J. Biochem. 124, 863-868 (1998)

Desensitization of Human Muscarinic Acetylcholine Receptor m2 Subtypes Is Caused by Their Sequestration/Internalization[1]

Hirofumi Tsuga,*,† Kimihiko Kameyama,* and Tatsuya Haga*,[2]

*Department of Neurochemistry, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033; and †Division of Health Effects Research, National Institute of Industrial Health, 6-21-1 Nagao, Tama-ku, Kawasaki, Kanagawa 214-8585

Received for publication, June 24, 1998

Desensitization of human muscarinic acetylcholine receptor m2 subtypes (hm2 receptors) stably expressed in chinese hamster ovary cells was measured as decreases in the carbamylcholine-stimulated [^{35}S]GTPγS binding activity in membrane preparations after pretreatment of cells with carbamylcholine. The extent of carbamylcholine-stimulated [^{35}S]-GTPγS binding activity was found to decrease to 64% following pretreatment of cells with 10 μM carbamylcholine for 30 min, and under the same conditions 51-59% of hm2 receptors were sequestered/internalized as assessed by decreases in the [^{3}H]*N*-methylscopolamine binding activity on the cell surface. A similar reduction in the carbamylcholine-stimulated [^{35}S]GTPγS binding activity was observed by pretreatment of cells with 5 nM propylbenzylylcholine mustard, which irreversibly bound to and inactivated 58% of the hm2 receptors. When the cells were pretreated with 10 μM carbamylcholine in the presence of 0.32 M sucrose, which is known to inhibit clathrin-mediated endocytosis, no sequestration/internalization of hm2 receptors was observed, and the extent of carbamylcholine-stimulated [^{35}S]GTPγS binding activity did not change. These results indicate that desensitization of hm2 receptors may be caused by reduction of receptor number on the cell surface through sequestration/internalization rather than by loss of the function of receptors.

Key words: desensitization, G protein-coupled receptor kinase 2, internalization, muscarinic acetylcholine receptor, sequestration.

Homologous desensitization of G protein-coupled receptors is defined as the reduction in responsiveness to subsequent agonist treatment following prolonged agonist exposure. Desensitization may result from the functional uncoupling of receptors from G proteins (desensitization in a narrower sense), the loss of receptors from the cell surface (sequestration/internalization), and/or the reduced levels of receptors (down-regulation). Recently, evidence has accumulated showing that agonist-dependent phosphorylation of G protein-coupled receptors by G protein-coupled receptor kinases (GRKs) is involved in each of these three processes (*1-4*).

A number of studies have indicated that the phosphorylation of β_2-adrenergic receptors by GRK2 (=β-adrenergic receptor kinase 1=βARK1) may be involved in the functional uncoupling of β_2-adrenergic receptors from G protein Gs. Phosphorylated forms of β_2-adrenergic receptors were reported to be uncoupled from G proteins because of their interaction with arrestin 2 (=β-arrestin) (*5*). The relationship between phosphorylation by GRKs and functional uncoupling has also been suggested for other G protein-coupled receptors including muscarinic acetylcholine receptors m2 subtypes (m2 receptors) (*6-8*). The m2 receptor is reported to be phosphorylated by GRK2 (*9, 10*) and other GRKs (*11, 12*), and to interact with arrestin 2 and arrestin 3 (*13-15*).

The phosphorylation by GRKs of β_2-adrenergic receptors was originally thought to be concerned with only functional uncoupling and not with the sequestration/internalization (for review, see Ref. *3*). Recently, however, Ferguson *et al.* reported that the sequestration/internalization of a β_2-adrenergic receptor mutant is facilitated by its phosphorylation by GRKs (*16, 17*), and suggested that sequestration/internalization of β_2-adrenergic receptors is also facilitated by phosphorylation by means of the interaction of arrestin 2 (*18*). Furthermore, Goodman *et al.* (*19*) have demonstrated the interaction of arrestin 2/arrestin 3 with clathrin, a major protein of coated vesicles.

We have shown that agonist-dependent phosphorylation and sequestration/internalization of hm2 receptors expressed in COS-7 cells are facilitated by coexpression of GRK2 and attenuated by coexpression of a dominant-nega-

[1] This work was supported in part by grants from Japan Society for the Promotion of Science, the Ministry of Education, Science, Sports and Culture of Japan, Japan Health Science Foundation, and Japan Science and Technology Corporation (CREST).

[2] To whom correspondence should be addressed at: Department of Neurochemistry, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033. Tel: +81-3-5689-7331, Fax: +81-3-3814-8154, E-mail: haga@m.u-tokyo.ac.jp

Abbreviations: hm2 receptor, human muscarinic acetylcholine receptor m2 subtype; GRK, G protein-coupled receptor kinase; G protein, guanine nucleotide-binding regulatory protein; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate; PBS, phosphate-buffered saline; PrBCM, propylbenzylylcholine mustard.

tive mutant of GRK2 that lacks kinase activity (*20*). We have also shown that coexpression of GRK2 facilitates both sequestration/internalization and down-regulation of hm2 receptors stably expressed in CHO-K1 cells (*21*), and that sequestration of m2-m5 receptors in COS-7 and BHK-21 cells is facilitated by GRK2 and other GRKs (*22*). Pals-Rylaarsdam *et al.* have indicated that the phosphorylation by GRK2 of hm2 receptors expressed in HEK293 cells may be involved in their desensitization but not in their sequestration (*6*). Recently, however, they have reported that both sequestration and desensitization of hm2 receptors are attenuated by deletion of phosphorylation sites in GRK2 (*7*), and that sequestration is facilitated by coexpression of arrestin 2/arrestin 3 (*15*). Schlador and Nathanson have also shown that overexpression of GRK2 and β-arrestin synergistically enhance both sequestration and desensitization of porcine m2 receptors in JEG-3 cells (*8*). These results indicate that the phosphorylation by GRK2 of m2 receptors is involved in both sequestration and desensitization through facilitation of their interaction with β-arrestin/arrestin 3.

The relationship between desensitization and sequestration, however, has not yet been clarified. If the number of receptors on the cell surface is a limiting factor, the sequestration/internalization of receptors from the cell surface should result in the reduced activation of G proteins, that is desensitization in a broader sense. In fact, desensitization of m3 muscarinic receptors expressed in HEK293 cells was reported to be caused by their sequestration/internalization (*23*). On the other hand, a m2 receptor mutant expressed in HEK293 cells is reported not to be desensitized under conditions where 75% of the receptor was sequestered/internalized (*7*). Sequestration/internalization of β_2-adrenergic receptors has been reported to contribute to their resensitization rather than their desensitization, and it was proposed, although not proven, that the phosphorylated- and desensitized-receptors are dephosphorylated and resensitized in internalized vesicles (*24, 25*). Bogatkewitsch *et al.*, however, have reported that sequestration/internalization of m4 muscarinic receptors delays the process of resensitization of desensitized receptors, in contrast to the case of β_2-adrenergic receptors (*26*). Thus sequestration/internalization of receptors appears to cause both desensitization and resensitization, depending on the receptor species or experimental conditions.

In the present studies, we have examined the relationship between sequestration/internalization and desensitization of m2 receptors stably expressed in CHO cells, and provide evidence that the sequestration/internalization of m2 receptors causes their desensitization through physical uncoupling from G proteins.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]NMS (specific activity of 71.3 Ci/mmol), [^{3}H]QNB (specific activity of 36.4 Ci/mmol), and GTPγS (specific activity of 30-40 cpm/fmol) were purchased from Du Pont-New England Nuclear; restriction enzymes from Toyobo and Takara Shuzo. GRK2 cDNA was kindly donated by Dr. R.J. Lefkowitz (Duke University); cDNA encoding hm2 receptors by Dr. W. Sadée (University of California, San Francisco); mammalian expression vector for hygromycin resistant gene (pSV-hygro) from Dr. H. Okayama (The University of Tokyo); mammalian expression vector with neomycin resistant gene (pEF-neo) and mammalian expression vector pEF-BOS from Dr. S. Nagata (Osaka Bioscience Institute); chinese hamster ovary CHO-K1 cells from the Health Science Research Resources Bank.

Construction of Stable Transfectants Expressing hm2 Receptors and GRK2—The construction of mammalian expression vectors for c-Myc epitope-tagged hm2 receptor (pEF-Myc-hm2) and GRK2 (pEF-GRK2) was described previously (*20*). CHO-K1 cells (5×10^4 cells) were transfected with 18 μg of pEF-Myc-hm2 expression vector and 2 μg of pEF-neo using the calcium phosphate precipitation method (*27*). Stable transfectants were selected in the presence of 400 μg/ml of geneticin (Life Technologies) and subcloned by limiting dilution. Expression of receptors was detected by [^{3}H]QNB binding. The [^{3}H]QNB-binding sites in these cells were estimated to be 165 fmol/mg protein in homogenates. The transfectants were cultured in F-12 nutrient mixture (Ham's) (Life Technologies) supplemented with 10% fetal bovine serum (Cansera International), 40 units/ml penicillin G (Meiji Seika), 40 mg/ml streptomycin sulfate (Meiji Seika), and 100 μg/ml geneticin at 37°C in 95% air and 5% CO_2. 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 transfectants were selected in the presence of 300 μg/ml of hygromycin B (Boehringer Mannheim) and subcloned by limiting dilution. Expression of GRK2 was detected with use of Western blotting as described previously (*20*). The [^{3}H]QNB-binding sites of these cells were estimated to be 330 fmol/mg protein in homogenates, and expressed amounts of GRK2 were estimated by immunostaining with anti-GRK2 antibodies to be 300-600 fmol/mg in the supernatant protein. The transfectants 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% CO_2.

Measurements of Sequestration/Internalization of hm2 Receptors—Agonist-dependent sequestration/internalization of hm2 receptors was assessed as the loss of [^{3}H]NMS binding activity from the cell surface as follows. CHO cells (1×10^4 cells/well) were plated onto 12-well culture dishes. Forty to 48 h after plating, 10^{-5} M of carbamylcholine were added to the culture media. After incubation with carbamylcholine for 30 min, cells were washed three times with 1 ml per well of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5) and incubated with 1.2-1.6 nM [^{3}H]NMS in Hepes-buffered saline (25 mM Hepes, 113 mM NaCl, 6 mM glucose, 3 mM $CaCl_2$, 3 mM KCl, 2 mM $MgSO_4$, and 1 mM NaH_2PO_4, pH 7.4; 0.5 ml per well) at 4°C for 4 h. After incubation, cells were washed three times with 1 ml per well of ice-cold PBS. After washing, cells were dissolved in 0.3 ml of 1% Triton X-100 (w/v), mixed with 4.5 ml of Triton-Toluene cocktail containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(methyl-5-phenyloxazolyl)-benzene, and the radioactivity was measured. Triplicate samples were assayed for each point. In some experiments, cells were treated with carbamylcholine in the hypertonic medium containing 0.32 M sucrose besides normal constituents. For assay of total amounts of hm2 receptors,

[^{3}H]NMS was replaced by [^{3}H]QNB. [^{3}H]NMS, a quaternary amine, cannot penetrate the cell membrane, whereas [^{3}H]QNB, a tertiary amine, can penetrate it.

Measurement of Uncoupling of hm2 Receptors from G Proteins—The function of hm2 receptors was measured as the agonist-stimulated [^{35}S]GTPγS binding activity of the membrane preparation. [^{35}S]GTPγS binding assay was carried out as described by Lazareno *et al.* (*28*). Semi-confluent CHO cells cultured in a 15-cm diameter dish were treated with 10^{-5} M carbamylcholine or with 5×10^{-9} M propylbenzylylcholine mustard (PrBCM) for 30 min, then washed with 10 ml of ice-cold PBS three times. In some experiments, the medium was changed to one containing 0.32 M sucrose 10 min before treatment with carbamylcholine. The washed cells were scraped free, suspended in HMEE buffer (20 mM Hepes-KOH, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA, pH 7.4) and homogenized with Potter-Elvehjam homogenizer. The homogenate was centrifuged at 1,500 rpm for 10 min at 4°C, and the supernatant was centrifuged at $100,000\times g$ for 30 min at 4°C. The pellet was resuspended in HMEE buffer, and an aliquot containing 50 μg of protein was incubated in 0.2 ml of HENDM buffer (20 mM Hepes-KOH, 2 mM $MgCl_2$, 1 mM EDTA, 160 mM NaCl, 1 mM dithiothreitol, pH 7.4) supplemented with 1 μM GDP, 0.1 nM [^{35}S]GTPγS, and various concentrations of carbamylcholine at 30°C for 20 min. After incubation, 0.8 ml of ice-cold HEN buffer supplemented with 0.1 mM GTP was added to the reaction mixture, then membranes were trapped onto Whatman GF-B glass fiber filters, washed four times with 1 ml of washing buffer (20 mM Tris, 100 mM NaCl, and 25 mM $MgCl_2$, pH 8.0), and radioactivity was determined.

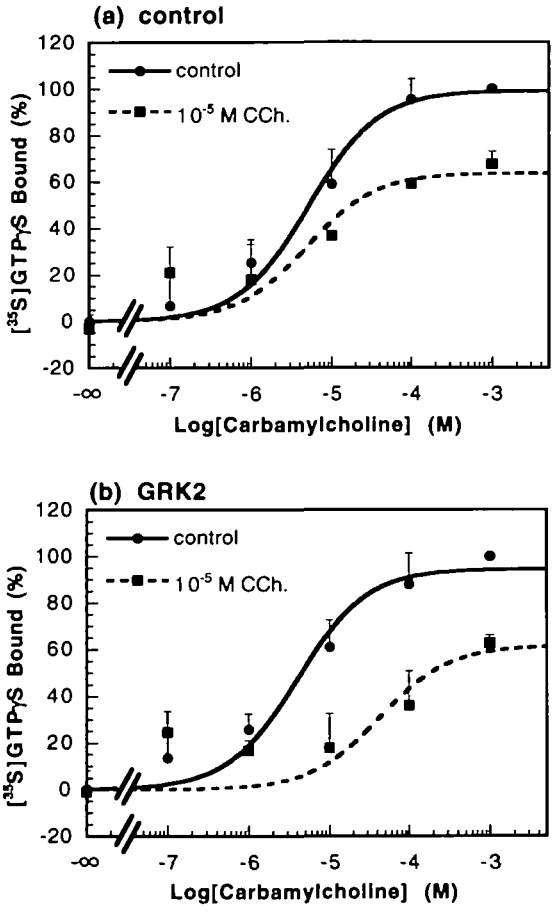

Fig. 1. **Desensitization of hm2 receptors in CHO cells treated with 10^{-5} M carbamylcholine for 30 min.** CHO cells expressing hm2 alone (a) or hm2 plus GRK2 (b) were incubated with or without 10^{-5} M carbamylcholine for 30 min, followed by preparation of membrane fractions. The membrane preparation containing 50 μg of protein was subjected to [^{35}S]GTPγS binding assays in the presence of different concentrations of carbamylcholine, as described under "EXPERIMENTAL PROCEDURES." Results are shown as mean ± SD from three to five independent experiments. Dose response curves were fitted to the equation $B_{max}\times$[Carbamylcholine]/(EC_{50} + [Carbamylcholine]), where the value in the presence of 10^{-3} M carbamylcholine for cells treated without carbamylcholine was taken as 100%. The B_{max} values were estimated to be (a) 64 and 99%, (b) 62 and 95% for cells treated or not treated with 10^{-5} M carbamylcholine, respectively. The EC_{50} values were estimated to be (a) 4.9 and 5.2 μM, (b) 43 and 4.0 μM for cells treated or not treated 10^{-5} M carbamylcholine, respectively. Some points, especially at low concentrations of carbamylcholine, did not fit above equation, but the reason remains unknown. Absolute values for 0 and 100% are (a) 460-1,350 cpm and 820-2,100 cpm, (b) 730-1,200 cpm and 1,130-1,950 cpm, respectively.

RESULTS AND DISCUSSION

We have examined the effect of agonist-treatment on the coupling between hm2 receptors and G proteins using a [^{35}S]GTPγS binding assay in membrane preparations of CHO cells expressing hm2 receptors. Carbamylcholine stimulates the [^{35}S]GTPγS binding to G proteins G_i and G_o of membrane preparations in the presence of unlabeled GDP, because carbamylcholine facilitates the dissociation of GDP from G proteins and lowers the affinity for GDP of G proteins (*28, 29*). We prepared crude membrane fractions from CHO cells which had been treated with or without 10^{-5} M carbamylcholine for 30 min, and measured the [^{35}S]GTPγS binding activity in the presence of different concentrations of carbamylcholine, 0.1 nM [^{35}S]-GTPγS, and 1 μM GDP. As shown in Fig. 1a, the extent of carbamylcholine-stimulated [^{35}S]GTPγS binding activity was decreased to 64% by the pretreatment of cells with carbamylcholine. In cells expressing both hm2 and GRK2, the concentrations of carbamylcholine required to stimulate [^{35}S]GTPγS binding activity were also increased by the pretreatment (Fig. 1b).

Following the same pretreatment with carbamylcholine,

TABLE 1. **Sequestration/internalization and down-regulation of hm2 receptors in CHO-K1 cells expressing hm2 receptors with or without GRK2.** CHO-K1 cells expressing hm2 receptors with or without GRK2 were incubated with 10^{-5} M carbamylcholine for 30 min in normal medium or hypertonic medium containing 0.32 M sucrose. Proportions of sequestered or down-regulated receptors were determined as decrease in the [^{3}H]NMS-binding sites on the cell surface or in the [^{3}H]QNB-binding sites in total cells, respectively. Results are shown as means ± SD from three independent experiments. Significant differences compared with control cells with $p<0.05$ are labeled with *.

	In normal medium		In hypertonic medium	
	Sequestered receptors (%)	Down-regulated receptors (%)	Sequestered receptors (%)	Down-regulated receptors (%)
Control	50.9±6.9	4.8±2.8	0.0±8.8	2.7±6.7
GRK2	59.3±4.0*	1.4±5.7	1.2±3.9	−2.1±3.8

50.9 and 59.3% of hm2 receptors were sequestered/internalized from the cell surface of CHO cells expressing hm2 receptors alone and hm2 receptors plus GRK2, respectively (Table I). Under the same conditions, the [^{3}H]QNB binding activity was not affected by the pretreatment in either type of cells (Table I), indicating that hm2 receptors were internalized from the cell surface but not down-regulated. To determine whether the apparent desensitization shown in Fig. 1 is due to the functional uncoupling of receptors or to the loss of receptors from the cell surface, we pretreated the cells with carbamylcholine in the presence of 0.32 M sucrose, then measured the [^{35}S]GTPγS binding activity. This was done because hypertonic medium is known to inhibit the internalization *via* clathrin-coated vesicles (*30*). In fact, no sequestration/internalization was observed upon treatment of CHO cells with 10^{-5} M carbamylcholine in the hypertonic medium in the presence of 0.32 M sucrose (Table I). The carbamylcholine-stimulated [^{35}S]GTPγS binding activity was also found not to be affected by pretreatment with carbamylcholine in hypertonic medium containing 0.32 M sucrose for cells expressing hm2 receptors alone, except that the dose-response curve was slightly shifted to the right (Fig. 2a). For cells expressing hm2 receptors and GRK2, a slight decrease in carbamylcholine-stimulated [^{35}S]GTPγS binding activity was observed, and the extent of shift in the dose-response curve was greater than for cells expressing hm2 receptors alone (Fig. 2b). These changes were reproducibly observed, but the differences were not statistically significant. These results suggest that the apparent desensitization shown in Fig. 1 was due to primarily the loss of surface receptors rather than functional uncoupling. Alternatively, there is a possibility that the functional desensitization might have been abolished by the treatment of cells with the hypertonic medium independent of sequestration/internalization. We therefore took another approach to test the hypothesis that the desensitization is caused by receptor loss. We reduced the receptor number by treating cells with an irreversible ligand, PrBCM, and examined if the apparent desensitization would be caused by the loss of receptors.

As shown in Fig. 3, carbamylcholine-stimulated [^{35}S]-GTPγS binding activity was reduced to 44% by the pretreatment of cells with 5×10^{-9} M PrBCM for 30 min, which resulted in the loss of 58% of [^{3}H]NMS binding activity. Thus, treatment of cells with 10^{-5} M carbamylcholine and 5×10^{-9} M PrBCM caused very similar reductions in the amount of surface receptors (51 *vs.* 58% loss) and in the carbamylcholine-stimulated [^{35}S]GTPγS binding activities (compare Fig. 1a and Fig. 3). These results are consistent with the hypothesis that the apparent desensitization of hm2 receptors induced by the pretreatment of cells with 10^{-5} M carbamylcholine could have been caused mostly, if not completely, by sequestration/internalization of hm2 receptors. These results, however, do not exclude the possibility that the functional uncoupling of hm2

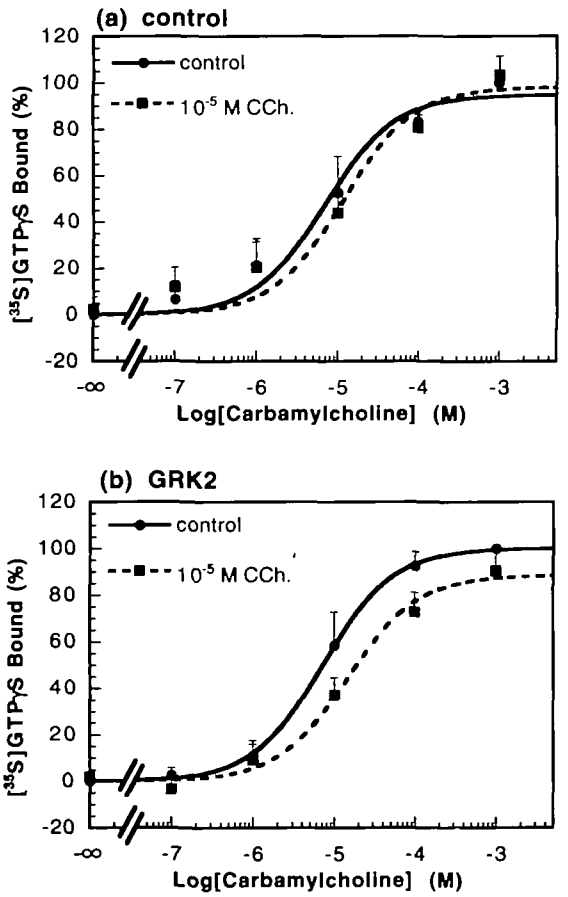

Fig. 2. **Desensitization of hm2 receptors in CHO cells treated with 10^{-5} M carbamylcholine in the presence of 0.32 M sucrose.** Experimental procedures are the same as described in the legend to Fig. 1 except that 0.32 M sucrose was included in the medium for the pretreatment. Results are shown as means ± SD from three independent experiments. The B_{max} values were estimated to be (a) 9 and 95%, (b) 89 and 100% for cells treated or not treated with 10^{-5} M carbamylcholine, respectively. The EC_{50} values were estimated to be (a) 12 and 6.9 μM, (b) 15 and 7.3 μM for cells treated or not treated with 10^{-5} M carbamylcholine, respectively. Absolute values for 0 and 100% are (a) 730-790 cpm and 1,400-1,590 cpm, (b) 770-1,000 cpm and 1,350-2,050 cpm, respectively.

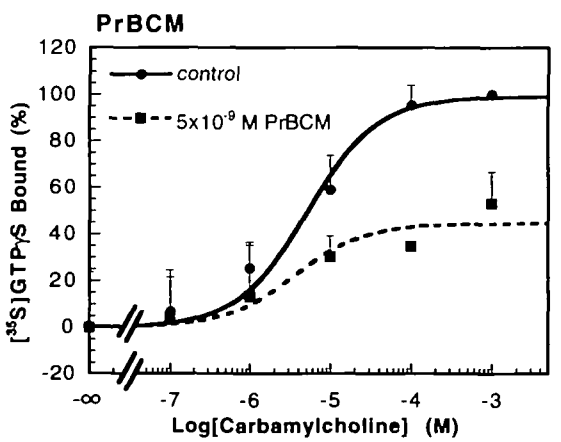

Fig. 3. **Apparent desensitization of hm2 receptors in CHO cells treated with 5×10^{-9} M PrBCM.** Experimental procedures are the same as described in the legend to Fig. 1 except that CHO cells expressing hm2 receptors alone were pretreated with or without 5×10^{-9} M PrBCM for 30 min in the absence of carbamylcholine. Results are shown as means ± SD from three to five independent experiments. The B_{max} values were estimated to be 44 and 99% for cells treated without or with 5×10^{-9} M PrBCM, respectively. The EC_{50} values were estimated to be 3.6 and 5.2 μM for cells treated or not treated with 5×10^{-9} M PrBCM, respectively. Absolute values for 0 and 100% are 460-1,350 cpm and 820-2,100 cpm, respectively.

receptors from G proteins occurs independently of their sequestration/internalization. The rightward shifts of the dose-response curves for the carbamylcholine-stimulated [^{35}S]GTPγS binding, which was more apparent for cells expressing GRK2, might represent the functional uncoupling of hm2 receptors from G proteins (Fig. 2).

In the present study, the desensitization of hm2 receptors expressed in CHO cells was found to be mostly due to the loss of surface receptors by sequestration/internalization. On the other hand, Pals-Rylaarsdam *et al.* reported that the desensitization of hm2 receptors expressed in a clone of HEK293 cells was independent of their sequestration/internalization (*6, 7*). There are several possible reasons why we could detect an effect of sequestration/internalization on desensitization. We have measured a direct function of hm2 receptors, *i.e.* activation of G proteins, and this assay could be more sensitive to the loss of receptors than the assay of cAMP-decrease used by Pals-Rylaarsdam *et al.* (*6, 7*). The cAMP assay might be unaffected by a partial loss of receptors when the number of activated G proteins is not a limiting factor. Alternatively, the differences in the cell types and the densities of expressed receptors might be reasons. The densities of hm2 receptors in HEK293 cells are reported to be 1-3 pmol/mg protein (*7*), whereas those in CHO cells are 0.16-0.33 pmol/mg protein. Thus there may be spare receptors for HEK293 cells but not for CHO cells. It remains, however, to be examined if the expression revels of receptors actually affect the mode of desensitization. In this regard, it should be pointed out that desensitization caused by sequestration/internalization may play a role in intact tissues, where expression levels of receptors are generally lower than those in cultured cells.

In the present study we have shown that the apparent desensitization of hm2 receptors might be caused by their sequestration/internalization from cell surface.

We thank Dr. R.J. Lefkowitz for cDNA of GRK2, Dr. S. Nagata for the mammalian expression vector pEF-BOS and pEF-neo, Dr. H. Okayama for pSV-hygro, the Health Science Research Resources Bank for CHO-K1 cells, and Dr. D.W. Saffen for comments and editing the manuscript.

REFERENCES

1. Haga, T., Haga, K., and Kameyama, K. (1994) G protein-coupled receptor kinases. *J. Neurochem.* **63**, 400-412
2. Premont, R.T., Inglese, J., and Lefkowitz, R.J. (1995) Protein kinase that phosphorylate activated G protein-coupled receptors. *FASEB J.* **9**, 175-182
3. Ferguson, S.S.G., Barak, L.S., Zhang, J., and Caron, M.G. (1996) G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. *Can. J. Physiol. Pharmacol.* **74**, 1095-1110
4. Böhm, S.K., Grady, E.F., and Bunnett, N.G. (1997) Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem. J.* **322**, 1-18
5. Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G., and Lefkowitz, R.J. (1990) β-Arrestin: a protein that regulates β-adrenergic receptor function. *Science* **248**, 1547-1550
6. Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J.L., and Hosey, M.M. (1995) Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. *J. Biol. Chem.* **270**, 29004-29011
7. Pals-Rylaarsdam, R. and Hosey, M.M. (1997) Two homologous phosphorylation domains differentially contribute to desensitization and internalization of the m2 muscarinic acetylcholine receptor. *J. Biol. Chem.* **272**, 14152-14158
8. Schlador, M.L. and Nathanson, N.M. (1997) Synergistic regulation of m2 muscarinic acetylcholine receptor desensitization and sequestration by G protein-coupled receptor kinase-2 and β-arrestin-1. *J. Biol. Chem.* **272**, 18882-18890
9. Richardson, R.M., Kim, C., Benovic, J.L., and Hosey, M.M. (1993) Phosphorylation and desensitization of human m2 muscarinic cholinergic receptors by two isoforms of the β-adrenergic receptor kinase. *J. Biol. Chem.* **268**, 13650-13656
10. Kameyama, K., Haga, K., Haga, T., Moro, O., and Sadée, W. (1994) Activation of a GTP-binding protein and a GTP-binding-protein-coupled receptor kinase (β-adrenergic-receptor kinase-1) by a muscarinic receptor m2 mutant lacking phosphorylation sites. *Eur. J. Biochem.* **226**, 267-276
11. Kunapuli, P., Onorato, J.J., Hosey, M.M., and Benovic, J.L. (1994) Expression, purification, and characterization of the G protein-coupled receptor kinase GRK5. *J. Biol. Chem.* **269**, 1099-1105
12. Loudon, R.P. and Benovic, J.L. (1994) Expression, purification, and characterization of the G protein-coupled receptor kinase GRK6. *J. Biol. Chem.* **269**, 22691-22697
13. Gurevich, V.V., Richardson, R.M., Kim, C.M., Hosey, M.M., and Benovic, J.L. (1993) Binding of wild type and chimeric arrestins to the m2 muscarinic cholinergic receptor. *J. Biol. Chem.* **268**, 16879-16882
14. Gurevich, V.V., Dion, S.B., Onorato, J.J., Ptasienski, J., Kim, C.M., Sterne-Marr, R., Hosey, M.M., and Benovic, J.L. (1995) Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, β_2-adrenergic, and m2 muscarinic cholinergic receptors. *J. Biol. Chem.* **270**, 720-731
15. Pals-Rylaarsdam, R., Gurevich, V.V., Lee, B.K., Ptasienski, J.A., Benovic, J.L., and Hosey, M.M. (1997) Internalization of the m2 muscarinic acetylcholine receptor: arrestin-independent and -dependent pathways. *J. Biol. Chem.* **272**, 23682-23689
16. Ferguson, S.S.G., Ménard, L., Barak, L.S., Koch, W.J., Colapietro, A.M., and Caron, M.G. (1995) Role of phosphorylation in agonist-promoted β_2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by βARK1. *J. Biol. Chem.* **270**, 24782-24789
17. Ménard, L., Ferguson, S.S.G., Barak, L.S., Bertrand, L., Premont, R.T., Colapietro, A.M., Lefkowitz, R.J., and Caron, M.G. (1996) Members of the G protein-coupled receptor kinase family that phosphorylate the β_2-adrenergic receptor facilitate sequestration. *Biochemistry* **35**, 4155-4160
18. Ferguson, S.S.G., Downey, W.E., Colapietro, A.M., Barak, L.S., Ménard, L., and Caron, M.G. (1996) Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* **271**, 363-366
19. Goodman, O.B., Krupnick, J.G., Santini, F., Gurevich, V.V., Penn, R.B., Gagnon, A.W., Keen, J.H., and Benovic, J.L. (1996) β-Arrestin acts as a clathrin adaptor in endocytosis of the β_2-adrenergic receptor. *Nature* **383**, 447-450
20. Tsuga, H., Kameyama, K., Haga, T., Kurose, H., and Nagao, T. (1994) Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant negative mutant of GRK2. *J. Biol. Chem.* **269**, 32522-32527
21. Tsuga, H., Kameyama, K., Haga, T., Honma, T., Lameh, J., and Sadée, W. (1998) Internalization and down-regulation of human muscarinic acetylcholine receptor hm2 subtypes: role of third intracellular m2 loop and G protein-coupled receptor kinase 2. *J. Biol. Chem.* **273**, 5323-5330
22. Tsuga, H., Okuno, E., Kameyama, K., and Haga, T. (1998) Sequestration of human muscarinic acetylcholine receptor hm1-hm5 subtypes: effect of G protein-coupled receptor kinases, GRK2, GRK4, GRK5 and GRK6. *J. Pharmacol. Exp. Ther.* **284**, 1218-1226
23. Yang, J., Williams, J.A., Yule, D.I., and Logsdon, C.D. (1995) Mutation of carboxyl-terminal threonine residues in human m3 muscarinic acetylcholine receptor modulates the extent of se-

questration and desensitization. *Mol. Pharmacol.* **48**, 477-485
24. Yu, S.S., Lefkowitz, R.J., and Hausdorff, W.P. (1993) β-Adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J. Biol. Chem.* **268**, 337-341
25. Pipig, S., Andexinger, S., and Lohse, M.J. (1995) Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* **47**, 666-676
26. Bogatkewitsch, G.S., Lenz, W., Jakobs, K.H., and Van Koppen, C.J. (1996) Receptor internalization delays m4 muscarinic acetylcholine receptor resensitization at the plasma membrane. *Mol. Pharmacol.* **50**, 424-429
27. Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752
28. Lazareno, S., Farries, T., and Birdsall, N.J.M. (1993) Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors m1-m4. *Life Sci.* **52**, 449-456
29. Shiozaki, K. and Haga, T. (1992) Effects of magnesium ion on the interaction of atrial muscarinic acetylcholine receptors and GTP-binding regulatory proteins. *Biochemistry* **31**, 10634-10642
30. Heuser, J.E. and Anderson, R.G.W. (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J. Cell. Biol.* **108**, 389-400