Okayama/Berg pCDI expression vector [17] carrying the SV 40 promoter. Media (DMEM) were changed 24 h after transfection, and cell membranes were obtained 48 h later as described previously [18]. Binding buffer consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM $MgCl_2$. For direct binding assays, 6–8 different concentrations (0.025–3.2 nM) of N-[^{3}H]methylscopolamine ([^{3}H]NMS; 85 Ci/mmol, NEN) were used. In the carbachol displacement experiments, 10 different carbachol concentrations (0.05–1000 μM) were employed. The [^{3}H]NMS concentration used in these experiments was 0.8 nM (m2, m2/m3-i3, m2/m3-17aa) or 0.2 nM (m3), respectively. Nonspecific binding was defined by 1 μM atropine. Incubations were carried out at room temperature for 2 h. Binding data were analyzed by a nonlinear least-squares curve-fitting procedure as detailed in [18] using the program DATAPLOT run on a VAX II computer.

Protein concentrations were determined according to the method of Bradford [19] using a Bio-Rad protein assay kit.

2.3. *PI assay*

COS-7 cells were incubated in 10 cm plates with 3 μCi/ml [^{3}H]*myo*-inositol (ARC; 23 Ci/mmol) for 48 h starting 24 h after transfection. The cells were then washed twice with phosphate-buffered saline (PBS) and lifted from the dish bottoms with 2 ml of divalent cation-free DMEM containing 1 mM EDTA. After centrifugation, cells were resuspended in 18 ml of DMEM containing 25 mM Hepes and 10 mM LiCl and incubated at room temperature for 10 min. Aliquots (1 ml) of the cell suspension were then put into 13 × 100 mm glass tubes. After addition of various concentrations of carbachol (0.1–1000 μM), cells were incubated for 1 h at 37°C. Cells were then collected by centrifugation and incubated on ice with 1 ml of 10% trichloroacetic acid (TCA) for 30 min. The TCA was extracted with diethylether (4 × 4 ml) and levels of IP_1 were determined using anion exchange chromatography [20] and a LKB liquid scintillation counter.

3. RESULTS

3.1. *Binding studies*

While [^{3}H]NMS showed no detectable binding to non-transfected COS-7 cells, specific [^{3}H]NMS binding could be demonstrated after transfection with the various chimeric receptor genes. m2/m3-i3 and m2/m3-17aa bound [^{3}H]NMS with affinities similar to that of m2 (K_D ~ 0.8 nM), whereas m3, m3/m2-i3 and m3/m2-16aa displayed an about 3–5 fold higher affinity (K_D ~ 0.2 nM) (table 1).

The B_{max} values obtained for m3/m2-i3 and m3/m2-16aa were considerably lower than those determined for the other receptors (table 1). Thus, the relative inability of these two receptor chimeras to effi-

Table 1

Functional expression of chimeric m2/m3 receptors in COS-7 cells

	PI assay		[^{3}H]NMS binding		Carbachol binding	
	Maximum increase in IP_1 levels (%)	EC_{50} (μM)	K_D[^{3}H]NMS (nM)	B_{max} (fmol/mg protein)	IC_{50} (μM)	^{n}H (Hill coefficient)
m2	41 ± 6	17.0 ± 7.5	0.79 ± 0.11	1549 ± 118	6.1 ± 1.2	0.89 ± 0.11
m2/m3-i3	233 ± 43	0.55 ± 0.18	0.79 ± 0.13	2651 ± 114	45.9 ± 7.9	0.67 ± 0.09
m2/m3-17aa	137 ± 23	5.1 ± 0.6	0.80 ± 0.21	1643 ± 324	4.1 ± 2.2	0.75 ± 0.03
m3	141 ± 24	0.29 ± 0.11	0.17 ± 0.03	739 ± 114	41.8 ± 4.2	0.71 ± 0.05
m3/m2-i3	38 ± 12	*	0.25 ± 0.13	135 ± 60	n.d.	n.d.
m3/m2-16aa	24 ± 6	*	0.25 ± 0.14	171 ± 91	n.d.	n.d.

The values represent means of 2-4 independent experiments each performed in duplicate. In case of [^{3}H]NMS binding, Hill coefficients were not significantly different from unity; n.d., not determined

*not determinable with sufficient accuracy

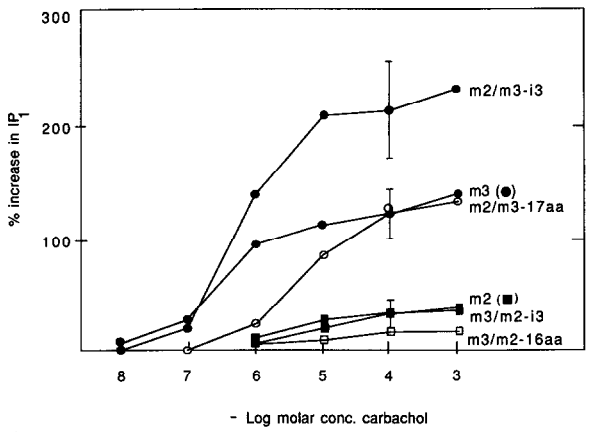

Fig.3. Carbachol-induced increases in IP_1 levels in transfected COS-7 cells transiently epressing m2, m3 and m2/m3 receptor hybrids. The values represent means of 3–4 independent experiments each performed in duplicate. Typical standard errors are shown for a carbachol concentration of 100 μM.

ciently stimulate PI hydrolysis (see below) cannot be ascribed with certainty to their primary structures.

Carbachol displacement experiments were performed with those receptors which were expressed at high levels in COS-7 cells (m2, m3, m2/m3-i3 and m2/m3-17aa). Carbachol inhibited [^{3}H]NMS binding to m2/m3-17aa with a potency similar to that of m2, while m2/m3-i3 gave an about 8-fold greater IC_{50} value (table 1).

3.2. *PI assay*

The ability of the chimeric receptors to stimulated PI hydrolysis was determined by studying carbachol-induced increases in inositol monophosphate (IP_1) levels in transiently transfected COS-7 cells. In non-transfected cells, carbachol treatment had no detectable effect on IP_1 levels. As shown in fig.3, carbachol stimulation resulted in dose-dependent increases in IP_1 levels in native m2 and m3 as well as in all hybrid receptors studied. These effects could be completely blocked by 10 μM of atropine.

Large quantitative differences in the ability of the individual receptors to stimulate PI hydrolysis were observed. The maximum increases in IP_1 levels were 3–10 times higher for m3, m2/m3-i3 and m2/m3-17aa than for m2, m3/m2-i3 and m3/m2-16aa (fig.3, table 1). The carbochol EC_{50} values were considerably lower for m3 and m2/m3-i3 than for the other receptors (fig.3, table 1). While m2/m3-i3 gave a carbachol EC_{50} value comparable to that of m3, this value was about 10–20 fold higher for m2/m3-17aa (table 1).

4. DISCUSSION

In this study cDNAs encoding chimeric m2/m3 receptors were constructed and transiently expressed in COS-7 cells in order to identify intracellular domains responsible for the selective coupling of the m3 receptor to PI hydrolysis [7,10,11].

In accordance with previous binding studies [18], [^{3}H]NMS showed a 4–5 fold higher affinity to m3 than to m2. This difference in binding affinities remained essentially unaltered when the i3 region or parts of it were exchanged between m2 and m3 (table 2). This finding corroborates the view that the i3 domain is not involved in the selective binding of muscarinic antagonists [13].

As previously described [7,10,11,12], we observed that m3 mediates a strong stimulation of PI metabolism while m2 is only poorly linked to this effector system (fig.3, table 1). Replacement of the i3 domain of m2 by the corresponding m3 sequence led to a receptor chimera (m2/m3-i3) which, similar to m3, induced a strong PI response with an ED_{50} also comparable to that of m3 (fig.3, table 1). The fact that the maximum response generated by m2/m3-i3 was even higher than that of native m3 may be due to its very high levels of expression (table 1). This finding indicates that the i3 segment of the muscarinic receptors plays a pivotal role in determining how efficiently the different muscarinic receptors are linked to PI hydrolysis.

Our data are consistent with a recent study on m1/m2 receptor chimeras expressed in *Xenopus* oocytes showing that the i3 segment is responsible for the selective coupling of m1 and m2 to different electrophysiological responses [13]. Since it has been shown that inositol 1,4,5-triphosphate (IP_3), a major product of PI hydrolysis, mimics the current responses induced by m1 and m3 [21], it becomes clear that the i3 domain of these receptors specifies both the biochemical and the electrophysiological responses. Altogether, these data, in conjunction with a recent biochemical investigation of $\alpha 2/\beta 2$ receptor hybrids [22], suggest that the i3 domain may generally determine the coupling selectivity of G protein-linked receptors.

Our result also indicate that a short stretch of 16–17 amino acids at the N-terminal portion of the i3 loop is a major determinant of how efficiently the individual muscarinic receptors are coupled to PI breakdown. Introduction of this small segment from m3 into m2 yielded a receptor hybrid (m2/m3-17aa) which mediated a pronounced increase in IP_1 levels comparable in magnitude to that of m3 (table 1). However, m2/m3-17aa displayed an about 20-fold higher EC_{50} for carbachol than m3 in inducing this response (table 1). Since the binding data (table 1) suggest that this reduction in potency is not due to a decrease in carbachol affinity, it appears that other yet unidentified domains of the i3 loop (most likely the C-terminal portion) are involved in conferring full coupling efficiency. This view is in agreement with the finding that deletion mutants of the $\beta 2$ receptor which lacked short amino or carboxy terminal sequences of the i3 domain were no longer able to mediate a stimulation of adenylate cyclase [23].

To provide a more thorough examination of the mechanisms controlling second messenger selectivity of the different muscarinic receptors, we are presently establishing cell lines stably expressing the chimeric receptors described here as well as creating chimeras involving the 20 amino acids at the C-terminus of i3. The stable cell lines should allow us to better control receptor numbers and to determine if there are reciprocal effects in coupling to inhibition of adenylate cyclase.

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