Internalization and Down-regulation of Human Muscarinic Acetylcholine Receptor m2 Subtypes

ROLE OF THIRD INTRACELLULAR m2 LOOP AND G PROTEIN-COUPLED RECEPTOR KINASE 2*

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Internalization and down-regulation of human muscarinic acetylcholine m2 receptors (hm2 receptors) and a hm2 receptor mutant lacking a central part of the third intracellular loop (I3-del m2 receptor) were examined in Chinese hamster ovary (CHO-K1) cells stably expressing these receptors and G protein-coupled receptor kinase 2 (GRK2). Agonist-induced internalization of up to 80–90% of I3-del m2 receptors was demonstrated by measuring loss of [3H]N-methylscopolamine binding sites from the cell surface, and transfer of [3H]quinuclidinyl benzilate binding sites from the plasma membrane into the light-vesicle fractions separated by sucrose density gradient centrifugation. Additionally, translocation of hm2 receptors with endocytic vesicles were visualized by immunofluorescence confocal microscopy. Agonist-induced down-regulation of up to 60–70% of hm2 receptors was demonstrated by determining the loss of [3H]quinuclidinyl benzilate binding sites in the cells. The half-time (t1/2) of internalization and down-regulation in the presence of 10−4 M carbachol was estimated to be 9.5 min and 2.3 h, respectively. The rates of both internalization and down-regulation of hm2 receptors in the presence of 10−6 M or lower concentrations of carbachol were markedly increased by coexpression of GRK2. Agonist-induced internalization of I3-del m2 receptors was barely detectable upon incubation of cells for 1 h, but agonist-induced down-regulation of up to 40% of I3-del m2 receptors occurred upon incubation with 10−4 M carbachol for 16 h. However, the rate of down-regulation was lower compared with wild type receptors (t1/2 = 9.9 versus 2.3 h). These results indicate that rapid internalization of hm2 receptors is facilitated by their phosphorylation with GRK2 and does not occur in the absence of the third intracellular loop, but down-regulation of hm2 receptors may occur through both GRK2-facilitating pathway and third intracellular loop-independent pathways.

Loss of the cell’s response to agonist acting at G protein-coupled receptors can occur in three phases: uncoupling from G proteins, sequestration/internalization, and down-regulation of the receptors. Many G protein-coupled receptors are phosphorylated by G protein-coupled receptor kinases (GRKs) in an agonist-dependent manner as a major mechanism in receptor regulation (for review, see Refs. 1 and 2). Muscarinic acetylcholine receptor m2 subtypes have also been shown to be phosphorylated by GRK2 (β-adrenergic receptor kinase 1) (3, 4) and other GRKs (5, 6). In addition, phosphorylation of β2-adrenergic receptors by GRK2 may be involved in the uncoupling of β2-adrenergic receptors from G protein Gs (1, 2). The phosphorylated β2-adrenergic receptors were reported to be uncoupled from G proteins because of their interaction with β-arrestin (1, 2, 7). Indeed, phosphorylation by GRKs has been reported to correlate with uncoupling for several G protein-coupled receptors including muscarinic m2 (8), α1D-adrenergic (9), α2-adrenergic (10), thrombin (11), dopamine D1A (12), and thyrotropin receptors (13). β-Arrestin and arrestin 3 (β-arrestin 2) were shown to interact with m2 muscarinic as well as β2-adrenergic receptors (14, 15).

Sequestration/internalization of β2-adrenergic receptors seemed to be independent of phosphorylation by GRK2 on the basis of results with β2-adrenergic receptor mutants lacking phosphorylation sites or GRK-specific inhibitors (16–20). On the other hand, the agonist-induced sequestration of hm2 receptors expressed in HEK293 cells is hampered by deletion of the third intracellular loop (13-loop) which includes the GRK2 phosphorylation sites (21, 22). Moreover, agonist-dependent phosphorylation and sequestration of m2 receptors expressed in COS-7 cells are facilitated by coexpression of GRK2 and attenuated by coexpression of a dominant-negative mutant of GRK2 (DN-GRK2) that lacks kinase activity (23). Recently, Ferguson et al. have reexamined the relationship between the phosphorylation by GRK2 and sequestration of β2-adrenergic receptors, demonstrating that phosphorylation by GRK2 (24) or other GRKs (25) facilitates sequestration of β2-adrenergic receptors. Phosphorylation facilitates β-arrestin binding to β2-adrenergic receptors (26) and thereby appears to enhance sequestration, possibly interacting with clathrin (27), a major

1 The abbreviations used are: GRK, G protein-coupled receptor kinase; hm2 receptor, human muscarinic acetylcholine receptor m2 subtype; DN-GRK2, dominant negative form of GRK2; 13-loop, the third intracellular loop; I3-del m2 receptor, a hm2 receptor mutant that lacks a central part of the third intracellular loop; G protein, guanine nucleotide-binding regulatory protein; NMS, N-methylscopolamine; QNB, quinuclidinyl benzilate; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.

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protein of coated pits. Pals-Ryalaardsam et al. (8, 28) have provided results showing that the phosphorylation by GRK2 of m2 receptors is involved in their internalization as well as in their uncoupling from G proteins in HEK293 cells. These results suggest that the phosphorylation by GRK2 of m2 muscarinic and β2-adrenergic receptors may be involved in both internalization and uncoupling through facilitation of their interaction with β-arrestin/arrestin 3.

No studies have been carried out on the relation between down-regulation and phosphorylation of G protein-coupled receptors, except that down-regulation of β2-adrenergic receptors has been reported to be independent of their phosphorylation by GRK2 (16, 18). It is also unclear whether the cellular pathway leading to down-regulation is distinct from that of internalization. If a portion of receptors in clathrin-coated vesicles translocates into lysosomes and is down-regulated, their phosphorylation with GRKs or the deletion of I3-loop should also affect down-regulation. However, if down-regulation occurs by a distinct pathway, receptor phosphorylation may not play a role. Alternatively, both phosphorylated and non-phosphorylated receptors may enter the clathrin-dependent internalization pathway, albeit at different rates. Finally, receptor phosphorylation could affect the rate of translocation between endosomes and lysosomes, or recycling to the cell surface.

Here, we provide evidence that down-regulation as well as internalization of hm2 receptors are facilitated by coexpression of GRK2. Moreover, deletion of I3-loop, which contains the GRK2 phosphorylation sites (22), suppressed rapid internalization and markedly reduced the rate of down-regulation.

**EXPERIMENTAL PROCEDURES**

**Materials—**[3H]NMS (specific activity of 71.3 Ci/mmol) and [3H]QNB (specific activity of 36.6 Ci/mmol) were purchased from NEN Life Science Products; restriction enzymes were from Toyobo Corp. and Takara Shuzo Co., Ltd.; Cy3-conjugated goat anti-mouse IgG antibody was from Jackson Laboratories. cDNA of GRK2 was kindly donated by Dr. R. J. Lefkowitz, mammalian expression vector for hygromycin-resistant gene (pSV-hygro) was from Dr. H. Okayama, and mammalian expression vector with neomycin-resistant gene (pEG-neo) and mammalian expression vector pEF-BOS were from Drs. S. Nagata and T. Shimizu. Hybridoma cells expressing 9E10 were obtained from the American Type Culture Collection; Chinese hamster ovary CHO-K1 cells were from the Japanese Cancer Research Resources Bank.

**Construction of Stable Transfected Expressing hm2 Receptors and GRK2—**The construction of mammalian expression vectors for c-Myc epitope-tagged hm2 receptor (pEF-My-hm2) and GRK2 (pEF-GRK2) was performed basically as described (23). CHO cells were transfected with 18 μg of expression vectors of pEF-My-hm2 and 2 μg of pEG-neo by the calcium phosphate precipitation method (29). Stable transfected cells were selected in the presence of 400 μg/ml Geneticin (Life Technologies, Inc.) and were subcloned by limiting dilution. Expression of receptors was detected by [3H]NMS binding. The [3H]NMS binding sites in these cells were estimated to be 165 fmol/mg of protein in total homogenate. The transfected cells were cultured in F-12 nutrient mixture (Ham’s) (Life Technologies Inc.) supplemented with 10% fetal bovine serum (Cansera International Inc.), 40 units/ml penicillin G (Meiji Pharmaceuticals Co., Ltd.), and 100 units/ml streptomycin sulfate (Meiji Seika, Kaisha Ltd.), 40 mg/ml streptomycin sulfate (Meiji Seika, Kaisha Ltd.), and 100 μg/ml Geneticin at 37 °C in 95% air and 5% CO2. One of the CHO cell clones expressing hm2 receptors was transfected with 18 μg of pEF-GRK2 and 2 μg of pSV-hygro, and stable transfected were selected in the presence of 300 μg/ml hygromycin B (Boehringer Mannheim) and subcloned by limiting dilution. Expression of GRK2 was detected with use of Western blotting as described previously (23). The [3H]QNB binding sites of these cells were estimated to be 330 fmol/mg of protein in total homogenate, and expressed amounts of GRK2 were estimated to be 300–600 fmol/mg of protein in the supernatant by immunostaining with anti-GRK2 antibody. The transfected cells were cultured in F-12 nutrient mixture (Ham’s) supplemented with 10% fetal bovine serum, 40 units/ml penicillin G, 40 mg/ml streptomycin sulfate, and 100 μg/ml hygromycin B at 37 °C in 95% air and 5% CO2. A mammalian expression vector for a m2 receptor mutant that lacks a central part of the third intracellular loop (I3-del m2 receptor) was constructed by inserting the Nhel/Xhol fragment of the pSG5/ 

Hm2(2324–381) (21) into the Nhel/Xhol site of pEF-My-hm2. 13-del m2 receptors were stably expressed in CHO-K1 cells as described above, and the [3H]QNB binding sites of these cells were estimated to be 260 fmol/mg of protein in total homogenate.

**Sucrose Density Gradient Centrifugation Experiments—**Sucrose density gradient centrifugation was performed as described by Harden et al. (30). Semiconfluent CHO cells cultured in a 15-cm diameter dish were treated with 10–5 M carbamylcholine for 20 min and then washed three times with 10 ml of ice-cold, phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.5). Washed cells were incubated with 10 ml of medium containing [3H]NMS (0.5 μCi/ml) for 30 min on ice, hypotonically lysed with 10 ml of lysis buffer (1 mM Tris, 2 mM EDTA, pH 7.4), and hypotonically lysed by incubation in 10 ml of lysis buffer for 20 min on ice. After removing the lysis buffer, cells were collected in a small volume of lysis buffer with rubber policeman. The lysate (1 ml) was layered on top of a sucrose density gradient comprising 55, 50, 47.5, 45, 42.5, 40, 37.5, 35, 32.5, and 30% of sucrose solution (0.9 ml each) buffered with 2 mM Tris-HCl (pH 8.0). The gradient was centrifuged in a Hitachi SW 27 rotor at 25,000 rpm for 2.5 h at 4 °C. Twenty 0.5-ml fractions were collected from bottom and then subjected to [3H]QNB binding assay. A portion of each fraction (0.1 ml) was mixed with 0.9 ml of HEN buffer (20 mM Hepes/KOH, 1 mM EDTA, 160 mM NaCl, pH 7.4) containing 0.36 μM [3H]QNB, and incubated at 30 °C for 1 h. After incubation, membranes were washed with GF-B glass fiber filter, washed with 1 ml of 20 mM potassium phosphate buffer (pH 7) four times, and radioactivity determined by scintillation counting.

[3H]NMS and [3H]QNB Binding Assay—CHO cells (1 × 106 cells/well) were plated onto 12-well culture dishes. Forty to forty-eight hours after plating, various concentrations of carbamylcholine were added to culture media. After incubation with carbamylcholine for 15 min to 16 h, cells were washed three times with 1 ml of ice-cold PBS/well and incubated with 1.2–1.6 nM [3H]NMS or [3H]QNB in HEN-buffered saline (25 mM Hepes, 113 mM NaCl, 6 mM glucose, 3 mM CaCl2, 3 mM KCl, 2 mM MgSO4, and 1 mM Na2HPO4, pH 7.4: 0.5 ml/well) at 4°C for 4 h. After incubation, cells were washed three times with 1 ml ice-cold PBS/well. After washing, cells were dissolved in 0.5 ml of 1% Triton X-100 in PBS containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(methyl-5-phenyloxazolyl)benzene, and the radioactivity measured. Quadruplicate samples were assayed for each point. In some experiments, cells were treated with carbamylcholine in the hypertonic medium containing 0.3 M sucrose besides normal constituents. Down-regulation in the hypertonic medium was examined for cells treated with carbamylcholine for 1–4 h, because more than 4 h in the hypertonic medium caused CHO cells to deteriorate.

**Immunofluorescence Confocal Microscopy of hm2 Receptors—**CHO cells expressing human c-Myc-tagged m2 receptors were grown overnight on plastic chamber slides (Nunc Inc.). Treatment with various concentrations of carbamylcholine was carried out at 37 °C for 10 min. At the end of drug treatment, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% paraformaldehyde in PBS, permeabilized in PBS containing 0.25% fish gelatin for 10 min at room temperature with 3.7% paraformaldehyde in PBS, permeabilized in PBS containing 0.25% fish gelatin, 0.04% saponin, and 0.05% NaN3. After permeabilization, cells were labeled with anti-Myc monoclonal antibody (9E10) (31) for 1 h, washed four times with PBS, incubated with Cy3 (indocarbocyanine)-conjugated goat anti-mouse secondary antibody, and then washed four times with PBS and once with water. Slides were mounted using Fluoromount G (Fisher Scientific) containing a trace amount of phenylenediamine and stored at 4°C. Samples were visualized using laser scanning confocal microscopy with a krypton-argon laser coupled with a Bio-Rad MRC-600 confocal head attached to an Optiphot II Nikon microscope with a Plan Apo 60 × 1.4 NA objective lens with 1.4 numeric aperture. Cy3 emission was detected with a yellow high sensitivity filter block.

**RESULTS**

**Sequestration of hm2 Receptors as Assessed by Loss of [3H]NMS Binding Sites from the Cell Surface—**CHO cells expressing hm2 receptors with or without GRK2 were treated with carbamylcholine for various times, and the [3H]NMS binding activity of intact cells was measured. Fig. 1 summarizes the effects of incubation time and concentrations of carbamylcholine on the sequestration of hm2 receptors, as assessed by loss of [3H]NMS binding activity from the cell surfaces. In the presence of 10–5 M or higher concentrations of carbamylcholine, 70–80% of [3H]NMS binding sites were se-
Questered with a half-life of approximately 10 min. In the presence of $10^{-2} \text{M}$ carbamylcholine, the rate of sequestration was slower, and less than 20% of $[\text{3H}]\text{NMS}$ binding sites were sequestered in 60 min. Coexpression of GRK2 markedly increased the rate of sequestration in the presence of $10^{-2} \text{M}$ carbamylcholine but only slightly increased the extent of sequestration in the presence of $10^{-5} \text{M}$ or higher concentrations of carbamylcholine (Fig. 1, A–C). Fig. 1D shows the $[\text{3H}]\text{NMS}$ binding sites remaining on the surface of cells pretreated with carbamylcholine for 60 min. Without GRK2 cotransfection, $10^{-6} \text{M}$ or higher carbamylcholine concentrations were required to elicit sequestration, whereas, with GRK2 cotransfection, $10^{-7} \text{M}$ carbamylcholine was sufficient to detect sequestration. Apparent EC$_{50}$ values of carbamylcholine in control and GRK2-expressing cells were estimated to be 2.4 and 0.37 $\mu\text{M}$, respectively. $[\text{3H}]\text{NMS}$ binding sites of control and GRK2-expressing cells were 190 and 310 fmol/well, respectively. 

FIG. 1. Effects of incubation time and carbamylcholine (CCh) concentrations on sequestration of $[\text{3H}]\text{NMS}$ binding sites on CHO cells. CHO cells expressing hm2 alone or hm2 plus GRK2 were incubated with $10^{-6} \text{M}$ (A), $10^{-5} \text{M}$ (B), and $10^{-4} \text{M}$ (C) carbamylcholine for the indicated times or with various concentrations of carbamylcholine for 60 min (D), and then subjected to $[\text{3H}]\text{NMS}$ binding assays at 4 °C for 4 h. In experiments shown in Fig. 1 (E), CHO cells were incubated with various concentrations of carbamylcholine for 60 min in the hypertonic medium containing 0.32 M sucrose. Results are shown as means ± S.D. from three independent experiments. Time-course and dose-response curves were fitted to the following equations: $B \times \exp(-0.69/t_{1/2}) + (100 - B)$ and $R_{\text{max}} \times \text{EC}_{50}/(\text{EC}_{50} + [\text{carbamylcholine}]) + (100 - R_{\text{max}})$, respectively. The values of $B$ and $t_{1/2}$ in control cells and GRK2-expressing cells were as follows: panel A, $10^{-6} \text{M}$ carbamylcholine, $B = 34\%$ (control) and 83% (GRK2), $t_{1/2} = 53$ min (control) and 42 min (GRK2); panel B, $10^{-5} \text{M}$ carbamylcholine, $B = 62\%$ (control) and 73% (GRK2), $t_{1/2} = 8.9$ min (control) and 11.2 min (GRK2); panel C, $10^{-4} \text{M}$ carbamylcholine, $B = 69\%$ (control) and 76% (GRK2), $t_{1/2} = 9.5$ min (control) and 10.7 min (GRK2). The values of EC$_{50}$ for carbamylcholine in control and GRK2-expressing cells were estimated to be 2.4 and 0.37 $\mu\text{M}$, respectively. $[\text{3H}]\text{NMS}$ binding sites of control and GRK2-expressing cells were 190 and 310 fmol/well, respectively.

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greater (21). It should be noted that the portion of sequestered m2 receptors was higher for CHO cells (80%) than for COS-7 cells (40%) or BHK-21 cells (20–25%).

Many membrane proteins including G protein-coupled receptors have been shown to be internalized through coated vesicles (26, 27, 32, 33), whereas some receptors including m2 muscarinic receptors have also been reported to be internalized via caveolae (34, 35). To determine which process is involved in the sequestration of hm2 receptors expressed in CHO cells, we have examined the effect of hypotonic medium on the sequestration, because the hypotonic medium is known to inhibit the internalization through clathrin-coated vesicles but not the internalization through caveolae (33, 34, 36, 37). Sequestration of hm2 receptors in the presence of 10^{-4} M or lower concentrations of carbamylcholine was completely suppressed in the hypotonic medium containing 0.32 M sucrose (Fig. 1F), indicating that hm2 receptors are internalized through clathrin-coated vesicles. The inhibition of sequestration by the hypotonic medium was observed whether GRK2 was coexpressed or not, excluding the possibility that the coexpression of GRK2 facilitated the internalization of hm2 receptors through the pathway different from the coated vesicle-mediated pathway.

Assessment of Internalization of hm2 Receptors by Sucrose Density Gradient Centrifugation and Confocal Microscopy—Sequestration of muscarinic receptors as assessed by the loss of [3H]NMS binding sites from the cell surface is generally thought to represent internalization of receptors in the form of endocytosed vesicles. We confirmed internalization of hm2 receptors expressed in CHO cells with two different methods: sucrose density gradient centrifugation and confocal microscopy. Sucrose density gradient centrifugation was carried out as described by Harden et al. (30). The carbamylcholine-treated cells were incubated with concanavalin A, hypotonically lysed, and then subjected to sucrose density gradient centrifugation as described under “Experimental Procedures.” After centrifugation, [3H]QNB binding assays were carried out on each fraction. The data are presented as the percentage of total [3H]QNB binding activity. Experiments were repeated three times with essentially the same results, and results of one typical experiment are shown. Total [3H]QNB binding sites of control and carbamylcholine-treated cells were 5.3 and 7.0 pmol, respectively. The inhibition of sequestration by the hypertonic medium containing 0.32 M sucrose (Fig. 1A) was much more marked than the decrease in [3H]NMS binding sites. Hypotonic medium containing 0.32 M sucrose (Fig. 1F) indicated that the transfer of [3H]QNB binding sites represents the translocation of [3H]NMS binding sites to the heavy membrane fraction containing cell surface membranes and a light fraction containing intracellular vesicles (endosomes). As shown in Fig. 2, the peak of [3H]QNB binding sites shifted from the heavy to light fraction by treatment of cells with 10^{-6} M carbamylcholine for 20 min. This result is consistent with the interpretation that the sequestered [3H]NMS binding sites corresponding to approximately 50% of total hm2 receptors were transferred from cell membranes to light vesicle fractions.

We have also followed internalization using laser scanning confocal microscopy. CHO cells expressing Myc-tagged hm2 receptors alone or Myc-tagged hm2 receptors together with GRK2 were labeled with anti-Myc monoclonal antibody (9E10) as described previously by Tolbert and Lameh (32). In the absence of agonist, hm2 receptors can be observed only at the cell surface (Fig. 3, A and C). When the cells were treated with 10^{-6} M carbamylcholine for 10 min, vesicles containing hm2 receptors were observed only in cells coexpressing GRK2 (Fig. 3D). In the cells expressing only hm2 receptors, no intracellular vesicles containing hm2 receptors were observed after agonist treatment (Fig. 3B).

These results provide evidence that the sequestration/internalization observed as the loss of [3H]NMS binding sites and the transfer of [3H]QNB binding sites represents the translocation of hm2 receptors from plasma membranes into cytoplasmic vesicles.

Down-regulation of hm2 Receptors as Assessed by the Decrease in [3H]QNB Binding Sites—The down-regulation of hm2 receptors was assessed as the agonist-induced decrease in [3H]QNB binding sites, as the tertiary amine [3H]QNB can penetrate the cell membranes and label total hm2 receptors, whereas the quaternary amine [3H]NMS cannot penetrate the cell membrane and labels only surface hm2 receptors. As shown in Fig. 4, the [3H]QNB binding sites decreased with slower rates compared with the decrease in [3H]NMS binding sites from the cell surface, and the rate was dependent on the carbamylcholine concentration. In cells expressing hm2 receptors alone, [3H]QNB binding sites decreased by 60% upon incubation with 10^{-6} M or higher concentration of carbamylcholine for 16 h. In cells expressing both hm2 receptors and GRK2, the loss of [3H]QNB binding site was similar initially, and enhanced down-regulation by 70% appeared only at 16 h pretreatment (Fig. 4B). In contrast, down-regulation was undetectable in the presence of 10^{-6} M carbamylcholine without GRK2, whereas significant down-regulation occurred with GRK2 coexpression (Fig. 4A). Apparent EC_{50} values of carba-
mycholine for the down-regulation of hm2 receptors after 16 h of treatment were estimated to be 0.7 and 6 \(\mu\)M for cells with or without coexpression of GRK2 (Fig. 4D). These results provide the first evidence that the down-regulation of G protein-coupled receptors is facilitated by coexpression of GRK2 and suggest that phosphorylation by GRK2 of hm2 receptors is directly or indirectly linked to their down-regulation.

Down-regulation of hm2 receptors, as well as their sequestration, was markedly inhibited in the hypertonic medium (Fig. 4E). When cells were treated with \(10^{-4}\) \(\mu\)M carbachol for 4 h, the proportions of down-regulated receptors were 17–18\% in the hypertonic medium, in contrast with 39–49\% in the normal medium. The inhibition was observed irrespective of the coexpression of GRK2 or not. The finding that both sequestration and down-regulation involve the same event, e.g. the internalization through coated vesicles.

Sequestration and Down-regulation of I3-del m2 Receptors—We have stably expressed I3-del m2 receptors in CHO

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**FIG. 4.** Effects of incubation time and carbachol (CCh) concentrations on down-regulation of [\(^3\)H]QNB binding sites on CHO cell. CHO cells expressing hm2 alone or hm2 plus GRK2 were incubated with \(10^{-5}\) \(\mu\)M (A), \(10^{-4}\) \(\mu\)M (B), and \(10^{-3}\) \(\mu\)M (C) carbachol for the indicated times or with various concentrations of carbachol for 16 h (D), and then subjected to [\(^3\)H]QNB binding assays at 4 \(^\circ\)C for 4 h. In experiments shown in Fig. 4 (E), CHO cells were incubated with \(10^{-3}\) \(\mu\)M carbachol for 2 or 4 h in the hypertonic medium containing 0.32 \(\mu\)M sucrose. Results are shown as means \(\pm\) S.D. from three independent experiments. Time-course and dose-response curves were fitted to the following equations: \(B \times \exp(-0.69t/t_{1/2}) + (100 - B) + R_{\max} \times \frac{EC_{50}}{EC_{50} + [carbachol]} + (100 - R_{\max})\), respectively. The values of \(B\) and \(t_{1/2}\), in control cells and GRK2-expressing cells were as follows: panel A, \(10^{-5}\) \(\mu\)M carbachol, \(B = 1.0\%\) (control) and 56\% (GRK2), \(t_{1/2} = 0.01\) h (control) and 9.9 h (GRK2); panel B, \(10^{-4}\) \(\mu\)M carbachol, \(B = 51\%\) (control) and 99\% (GRK2), \(t_{1/2} = 3.3\) h (control) and 9.4 h (GRK2); panel C, \(10^{-3}\) \(\mu\)M carbachol, \(B = 57\%\) (control) and 71\% (GRK2), \(t_{1/2} = 2.3\) h (control) and 2.9 h (GRK2). The values of \(EC_{50}\) for carbachol in control and GRK2-expressing cells were estimated to be 5.7 and 0.65 \(\mu\)M, respectively. [\(^3\)H]QNB binding sites of control and GRK2-expressing cells were 230 and 370 fmol/well, respectively.
cells and examined changes in [3H]NMS and [3H]QNB binding sites on cells treated with different concentrations of carbamylcholine for various times. Phosphorylation sites by GRK2 in hm2 receptors are known to be in the I3-loop (22), and I3-del m2 receptors are not phosphorylated by GRK2 (4). I3-del m2 receptors transiently expressed in HEK293 cells have been shown to sequester much less than hm2 receptors (21). Similarly, I3-del m2 receptors in CHO cells failed to sequester significantly upon treatment with carbamylcholine for 1 h (Fig. 5A). The [3H]NMS binding sites were gradually decreased upon prolonged incubation with carbamylcholine, but the rate of loss was much lower for I3-del m2 receptors (t1/2 = 8.4 h) than for wild type hm2 receptors (t1/2 = 9.5 min) (Fig. 5C).

Fig. 5B shows changes in [3H]QNB binding sites after incubation of cells expressing wild type and I3-del m2 receptors for 16 h with different concentrations of carbamylcholine. Unexpectedly, appreciable loss of [3H]QNB binding sites was observed even for I3-del m2 receptors, although the extent of loss was less for I3-del m2 receptors (44%) than for hm2 receptors (60%). The rate of loss was also much slower for I3-del m2 receptors than for hm2 receptors (t1/2 = 9.9 versus 2.3 h) (Fig. 5C). These results indicate that the presence of the I3-loop is not required for agonist-induced down-regulation, although it may accelerate the rate of down-regulation. The loss of [3H]QNB binding sites in the presence of 10^{-4} M carbamylcholine occurred in parallel with the loss of [3H]NMS binding sites for I3-del m2 receptors, (t1/2 = 9.9 and 8.4 h for the loss of [3H]QNB and [3H]NMS binding sites, respectively), in sharp contrast to the rates for hm2 wild type receptors (t1/2 = 2.3 h and 9.5 min, respectively) (Fig. 5C). These results indicate that the I3-del m2 receptors are down-regulated as soon as they are lost from the cell surface and that no appreciable amounts of I3-del m2 receptors exist in an internalized form, whereas 40–60% of hm2 receptors exist in an internalized form (Figs. 5C and 6).

DISCUSSION

In previous studies (23), we have shown that sequestration of m2 receptors transiently expressed in COS-7 and BHK-21 cells was facilitated by coexpression of GRK2, an effect of which was evident only at low concentrations of carbamylcholine. In the present study, a similar effect of coexpression of GRK2 was observed for the sequestration of hm2 receptors stably expressed in CHO cells. Furthermore, the sequestration assessed as the loss of [3H]NMS binding sites from the cell surface was confirmed to represent the internalization of hm2 receptors from plasma membranes into cytoplasmic vesicles by analyses involving sucrose density gradient centrifugation of membrane fractions and confocal microscopy. The fact that a similar effect was observed in three different cell lines suggests that facilitation by GRK of the internalization of hm2 receptors is a general phenomenon independent of cell species. On the other hand, Pals-Rylaarsdam et al. (8) have argued against the involvement of GRK2 in the internalization of hm2 receptors, based on the finding that the level of sequestration was not affected by coexpression of GRK2 or a DN-GRK2 in a clone of HEK293. They measured the sequestration of hm2 receptors in cells treated with only a high concentration of carbamylcholine (1 mM), and therefore could have missed the effect of GRK2 coexpression. Very recently, these authors have shown that a hm2 receptor mutant with alanine residues in the place of serine/threonine residues in the GRK2 phosphorylation sites was sequestered by a lower extent compared with the wild type receptor, and concluded that sequestration of hm2 receptors was promoted by their phosphorylation (28). As for the effect of coexpression of DN-GRK2, we have also failed to detect any effect on the sequestration of m2 receptors in CHO cells and BHK-21 cells (23), although the sequestration of m2 receptors expressed in COS-7 cells was significantly attenuated by coexpression of DN-GRK2. At present, we have not identified the species of endogenous GRKs or related kinases in these cells.
that the I3-loop may have other functions. Pals-Rylaasdam et al. reported that a hm2 mutant with a deletion (252–327) in the I3-loop was not phosphorylated by GRK2; yet 50% of the mutant receptors were sequestered by GRK2, and do not know the reason why the expression of DN-GRK2 affect the sequestration of m2 receptors in some cells but not in other cells.

In contrast to wild type hm2 receptors, I3-del m2 receptors (deletion 234–381), which lack phosphorylation sites by GRK2, failed to internalize rapidly. The simplest interpretation for this finding is that phosphorylation by GRK2 of serine or threonine residues in the I3-loop is a necessary step for rapid internalization. We cannot exclude, however, the possibility that the I3-loop may have other functions. Pals-Rylaasdam et al. reported that a hm2 mutant with a deletion (252–327) in the I3-loop was not phosphorylated by GRK2; yet 50% of the mutant stably expressed in HEK293 cells were sequestered by GRK2, and these authors have not examined the effect of different concentrations of agonist, and therefore, the ability of GRK2 to reduce the effective concentration might not have been noticed.

When hm2 receptor-expressing cells were treated with 10^{-4} M of carbamylcholine, hm2 receptors were rapidly internalized with a halftime of 9.5 min and slowly down-regulated with a halftime of 2.3 h. Thus, approximately 60% of receptors were down-regulated, 30% were in an internalized form, and 10% remained in the cell surface after a 16-h incubation (see Fig. 5C). In contrast, I3-del m2 receptors were lost from the cell surface and down-regulated with slower rates of halftime = 8.4 and 9.9 h, respectively, so that approximately 60% of receptors were down-regulated, no appreciable receptors were detectable in an internalized form, and 40% remained in the cell surface after a prolonged incubation (see Fig. 5C). These results indicate that down-regulation may occur without the I3-loop. However, the I3-loop is necessary for rapid internalization and accumulation of internalized receptors.

In Fig. 6, we have presented a tentative schema for the relationship between internalization and down-regulation of hm2 receptors. We assume in this schema that agonist-bound receptors are rapidly internalized and that internalized receptors are slowly down-regulated. This schema explains the present findings that both rapid internalization and down-regulation in the presence of low concentrations of carbamylcholine are accelerated in parallel by coexpression of GRK2; this explanation is based on the assumption that the amounts of phosphorylated hm2 receptors are increased by coexpression of GRK2, the rate of internalization is limited by the concentration of phosphorylated receptors, and the rate of down-regulation is limited by concentrations of internalized receptors. The finding that both sequestration and down-regulation are inhibited in the hypertonic medium supports the scheme and suggests that the rapid internalization occurs via coated vesicles. We, however, cannot exclude the possibility that the down-regulation occurs through multiple pathways. In contrast to hm2 receptors, the I3-del m2 receptors are lost from the cell surface and down-regulated with similar slow rates (see Fig. 5C), indicating that no appreciable amounts of receptors exist in an internalized form. It is possible that hm2 receptors may down-regulate via two independent pathways, the I3-loop-requiring and I3-loop-independent pathways, which do and do not involve the rapid internalization, respectively. The I3-loop-requiring and I3-loop-independent pathways may represent the coated vesicle-mediated and coated vesicle-independent pathways, respectively. This interpretation is consistent with the results that the internalization of hm2 receptors caused by 10^{-4} M carbamylcholine was completely suppressed in the hypertonic medium but the down-regulation was only partly suppressed, and that the proportions of down-regulated hm2 receptors in the hypertonic medium were similar to those of down-regulated I3-del hm2 receptors in normal medium (compare Figs. 4E and 5C). Another interpretation is that down-regulation of hm2 receptors occurs through a single step involving internalized vesicles and that the internalization step proceeds rapidly for hm2 receptors with intact I3-loop but greatly slows down and becomes the rate-limiting step for down-regulation for I3-del hm2 receptors. At present, the question remains open whether down-regulation of hm2 receptors occurs through a single route via internalized receptors or through multiple independent pathways.

In the present study, we have shown that both internalization and down-regulation of hm2 receptors are facilitated by coexpression of GRK2, and that the I3-loop is necessary for

**Fig. 6. Schema for a possible relationship between internalization and down-regulation of hm2 receptors.** The rates of internalization (9.5 min and 8.4 h) and down-regulation (2.3 and 9.9 h), and proportions of cell surface receptors, internalized receptors, and down-regulated receptors were estimated for cells treated with 10^{-4} M carbamylcholine for 16 h or more (Fig. 5C). R, receptor.
rapid internalization but not necessary for down-regulation, although the rate of down-regulation is reduced in its absence.

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