THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 19, Issue of July 5, pp. 13406-13412, 1992
Printed in U.S.A.

# Hm1 Muscarinic Cholinergic Receptor Internalization Requires a Domain in the Third Cytoplasmic Loop*

(Received for publication, December 9, 1991)

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**Selected regions of the Hm1 muscarinic cholinergic receptor were mutated to analyze the molecular mechanisms of agonist-induced receptor internalization (or sequestration). The wild-type and mutant Hm1 genes were expressed, using pSG5, in U293 human kidney cells. Whereas surface receptor density measured with the polar tracer *N*-[$^3$H]methylscopolamine was rapidly reduced by carbachol exposure, total receptor content measured with [$^3$H]quinuclidinyl benzilate did not decline for at least 24 h, indicating the absence of extensive receptor down-regulation in U293 cells. Carbachol stimulation of phosphatidylinositol turnover paralleled receptor internalization, both with $EC_{50}$ values of 10–20 μM. Furthermore, a D71N point mutation that prevented receptor activation also abolished carbachol-induced receptor internalization, indicating that receptor activation (but not necessarily second messenger stimulation) was required for internalization. Truncation of the COOH-terminal tail (K447 trunc) and point mutations of several potential Ser and Thr phosphorylation sites to Ala failed to affect receptor activation and internalization. In contrast, partial deletions of the third intracellular loop (i3) ($Tyr^{208}$–$Thr^{366}$) resulted in receptor mutants deficient in agonist-induced receptor internalization/sequestration. Various deletions caused either complete loss of internalization (d 232–358) or impaired internalization, ranging from 10 to 30% over 2 h, whereas wild-type Hm1 internalized to ~50%. Whereas the reason for the observed differences among the deficient deletion mutants remains unclear, the initial rate of *N*-[$^3$H]methylscopolamine binding loss from the cell surface was much slower than that of wild-type Hm1 in each case. The deletion of only one single domain, 284–292 (SMESLTSSE), in the middle of i3 was consistently associated with impaired internalization. Domain 284–292 is partially conserved among closely related muscarinic receptors, whereas most of the remainder of i3 is not (except for the i3 membrane junctions), and similar Ser- and Thr-rich regions are present in many other G protein-coupled receptors. We propose that a small receptor domain in the middle of the i3 loop of Hm1 is involved in agonist-induced receptor internalization.**

The activity of membrane-bound receptors is regulated by several processes, including desensitization, internalization/sequestration, recycling, and down-regulation. Receptor-mediated endocytosis, as a major pathway of internalization, involves the formation of coated pits and interactions among receptors, adaptins, and clathrin (1). Molecular domains required for internalization have been defined for growth factor receptors (1–3), the transferrin receptor (4, 5), and the low density lipoprotein receptor (6). Whereas a universal consensus sequence could not be derived, the results suggested that intracellular domains near the transmembrane helices containing an aromatic residue (especially tyrosine) are required for internalization (1–6). Furthermore, internalization pathways can be distinguished by their requirement for receptor phosphorylation (5). Internalized receptor may either recycle to the cell surface or proceed to a lysosomal compartment for destruction (down-regulation). Different molecular domains are thought to cause receptor internalization and down-regulation (1), whereas little is known about the requirements for receptor recycling.

Most members of the G protein-coupled receptor family also undergo internalization/sequestration, recycling, and down-regulation; however, the molecular mechanisms of these processes remain largely unexplored, and no receptor domains required for cellular trafficking had been identified as yet. Valiquette *et al.* (7) prepared mutants of the human $\beta_2$-adrenergic receptor by replacing $Tyr^{350}$ and $Tyr^{354}$ with alanine, but these point mutations failed to alter the rapid agonist-induced internalization process, whereas the subsequent step of down-regulation was reduced. Receptor internalization is tightly associated with receptor activation; however, the role of each of the consecutive activation steps is not understood, *e.g.* at the level of the receptor, the G protein, and the second messenger. On the basis of deletion analyses, Cheung *et al.* (8) postulated that the receptor regions of the $\beta_2$-receptor involved in functional coupling to $G_s$ are also required for agonist-promoted sequestration. In contrast, $\beta_2$-receptors that are functionally uncoupled or impaired because of genetic lesions of $G_s$ (9) or substitution mutations of the receptor domains involved in G protein coupling (10) still are able to sequester normally. Therefore, activation of the G protein may not have been a prerequisite for receptor internalization/sequestration.

Muscarinic cholinergic receptor internalization and down-regulation have been studied in intact cells with the use of *N*-[$^3$H]methylscopolamine ([$^3$H]NMS)[1] as the polar tracer with access to only the cell-surface receptor and [$^3$H]quinuclidinyl

* This work was supported by United States Public Health Service Research Grants GM 43102 and DA 04166 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: [$^3$H]NMS, *N*-[$^3$H]methylscopolamine; [$^3$H]QNB, [$^3$H]quinuclidinyl benzilate; PI, phosphatidylinositol; PAO, phenylarsine oxide; PMA, phorbol 12-myristate 13-acetate; GPCRs, G protein-coupled receptors.

benzilate ([$^3$H]QNB) as the lipophilic tracer for assay of total receptor content (11–14). In this study, we do not distinguish between receptor internalization and sequestration; the term "internalization" is subsequently used to indicate loss of [$^3$H] NMS cell-surface binding without concomitant loss of [$^3$H] QNB binding.

The muscarinic receptors m1, m3, and m5 are coupled to phosphatidylinositol turnover (15, 16), and stimulation of protein kinase C by phorbol esters causes muscarinic receptor phosphorylation (17) and rapid receptor internalization as observed in several studies (12–14, 18). However, protein kinase C activation may have no effect (19) or may even inhibit internalization in some tissues (20). An essential role for protein kinase C activation in internalization is therefore questionable. Using Y1 adrenal carcinoma cells transfected with the mouse m1 gene, Shapiro and Nathanson (21) found that receptor mutants with large deletions of the third intracellular loop (i3) internalize normally, but that the largest deletion mutant (d 221–343) shows less down-regulation upon carbachol treatment. We have constructed mutants of the human m1 gene (which is nearly identical to the mouse gene) with an even larger deletion of the i3 loop, and the deletion mutant (d 232–358) reaching closest to the COOH-terminal end of the i3 loop was no longer capable of agonist-induced internalization, although stimulation of PI turnover was unaffected (22). This result suggested a role for the i3 loop in receptor internalization, and it dissociates G protein activation from internalization.

In this study, the relationship between receptor internalization and activation of Hm1 was studied with a D71N point mutation that was previously shown to bind agonist and antagonist ligands, but that fails to activate PI turnover upon agonist stimulation (23). Furthermore, we analyzed the molecular mechanisms of Hm1 receptor activation and internalization with the use of multiple mutants involving the i3 loop (positions 208–366) and the COOH-terminal tail (positions 420–460). The results suggest a critical role for a domain in the i3 loop, whereas none of the potential Ser or Thr phosphorylation sites studied nor the COOH-terminal tail were found to be required in agonist-induced internalization. We propose that a small domain in the middle of the i3 loop of Hm1 is required for agonist-induced receptor internalization.

## EXPERIMENTAL PROCEDURES

*Materials*—[$^3$H]NMS (specific activity of 80 Ci/mmol) and [$^3$H] QNB (specific activity of 30 Ci/mmol) were obtained from Amersham Corp. or Du Pont-New England Nuclear. All other reagents were of analytical grade quality from common commercial sources (*e.g.* phenylarsine oxide (PAO), the $Ca^{2+}$ ionophore A23187, and phorbol 12-myristate 13-acetate (PMA)). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Bal-31 was purchased from New England BioLabs, Inc. (Beverly, MA), and restriction enzymes were from either Boehringer Mannheim or Bethesda Research Laboratories.

*Construction of Vectors Expressing Hm1 and Mutants*—The gene encoding Hm1 was obtained from a human placental genomic library in vector EMBL3 as described previously (22). Deletions of the i3 domain were introduced after cutting the Gem3/Hm1 vector at the unique *Stu*I restriction site, at position 712 (at codon for $Arg^{271}$), followed by digestion with the exonuclease Bal-31 for varying times at 4 °C (22). The blunt-ended product was self-ligated to yield mutants with deletions of varying size within the i3 domain ($Tyr^{208}$–$Thr^{366}$). In-frame mutants were transferred to pSG5 using *Bam*HI/*Eco*RI. For the construction of point mutations, Hm1 was transferred to M13mp18 using *Bam*HI/*Eco*RI, and single-stranded DNA incorporating dUTP was made (Kunkel method; for general protocols, see Ref. 24). The desired point mutations were then introduced with oligonucleotides (21–24-mers) containing one codon change. Similarly, small deletions in i3 were introduced with 30-mer oligonucleotides, complementary to 15 nucleotides adjacent to the deletion points. A truncation mutant was obtained by introducing a termination codon at $Lys^{447}$. Each mutant Hm1 gene was sequenced before transfer to pSG5 using *Bam*HI/*Eco*RI. The double deletion mutant d 219–231/d 267–293 was prepared by transferring the *Apa*I restriction fragment (nucleotides 475–796) of d 219–231 into d 267–293 using vector Gem3, which lacks *Apa*I sites. Similarly, the double deletion mutant d 267–293/d 344–358 was constructed by exchanging the *Bcl*I/*Bam*HI fragment. *Bcl*I cuts at nucleotide 903 and requires a plasmid DNA preparation grown in a methylation-deficient *Escherichia coli* strain. To delete sequence 283–293, we employed an inverse polymerase chain reaction (25) that introduces a *Nar*I restriction site into the deleted sequence (GGCGCC); therefore, 2 amino acids (Gly and Ala) were introduced into the deleted sequence. Thus, mutant d 284–293 was obtained because $Gly^{283}$ was reintroduced in addition to 1 Ala residue for deleted sequence 284–293. Further deletion mutants or double deletion mutants at the 5′- and 3′-sides of the newly introduced *Nar*I site were prepared by generating the requisite fragments of Hm1 from *Nar*I to *Bam*HI (5′) or *Eco*RI (3′) with the use of polymerase chain reaction (26) and reinserting the fragment into the vector Hm1 (d 284–293) cut with *Nar*I and either *Bam*HI or *Eco*RI. All mutations were verified by sequencing. To obtain standardized DNA preparations (~1 mg of DNA) for wild-type pSG5/Hm1 and all mutants, plasmid DNA was purified by LiCl/isopropyl alcohol and polyethylene glycol 8000 precipitation (24).

*Transfection of Human Kidney Cells (U293)*—U293 cells were transfected by the calcium phosphate precipitation method (22, 24). Briefly, $1.5 \times 10^6$ exponentially growing U293 cells were plated onto 10-cm tissue culture dishes. After 24 h, a precipitate of 6 μg of DNA with calcium phosphate was added to freshly fed cells. After 4 h of incubation at 37 °C and a dimethyl sulfoxide shock, the cells were allowed to recover, and the binding experiments or PI turnover analysis was performed after 48 h. For stable transfections with pSG5 containing the wild-type and mutant Hm1 d 232–358 genes (20 μg/plate), U293 cells were cotransfected with $pSV^{neo}$ (2 μg/plate). Cell clones resistant to the antibiotic G418 (~500 μg/ml) were isolated and screened for receptor expression with [$^3$H]NMS. One clone each expressing ~300,000 sites/cell (~1000 fmol/mg of protein) of Hm1 and d 232–358 was selected for further study.

*Receptor Binding and Internalization Assay*—For internalization assays, we modified the procedure described previously (22) to obtain more accurate results and to permit assay of relatively small changes. Dishes containing cells transfected with the appropriate receptor mutant were pooled 24 h after transfection. The pooled cells were replated onto 12-well cell culture dishes and allowed to attach overnight. Then the cells were incubated with or without 1 mM carbachol for up to 2 h and, in the case of stably transfected cells, up to 24 h. After drug treatment, the cells were cooled on ice and washed three times with ice-cold phosphate-buffered saline to remove residual carbachol remaining on the cells. The cells were then incubated in an isotonic buffer containing 1.5–2 nM [$^3$H]NMS (or 1 nM [$^3$H]QNB for total sites) at 12 °C (to prevent receptor recycling) for 90 min. At the end of the incubation, the cells were placed on ice, removed from the wells, and filtered quickly on glass-fiber filters (SS 32), followed by three rapid rinses with phosphate-buffered saline. Six replicate independent samples were assayed for each treatment condition (unless noted otherwise) to obtain estimates of variability and significance of differences. Pretreatment with agents other than carbachol (PAO, PMA, forskolin, pertussis toxin) was as described under "Results."

Displacement binding curves with carbachol (0.01–10 mM) were performed at a [$^3$H]NMS concentration of 0.1 nM. The resultant carbachol displacement curves were analyzed with the use of the logistic function $B = B_{max} - B_{min} * L/(IC_{50} + L) + NSB$, where $B$ = tracer bound (in disintegrations/minute), L = carbachol concentration, and NSB = nonspecific binding. The curves were fitted using nonlinear regression analysis of the unweighted data with the curve fitting program Minim 1.2 (R. O. Purves, Department of Pharmacology, School of Medicine, University of Otago, Dunedin, New Zealand).

*PI Turnover*—PI turnover was measured by the method of Berridge *et al.* (27) after labeling the cells with [$^3$H]myoinositol for 24 h. For the measurements of IP, inositol 1,4-bisphosphate, and inositol 1,4,5-triphosphate, individual dishes of 10-cm diameter were used; for the measurement of PI alone, which accounts for most of the inositol phosphate $^3$H activity, six-well cell culture dishes (well diameter to 3.5 cm) were used. Results were expressed as percent of total $^3$H activity in the inositol phosphates, and the percent values were compared between carbachol-treated and -untreated cells. The following concentrations of carbachol were used for dose-response curves

TABLE I

*Effect of mutations of Hm1 on [$^3$H]NMS binding, carbachol-induced internalization, carbachol binding, and carbachol-induced activation of PI turnover*

Transiently transfected cells were incubated with 1 mM carbachol for 2 h to induce internalization and for 30 min to stimulate PI turnover, measured as [$^3$H]IP release. The [$^3$H]NMS concentration was ~1.5 nM, except for measuring carbachol binding competition, when 0.1 nM [$^3$H]NMS and carbachol concentrations from 0.01 to 10 mM were used. All data are mean ± S.D.; number of replicates/experiment are shown at the top of the columns. Independent experiments are listed separately, or the number of experiments is indicated next to the results if all results are averaged.

| | [$^3$H]NMS ($n$ = 4–6) | Carbachol pretreatment (% of control [$^3$H]NMS binding) ($n$ = 6) | Carbachol binding $IC_{50}$ | Stimulation of [$^3$H]IP release (1 mM carbachol) over control (no carbachol) ($E_{max}$) ($n$ = 3) |
|---|---|---|---|---|
| | *fmol/mg protein* | | *mM* | |
| Nontransfected U293 cells | 32 ± 1.4 ($n$ = 2) | 56 ± 7 | | 2.7 ± 0.9 ($n$ = 5)[a] |
| Wild-type Hm1 | 792 ± 347 ($n$ = 23) | 55 ± 8 ($n$ = 14) | 0.44 ± 0.05 | 12.6 ± 2.7 ($n$ = 11) |
| D71N | 676 ± 34 | 91 ± 8 | | 3.5 ± 0.1[a] |
| K447 trunc | 485 ± 132 ($n$ = 3) | 67 ± 4, 63 ± 6, 66 ± 9, 52 ± 2 | 0.18 ± 0.05 | 11 ± 0.4 |
| d 219–231 | 814 ± 56 ($n$ = 2) | 49 ± 3, 64 ± 6 | | |
| d 344–358 | 580 ± 99 ($n$ = 2) | 57 ± 5, 67 ± 6 | | 9 ± 0.3 ($n$ = 2) |
| d 267–293 | 710 ± 222 ($n$ = 4) | 87 ± 8, 91 ± 12, 85 ± 4, 93 ± 10 | | 11 ± 2.5 ($n$ = 2) |
| d 267–292 | 912 ± 67 | 85 ± 7 | | |
| d 265–274 | 1045 ± 101 ($n$ = 2) | 49 ± 2, 60 ± 4 | | 13 ± 0.3 |
| d 276–282 | 295 ± 66 ($n$ = 2) | 51 ± 3, 64 ± 4 | | 11 ± 0.2 |
| d 284–293[b] | 1332 ± 35 ($n$ = 3) | 77 ± 7, 85 ± 13, 76 ± 9, 87 ± 9 | | 12 ± 0.7 |
| d 256–293[b] | 675 ± 54 | 70 ± 3 | | |
| d 284–303[b] | 282 ± 16 | 72 ± 7 | | |
| d 255–310 | 650 ± 138 ($n$ = 3) | 77 ± 8, 86 ± 5 | | 9 ± 2 ($n$ = 2) |
| d 247–304 | 567 ± 65 | 83 ± 6, 61 ± 8 | | 10.7 ± 0.3 ($n$ = 2) |
| d 242–312 | 389 ± 121 ($n$ = 2) | 85 ± 8, 76 ± 3 | | 12 ± 0.1 ($n$ = 2) |
| d 220–314 | 873 ± 112 ($n$ = 4) | 61 ± 3, 60 ± 4, 68 ± 4 | 0.74 ± 0.2 | 8.9 ± 0.3 ($n$ = 2) |
| d 219–333 | 387 ± 105 ($n$ = 5) | 80 ± 9 | | 11.2[c] |
| d 220–349 | 217 ± 18 | 76 ± 11 | 0.2[c] | 11[c] |
| d 232–358 | 770 ± 320 ($n$ = 7) | 94 ± 7, 107 ± 7, 103 ± 2 | 1.8[c] | 15 ± 6 ($n$ = 3)[c] |
| d 219–231/d267–293 | 867 ± 158 ($n$ = 2) | 87 ± 5, 86 ± 4 | | |
| d 267–293/d 344–358 | 1296 ± 239 ($n$ = 2) | 95 ± 7, 95 ± 9 | | |
| d 284–293/d 344–358[b] | 286 ± 23 | 67 ± 6, 68 ± 7 | | |

[a] Nontransfected U293 cells and cells transfected with the inactive receptor mutant D71N (20) showed significantly lower [$^3$H]IP release in the absence of carbachol compared with cells transfected with active receptor (wild-type Hm1 and all other mutants). Whereas carbachol stimulation in native cells and D71N cells was clearly measurable, absolute values of [$^3$H]IP release were greater by a factor of >10 in cells transfected with active receptors.

[b] These mutants were prepared by polymerase chain reaction using a *Nar*I restriction site, thereby introducing GA into the deletion site.

[c] These mutants were characterized in a previous paper (22).

(IP measured only): 3, 10, 30, 100, and 1000 μM. The data were fitted to the equation $E = E_{max} * L/E_{max} + L)$ where $E$ = stimulation of IP release over control values and $E_{max}$ = maximum stimulation. The results were fitted with the Minim 1.2 program.

## RESULTS

*Stably Transfected U293 Cells Expressing Hm1 and d 232–358*—The binding of [$^3$H]NMS (1 nM) and [$^3$H]QNB to cells transiently transfected with wild-type Hm1 was differently affected by exposure to 1 mM carbachol. Whereas [$^3$H]NMS binding was reduced to ~50% of control (Figs. 1 and 2), [$^3$H]QNB binding was not significantly affected (94 ± 7% compared to no carbachol pretreatment at 20 min).

To investigate the time course of [$^3$H]NMS and [$^3$H]QNB binding during exposure to carbachol for up to 24 h, U293 cells stably transfected with wild-type Hm1 and the d 232–358 mutant were selected (~300,000 sites/cell). Previous work had indicated that loss of [$^3$H]NMS binding caused by carbachol pretreatment was independent of receptor density over at least a 10-fold range and that the d 232–358 mutant, although active in PI turnover, did not internalize or sequester any [$^3$H]NMS-binding sites for up to 2 h (19). As in the transiently transfected cells (19), carbachol caused rapid loss of [$^3$H]NMS binding over 2 h, whereas no further loss was observed for up to 24 h (54 ± 3%) in the cells stably transfected with wild-type Hm1. This result was surprising as one would have expected additional down-regulation after the initial rapid loss of cell-surface receptors. Accordingly, [$^3$H]QNB binding to all Hm1 receptors present did not change substantially upon carbachol treatment over the entire assay period (84 ± 5% of control at 24 h). In contrast, binding of both [$^3$H]NMS and [$^3$H]QNB to the d 232–358 mutant receptor was unaffected by carbachol pretreatment over 5 h (98 ± 9 and 106 ± 3%, respectively), confirming the lack of receptor sequestration (22), but it actually increased over 24 h (148 ± 3 and 187 ± 21%, respectively). Because muscarinic receptor number may actually increase during prolonged exposure to carbachol, we were unable to study receptor down-regulation in this cell line, and agonist exposure was limited to 2 h or less in subsequent experiments. Furthermore, all subsequent experiments were performed in transiently transfected cells. In 23 experiments, variability of expression of Hm1 was 792 ± 347 fmol/mg of protein (±S.D.), which was acceptable for this study, yielding an average loss of [$^3$H]NMS binding to 55 ± 8% of control (14 experiments) after a 2-h treatment period with 1 mM carbachol (Table I).

*Modulation of Carbachol-induced [$^3$H]NMS Binding Loss by PAO, Pertussis Toxin, and PMA/A23187*—The metabolic poison PAO has been shown to inhibit agonist-induced recep-

tor internalization (28). Upon pretreatment of Hm1-transfected U239 cells with 100 μM PAO for 5 min, followed by addition of 1 mM carbachol for 2 h, [$^3$H]NMS receptor loss was significantly reduced, with 81 ± 9 and 83 ± 4% (±S.D., $n$ = 6, two experiments) of tracer binding remaining relative to no carbachol treatment. Without PAO, [$^3$H]NMS binding was reduced to 55 ± 8% (Table I) by carbachol. This result is consistent with the hypothesis that loss of [$^3$H]NMS binding represents an internalization process that may be in part energy-dependent. Pretreatment of U293 cells with pertussis toxin (1 μg/ml) for 6 h failed to affect 1 mM carbachol-induced receptor internalization. [$^3$H]NMS binding was 106% of control after pertussis treatment alone, whereas additional carbachol treatment for 30 min resulted in receptor reduction to 61 ± 3%. In this same experiment, [$^3$H]NMS binding was reduced to 57 ± 4% by carbachol alone, without pertussis treatment. Furthermore, pertussis toxin (100 ng/ml for 6 h) did not affect carbachol-stimulated PI turnover in U293 cells transfected with wild-type Hm1 (pertussis toxin alone: 1.2-fold stimulation of [$^3$H]IP release; 1 mM carbachol alone: 10.1-fold; carbachol + pertussis toxin: 11.9-fold).

The effects of exposure of the cells to both PMA (10 μM) and A23187 (30 μM) for 30 min were tested after transient transfection with wild-type Hm1 and deletion mutant d 232–358. In neither case was a significant change from control [$^3$H]NMS binding detectable (wild-type: 99 ± 3 and 99 ± 6%, respectively; d 232–358: 94 ± 7 and 89 ± 7%, respectively; ± S.D., $n$ = 6, two independent experiments).

*Lack of Internalization of Inactive Mutant D71N*—Point mutation of Asp$^{71}$ to Asn$^{71}$ results in a mutant (D71N) that binds [$^3$H]NMS and carbachol similar to the wild type, but is completely inactive in stimulating PI turnover (23). We have confirmed this result (Table I), achieving transient transfection efficiencies similar to those of the wild type. Furthermore, carbachol-stimulated PI turnover was indistinguishable from that caused by the native muscarinic receptor present in U293 cells (32 fmol/mg of protein). The approximately 3-fold stimulation of PI turnover in nontransfected and D71N-transfected U293 cells was readily distinguishable from that occurring in cells transfected with wild-type Hm1 and active receptor mutants that displayed a severalfold higher base line of [$^3$H]IP release and a ~10-fold stimulation after carbachol pretreatment (Table I, Footnote *a*). Hence, mutant D71N was inactive, and it was found to be also resistant to carbachol-induced receptor internalization (91 ± 8%) (Table I).

*Truncation of COOH Terminus*—We introduced a termination codon at Lys$^{447}$ to yield mutant K447 trunc. The truncation position was chosen to eliminate any putative Ser and Thr phosphorylation sites in the COOH-terminal tail while preserving receptor regions with possible functions in G protein activation. Mutant K447 trunc yielded productive [$^3$H]NMS binding comparable to that of the wild type, and $IC_{50}$ values for carbachol displacement of [$^3$H]NMS were similar. Repetitive measurements of carbachol $IC_{50}$ values for the wild-type receptor yielded values ranging from 0.3 to 1 mM, with slope factors varying from $n$ = 0.5 to 0.8. One possible cause for this fluctuation could be a variable extent of G protein association of transiently transfected Hm1 receptors. As a consequence, a small variation of the carbachol $IC_{50}$ values may not indicate any significant change of carbachol binding parameters. Mutant K447 trunc was also maximally active in carbachol-stimulated PI turnover, and it did internalize to an extent similar to that of the wild type (Table I). Finally, the time course of internalization after 15, 30, 60, and 120 min of carbachol (1 mM) exposure was indistinguishable from that of the wild type (data not shown).

*Deletion Analysis of i3 Loop Domains Involved with Hm1 Internalization*—Our previous results indicated that deletion of a large portion of i3 leads to reduced loss of [$^3$H]NMS binding upon carbachol pretreatment, although the mutant receptors are still fully active in stimulating PI turnover (22). Furthermore, the $EC_{50}$ values for carbachol stimulation of PI turnover are only marginally changed (22). Similarly, all tested deletion mutants of i3 listed in Table I were maximally effective in [$^3$H]IP release upon exposure to 1 mM carbachol. Complete dose-response curves were performed with several deletion mutants (d 219–231, d 344–358, d 219–333, and d 232–358), and in each case, the $EC_{50}$ values were not significantly different from that of the wild-type receptor (10–20 μM) (data not shown). Finally, carbachol binding was minimally affected where tested (Table I). Therefore, the remainder of this study was performed with 1 mM carbachol, which maximally activates PI turnover.

Among the three large deletions studied (d 232–358, d 220–349, and d 219–333), d 232–358 was unresponsive to carbachol pretreatment, whereas d 220–349 and d 219–333 were partially defective (22). With the use of a more precise binding assay, these results were confirmed for d 232–358 (102 ± 10%, $n$ = 16, [$^3$H]NMS binding after 2 h of carbachol treatment relative to control) and for d 220–349 and d 219–333, which showed an intermediate loss of [$^3$H]NMS binding (Table I). The difference between these latter two mutants and d 232–358 was significant.

To define relevant domains of the i3 loop, we tested smaller deletions obtained by Bal-31 exonuclease digestion of Hm1 cut at Arg$^{271}$ by *Stu*I. Surprisingly, four smaller deletion mutants tested (d 220–314 to d 255–310) were still partially defective in their response to carbachol, and they internalized to an extent similar to that of d 220–349 and d 219–333 (Table I). We thus selected even smaller deletions, and an unexpected result was obtained: mutants d 267–293 and d 267–292 were again nearly unresponsive to carbachol in the [$^3$H]NMS binding assay. Taken all measurements together, the difference between [$^3$H]NMS binding loss of d 267–293 (88 ± 9%, ± S.D., $n$ = 25, 2 h of carbachol treatment) and control binding was highly significant ($p \leq 0.0001$, $t$ test), as was the difference between d 267–293 and the unresponsive mutant d 232–358 (see above). Therefore, d 267–293 still showed a statistically significant, but overall rather small, net loss of [$^3$H]NMS binding. We then prepared three small deletion mutants within sequence 267–283. Whereas d 265–274 and d 276–282 were indistinguishable from wild-type Hm1, d 284–293 was again deficient in agonist-induced internalization (Table I). These results indicate that domain 284–292 is required for normal internalization. There was a small difference between d 267–293 and d 284–293, which suggested that different or additional mechanisms may play a role. However, a slightly larger deletion (d 256–293) was similarly deficient compared with d 284–293. Therefore, the small difference between d 267–293 and the other intermediate deletion mutants could not be associated with any specific receptor domain.

The difference between all deletion mutants with intermediate loss of [$^3$H]NMS binding and d 232–358 with no apparent [$^3$H]NMS loss upon carbachol exposure suggested two hypotheses. First, sequence 219–231 could serve to prevent internalization or mediate recycling, or second, sequence 349–358 could mediate an additional, slower internalization pathway or prevent recycling. We therefore tested the behavior of the small deletion mutants d 219–231 and d 344–358. Loss of [$^3$H]NMS binding upon carbachol exposure was similar to the wild type in each case. However, any effect of these regions may not be detectable under the assay conditions. Therefore,

we also constructed the double deletion mutants d 219–231/d 267–293, d 267–293/d 344–358, and d 284–293/d 344–358. In each case, introduction of the small deletion at the junctions of the i3 loop had little overall effect on the internalization of mutants d 267–293 and d 284–293 (Table I). Therefore, we were unable to demonstrate that sequences 219–231 and 349–358 play a role in receptor trafficking.

*Time Course of Carbachol-induced Internalization*—Several processes could contribute to loss of [$^3$H]NMS binding upon carbachol exposure. We therefore studied the time course of [$^3$H]NMS binding after carbachol pretreatment from 15 min to 2 h (Fig. 1). The wild-type receptor rapidly reached maximum internalization at 30–60 min, with 55% of control reached as early as 15 min after carbachol addition. Mutant d 232–358 did not show any loss of [$^3$H]NMS binding (30 min and 2 h) or only minimal internalization (15- and 60-min time points) (Fig. 1*A*). Mutant d 267–293 did internalize to a small extent at all time points ($p < 0.05$ at 60 and 120 min) (Fig. 1*B*). Three mutants with intermediate deletions were also selected for the initial time course study. All three (d 220–314, d 247–304, and d 219–333) were strongly impaired in carbachol-induced internalization over the initial 30 min compared to the wild type, but these receptor mutants continued to disappear from the cell surface (Fig. 1*B*), so that after 60–120 min, net [$^3$H]NMS binding loss was intermediate between the wild type and d 267–293 (differences among curves significant, $p < 0.05$). We then compared the time course of [$^3$H]NMS binding loss for the smallest deficient deletion mutant (d 284–293) with that of the wild-type Hm1 receptor (Fig. 2). Whereas wild-type Hm1 again internalized rapidly over the first 15 min, measurable [$^3$H]NMS binding loss to ~80% of control occurred after only 1–2 h for d 284–293. This behavior of d 284–293 is similar to that of the intermediate deletion mutants, whereas the overall extent of [$^3$H]NMS binding loss over 2 h was variable.

*Lack of Evidence for Role of Receptor Ser and Thr Phosphorylation in Internalization*—A series of Ser and Thr mutations of Hm1 were constructed to test whether phosphorylation of any of these sites plays a role in receptor internalization (Table II). Replacement of Ser and Thr at various sites of Hm1 with Ala did not significantly affect carbachol-induced internalization. Three Ser/Thr residues in the COOH-terminal tail were eliminated by truncation at Lys$^{447}$, with no effect on the extent or rate of agonist-induced internalization (see above).

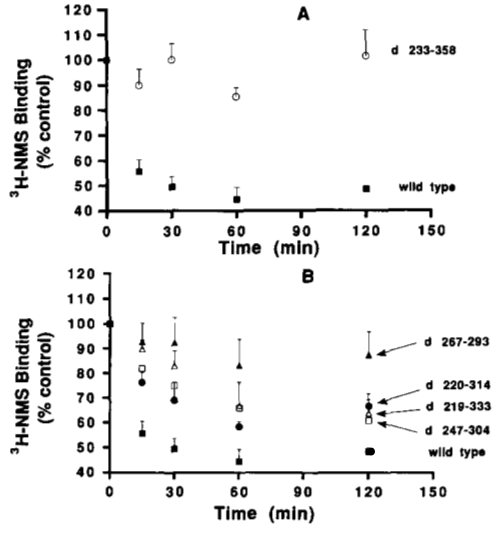


FIG. 1. **Time course of carbachol-induced loss of [$^3$H]NMS-binding sites of Hm1 and selected mutants.** Transiently transfected U293 cells were incubated with 1 mM carbachol for 15–120 min, and [$^3$H]NMS (1.5 nM) binding was measured after carbachol removal. For comparison, the wild-type Hm1 data were plotted in both *A* and *B*. *Error bars* are ±S.D., with $n = 4$, except for mutants d 232–358 and d 267–293, where $n = 10$–16 (several experiments were pooled).

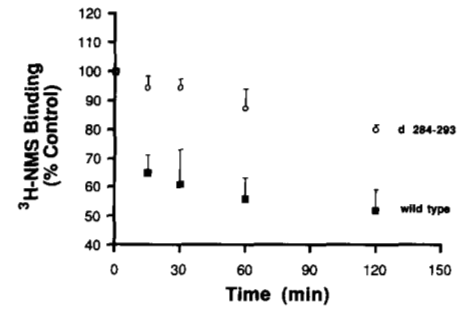


FIG. 2. **Time course of carbachol-induced loss of [$^3$H]NMS-binding sites of Hm1 and smallest deletion mutant with deficient internalization (d 284–293).** The experiment was performed as described for Fig. 1. *Error bars* are ±S.D. ($n = 4$).

TABLE II

*Mutations of Ser and Thr residues in Hm1*

Transiently transfected U293 cells were exposed to 1 mM carbachol for 2 h (or 30 min for T215A and T330A), and [$^3$H]NMS binding was measured. Overall [$^3$H]NMS binding was comparable to that of wild-type Hm1-transfected cells for each mutant.

| | % of control [$^3$H]NMS binding (±S.D., $n = 6$) |
|---|---|
| T215A | 55 ± 4 |
| S228A | 52 ± 4 |
| S322A | 52 ± 2 |
| T330A | 56 ± 7 |
| T354A | 62 ± 7, 58 ± 13, 55 ± 4 |
| S356A | 60 ± 7, 64 ± 3 |
| S368A | 58 ± 4 |
| T428A | 53 ± 4 |

## DISCUSSION

*Receptor Activation and Internalization*—Our results shed further light on the steps required for Hm1 receptor internalization following agonist exposure. The finding that the nonfunctional receptor mutant D71N (23) does not undergo agonist-induced internalization/sequestration suggests that receptor activation (but not necessarily G protein activation) is essential to this process. Previous work with $\beta_2$-receptors, on the other hand, had suggested that receptor internalization/sequestration still proceeds even if the receptors are functionally uncoupled from the second messenger pathway (9, 10). Indeed, independent activation of protein kinase C by PMA and enhancement of $Ca^{2+}$ flux by A23187 had no effect on [$^3$H]NMS binding to cell-surface receptor in U293 cells, suggesting that these downstream events of PI turnover do not play a role in receptor internalization/sequestration in this case.

The hypothesis of Cheung *et al.* (8) that the receptor regions involved in functional coupling to $G_s$ are also required for agonist-promoted sequestration does not apply to the Hm1 receptor as we were able to construct numerous mutants deficient in sequestration/internalization, but still maximally active in stimulating PI turnover. The failure of pertussis toxin to suppress both PI turnover and sequestration/internalization demonstrates that the G protein involved in activation is pertussis toxin-insensitive. Whereas internalization of the $\beta_2$-receptor was found to involve internalization from

its G protein (29), a muscarinic receptor internalized in astrocytoma cells as a G protein-receptor complex (11). Because of these divergent results, it is currently not possible to determine whether activation of the associated G protein is also required for agonist-induced internalization of Hm1.

*COOH-terminal Tail of Hm1 and Ser/Thr Phosphorylation Sites*—No evidence for an involvement of the COOH-terminal tail (truncated at $Lys^{447}$) in receptor internalization was evident. Point mutation of $Thr^{428}$ to yield T428A further ascertained that none of the Ser/Thr residues in the COOH-terminal tail plays an essential role in this process. Similarly, the lack of a role of the larger COOH-terminal tail of the $\beta_2$-receptor in internalization/sequestration was suggested by Strader *et al.* (31), and removal of Ser/Thr phosphorylation sites affected $\beta_2$-receptor desensitization, but not internalization (10, 32). Our subsequent studies therefore focused on the i3 loop of Hm1.

*Deletion Mutations of i3 Loop of Hm1*—On the basis of our previous results that large deletions of the i3 loop impair Hm1 internalization (22), we constructed a series of deletion mutants by Bal-31 digestion from the *Stu*I site ($Arg^{267}$) to define the responsible receptor domain(s) (see Fig. 3 for the i3 sequence). All of these deletions impaired internalization, and two rather small deletions in the middle of the i3 loop (d 267–292 and d 267–293) allowed only a minimal degree of internalization, suggesting that receptor sequence 267–292 contains a domain responsible for rapid internalization. This region can be further narrowed to domain 284–292 (Fig. 3) because of the wild-type behavior of the adjacent small deletion mutants (d 265–274 and d 276–282) and the strongly impaired internalization of d 284–293 ($Gly^{283}$ was reintroduced by an inverse polymerase chain reaction) (Fig. 2). The general significance of this sequence will be discussed at the end of this paper.

Lack of net internalization of d 232–358, compared with the partial defect of other deletion mutants, suggested a possible role of either sequence 219–231 to inhibit net internalization or of sequence 349–358 to promote a slow internalization process. However, deletion of sequences 219–231 or d 344–358 from wild-type Hm1 or from several internalization-defective mutants failed to affect the behavior of either the wild type or defective mutants (Table I). Therefore, sequences 219–231 and 349–358 do not appear to play a direct role in receptor trafficking; however, any function in modulating the wild-type behavior cannot yet be ruled out.

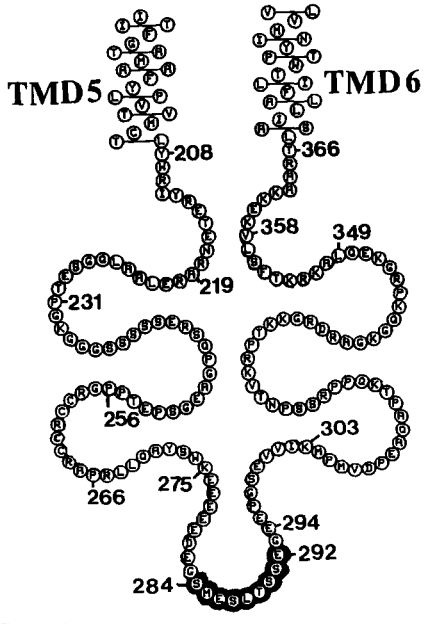


FIG. 3. **Graphic representation of i3 loop of Hm1 ($Tyr^{208}$–$Thr^{366}$).** The cloverleaf design does not imply any secondary structure of the i3 loop. The region proposed to be involved in regulating receptor internalization is *highlighted*. *TMD*, transmembrane domain.

A paradoxical result arose with the finding that the internalization of d 232–358 and d 267–293 was either absent or barely detectable, whereas the intermediate mutants showed appreciable net loss of [$^3$H]NMS-binding sites over 2 h of carbachol exposure. A study of the time course of internalization indicated that the intermediate deletion mutants equilibrated more slowly during carbachol stimulation (Fig. 1) so that the initial rates of internalization were considerably slower than those of wild-type Hm1. The large difference in the initial rate of [$^3$H]NMS binding loss is best seen when comparing d 284–293 and wild-type Hm1 (Fig. 2). However, overall net loss of [$^3$H]NMS binding loss over 2 h could approach that of the wild type (see d 220–314 in Table I). It is therefore possible that the internalization defect was not recognized in a previous report describing similar deletion mutants (21). The slow kinetic behavior of the intermediate deletion mutant is consistent with the hypothesis of an i3 region playing a role in receptor recycling, and such a region may be located immediately adjacent to d 267–293. Considering the location of all intermediate deletions, a putative region directing a recycling process can be narrowed to sequence 256–266 or 294–303. The deletion mutants d 256–293 and d 283–303 were constructed to test this hypothesis, but they also showed an intermediate degree of receptor internalization, as did the small deletion mutant d 284–293. Therefore, the most defective mutants (d 232–358 and d 267–293 (and d 267–292)) showed an anomalous behavior that could not be definitively traced to any discrete domain of the i3 loop. It is possible that more than one mechanism contributes to the loss of [$^3$H]NMS binding from the cell's surface. Overall, the only deletion consistently associated with defective internalization in each case was domain 284–292. Deletions cause changes in the receptor structure that can account for the observed phenotypic changes; however, because of the large variety of deletions analyzed, rather strong evidence implicates domain 284–292 as being required in cellular processing of Hm1.

*Significance of Domain 284–292 in Hm1 Cellular Trafficking*—This report is the first to propose a discrete receptor domain to be involved in agonist-induced receptor internalization/sequestration for any of the numerous G protein-coupled receptor (GPCRs). In contrast to the case of the low density lipoprotein receptor and growth factor receptors that carry such domains adjacent to their transmembrane helix (1–6), the Hm1 domain is located in the middle of the large i3 loop (Fig. 2). Among the cloned GPCRs, the i3 loop is highly diverse in sequence and size; therefore, applicability of this proposed domain to GPCR trafficking in general is not immediately obvious. On the other hand, there are also pronounced differences in the response of various GPCRs to agonist exposure, ranging from no detectable internalization to rapid and profound receptor loss from the surface and the cell (*e.g.* Ref. 33). One would therefore not expect functional domains involved in trafficking in each GPCR.

The location of domain 284–292 is surprising because little overall sequence identity can be observed for the i3 loop even among closely related GPCRs (34), and yet the various muscarinic receptor subtypes are all known to undergo internalization (35). Careful sequence comparison of the middle do-

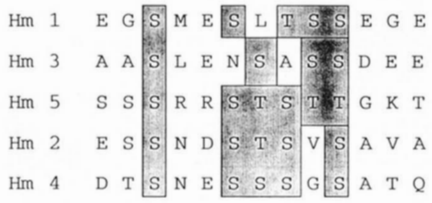


FIG. 4. **Sequence of domain 284–292 in Hm1 aligned with similar sequences in Hm2–Hm5, also located in the middle of their respective i3 loops.**

main of the i3 loop reveals a low but detectable degree of identity, already noted before (35), with the presence of multiple Ser and Thr residues (Fig. 4). Serine 284 of Hm1 is present in equivalent positions in Hm2–Hm5, and 2 amino acids downstream, there is a box of 5 amino acids with 3–5 Ser and Thr residues. It is therefore possible that the internalization domain identified in Hm1 is also functional in other muscarinic subtypes, and it may have been introduced by shuffling of small exons. In fact, when introns occur in GPCR genes, they are usually found in the i3 loop. For example, a *Drosophila* muscarinic receptor gene contains three introns in the i3 loop (36). Thus, functional domains could have been added from different gene fragments, and we will test the general relevance of domain 284–292 of Hm1 for other GPCRs.

The presence of multiple Ser and Thr residues suggests the possibility that phosphorylation events may play a role in the internalization process. Hausdorff *et al.* (30) have recently identified several Ser and Thr residues of a proximal portion of the carboxyl-terminal tail of the $\beta_2$-adrenoceptor (mutant S355–364) that are involved in its rapid regulation, including phosphorylation, desensitization, and sequestration. However, in view of further Ser and Thr point mutations of the COOH-terminal tail, these authors suggested that sequence 355–364 is not directly involved, but rather is critical for the COOH-terminal tail to assume a suitable conformation for these regulatory processes. It remains to be seen whether Hm1 domain 284–292 in the i3 loop is phosphorylated upon receptor activation to allow internalization to occur. Regions similar to domain 284–292 with multiple Ser and Thr residues are found in the i3 loop or COOH-terminal tail of many other G protein-coupled receptors.

*Acknowledgments*—Dr. Sadaaki Maeda (Department of Pharmacology, Faculty of Dentistry, Osaka University, Osaka, Japan) contributed to this study by identifying several in-frame deletion mutants, and Dr. Osamu Nagata (Hokoriku Seiyaka Co., Ltd., Fukui, Japan) contributed data on the time course of receptor internalization.

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