Internalization of the Hm1 muscarinic cholinergic receptor involves the third cytoplasmic loop

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The m1 muscarinic receptor was previously shown to stimulate phosphatidyl inositol (PI) turnover and to internalize rapidly upon agonist activation. Three receptor mutants with large deletions of the third cytoplasmic loop (i3) of human Hm1, leaving only 11 and 8 amino acids at the amino and carboxy terminal junctions of i3, respectively, retained full ability to stimulate PI turnover, when expressed in U293 cells, but receptor internalization was greatly reduced in two mutants with deletions reaching close to the NH_2 terminal of i3. We propose that a receptor domain located toward the amino terminal junction of i3 plays a role in Hm1 internalization.

Muscarinic cholinergic receptor Hm1; Deletion mutant; Receptor internalization; Phosphatidyl inositol turnover; Carbachol

1. INTRODUCTION

The muscarinic cholinergic receptors belong to the family of G protein coupled receptors and contain a large third intracellular loop (i3) [1,2]. The i3 loops of m1 and m2 direct the specificity of G protein coupling along different pathways [3], and preliminary results indicate that the i3 junctions adjacent to transmembrane domains 5 and 6 (TMD 5 and 6) are responsible for G protein coupling [4], as previously demonstrated for the adrenergic receptors (e.g. [5]). However, any other functions of the muscarinic i3 loop remained to be studied. Cholinergic agonists cause rapid internalization of m1 and m3 [6-8], which are coupled to phosphatidyl inositol (PI) turnover [1,2]. Recently, Shapiro and Nathanson [9] have demonstrated that 75% of the i3 loop of the mouse m1 receptor can be deleted without affecting coupling to PI turnover and rapid receptor internalization, although the slower m1 receptor downregulation was defective. Because of the large size of the i3 loop (208-366) the remaining 25% of i3 may contain important receptor functions. In the present study, we have constructed mutants of the human m1 (Hm1) receptor with large deletions in the i3 loop and tested their ability to stimulate PI turnover and to internalize upon agonist activation.

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2. MATERIALS AND METHODS

2.1. *Construction of vectors expressing Hm1 and deletion mutants*

The gene encoding for Hm1 was obtained from a human placental genomic DNA library in vector EMBL3 (from Clontech Labs, Palo Alto, CA), using a specific oligonucleotide probe, followed by amplification of the coding sequence with the polymerase chain reaction [10] using two oligonucleotide primers immediately adjacent to the start and stop codons of the Hm1 gene. The amplified gene product was then cloned into pGem™ – 3 (Promega, Madison, WI), using the Sma-I restriction site, and subsequently transferred into the mammalian expression vector pSG5 (Stratagene, La Jolla, CA). Restriction fragments were subcloned into m13 and sequenced using the Applied Biosciences Automatic Sequencer. No deviations of the obtained sequence from the published sequence were observed within the coding frame which does not contain any introns [1,2]. Deletions of the i3 domain were introduced, after cutting the Gem-3/Hm1 vector with the unique Stu-I restriction site at position 812 (at codon for R-271), by digestion with the exonuclease Bal-31 for varying times at 4°C. The blunt-ended product was then self-ligated. Mutants with deletions roughly equivalent in size to the i3 loop (from Y-208 to T-366) were selected, transferred to pSG5, using *Bam* HI and *Eco* RI, and expressed in human embryonic kidney U293 cells with the calcium phosphate precipitation method [2]. Three clones which gave the desired deletion size and yielded productive muscarinic tracer binding upon transfection, were sequenced and termed d219-333, d220-349 and d232-358, to indicate the deleted amino acid sequences. None of the three mutants contained a new amino acid at the ligation junctions.

2.2. *Transfection, receptor binding, and PI turnover*

HM1 and the deletion mutants, placed in pSG5, were transfected (6 μg DNA), using the calcium phosphate precipitation method, into U293 [2] cells plated out in 10 mm plastic Petri dishes. After 2 days incubation, muscarinic receptor density rose from a background of <30 fmol/mg protein to over 1000 fmol/mg protein. Cells were then brought into suspension with PBS/EDTA, washed, and replated into

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6 well tissue culture plates. After 2 h at 37°C, the cells had reattached and receptor binding assays were then performed with either cell homogenates (to determine [^{3}H]NMS saturation or carbachol displacement curves ± GppNHp) or with the intact cells according to the method of Liles et al. [6]. In order to assess receptor internalization, the intact cells were pretreated with 1 mM carbachol or 100 nM phorbol 12-myristate 13-acetate (PMA)/300 nM A23187 [6], for either 30 min or 2 h immediately prior to the receptor assay. For ligand displacement studies, 0.1 nM N [^{3}H]methylscopolamine ([^{3}H]NMS) was used, whereas for the internalization assay, 1.0 nM [^{3}H]NMS was used. The higher tracer concentration produces a large degree of receptor saturation, and the total tracer binding was taken to approximate receptor density for the wild-type Hm1 receptor. Phosphatidyl inositol (PI) turnover was measured by the method of Berridge et al. [11] after labeling of the cells with ^{3}H-inositol for 24 h.

3. RESULTS AND DISCUSSION

Expression of the cloned Hm1 gene, using pSG5, gave abundant ^{3}H-NMS binding in transiently transfected U293 cells (3042 ± 413 fmol/mg protein ($n = 10$) over a background of 9–15 fmol/mg protein in nontransfected cells. The K_d for NMS of 0.14 nM is consistent with previous results [6]. Further, carbachol displaced [^{3}H]NMS with an IC_{50} of 0.4 mM in membrane homogenates, and its affinity was not affected by the presence of 100 μM GppNHp. Lack of guanyl nucleotide effects on carbachol binding to m1 is also consistent with earlier reports [9,12], and it is not indicative of failure to couple to G proteins. The EC_{50} value for carbachol in stimulating PI turnover was approximately 20 μM, and 1 mM carbachol produced maximum effect. Using lower amounts of plasmid DNA for transfection to vary m1 receptor expression in U293 cells, it was found that 1 mM carbachol produced maximum stimulation when receptor density exceeded 300–500 fmol per mg protein. Among a large number of Hm1 deletion mutants, three were selected that gave comparable [^{3}H]NMS binding upon transfection of U293 cells (bound after incubation with 1 nM [^{3}H]NMS in fmol/mg protein: 1412 ± 251 (d219–333), 1783 ± 442 (d220–349), and 2385 ± 484 (d232–350), $n = 10$). [^{3}H]NMS equilibrium binding curves, performed with cell membranes transfected with d220–d349 and d232–358 gave the following respective values: IC_{50} 0.2 mM and 1.8 mM for carbachol and K_d 3.5 nM and 2.1 nM for NMS. Hence, both mutants displayed a 10 to 20-fold lower affinity for the antagonist NMS, while the carbachol affinity of d232–358 was 5-fold lower and that of d220–349 2-fold higher than in the wild-type. These results indicate some limited changes in overall receptor structure caused by the large deletions. Further, because of the lower affinity of NMS, the total number of expressed binding sites, estimated on the basis of the 1 nM ^{3}H-NMS incubations, is approximately 2 to 3-fold higher for d220–349 and d232–358 than for the wild-type receptor. Smaller deletions involving amino acids 221 to 343 [9] of the mouse m1 receptor were previously shown not to affect agonist or antagonist receptor binding. A carbachol dose response curve for d232–350 (PI turnover) gave an EC_{50} value of approximately 50 μM, a 2.5-fold shift from the wild-type (~ 20 μM), but 1 mM carbachol still yielded maximum stimulation of PI turnover. On the basis of this result, we compared the ability of m1 and the three deletion mutants to increase PI turnover when stimulated with 1 mM carbachol. Although the deletions range from positions 219 to 358, and thus, span most of the i3 loop (208 to 366), all three mutants were as effective as the undeleted Hm1 in stimulating PI turnover (Table I). This result limits the regions of i3 that can be involved with coupling to G proteins to 11 and 8 amino acids at the amino and carboxy terminal junctions of i3, respectively, whereas the large remainder of i3 does not appear to affect strongly the coupling process.

Table I

Stimulation of PI turnover by 1 mM carbachol.

	% of control	SE ($n = 3$–4)
U293 native	247	34
Hm1	1831	241
d219–333	2555	708
d220–349	1835	340
d232–358	2214	491

Cells were pretreated with [^{3}H]inositol and IP, IP_2 and IP_3 release was measured as percent of control (no carbachol). The percent increases for IP, IP_2 and IP_3 were averaged. Receptor density was measured simultaneously in one experiment and was similar in Hm1 undeleted and mutant transfected cells. Baseline release levels of IP, IP_2 and IP_3 averaged 10,514 ± 3,404, 520 ± 323 and 437 ± 201 dpm, respectively ($n = 22$).

Upon pretreatment of the transfected cells with carbachol, rapid internalization was observed with the quarternary tracer [^{3}H]NMS which does not penetrate the intact cells [6] (Fig. 1). The process was complete at 30 min pretreatment and did not continue beyond 55%

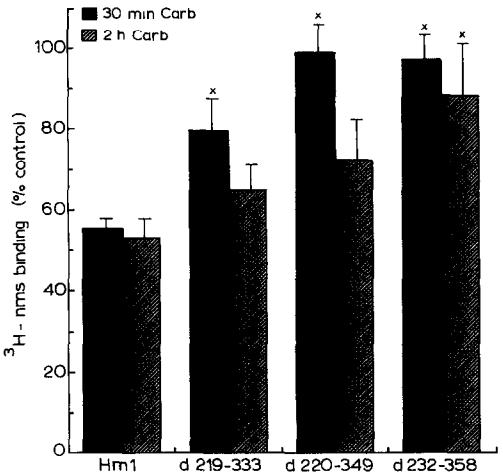

Fig. 1. Loss of surface ^{3}H-NMS binding sites upon preincubation of transfected U293 cells with 1 mM carbachol (A). *Significantly different when compared to preincubated wild-type Hm1, $P < 0.05$. Student t-test ($n = 4$), except for the value for d219–333 at 30 min, where $n = 3$).

internalization at 2 h. In contrast, no measurable internalization occurred with mutant d232–358 which contains the deletion closest to the carboxy terminal junction of i3. Mutant d220–349 measurably internalized only after 2 h carbachol pretreatment, whereas d219–333 showed some internalization already at 30 min and was not significantly different from the wild-type at 2 h. These results implicate the region from R334 to V358 of Hm1 as playing a role in receptor internalization. Since rapid internalization of a mouse m1 mutant with deletion to position 343 was also unaffected [9], this region may be further narrowed to R344 to V358, containing two potential phosphorylation consensus sites (T384 and S356). However, the large deletions of d220–349 and d232–358 had a small but measurable effect on overall receptor structure, and more of these mutants receptors were expressed at the cell surface than for the wild-type receptor. While the latter result is consistent with an impaired receptor internalization pathway, point mutations are now required to pinpoint the receptor domain responsible for rapid internalization upon agonist exposure.

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