Sequestration of Muscarinic Acetylcholine Receptor m2 Subtypes

FACILITATION BY G PROTEIN-COUPLED RECEPTOR KINASE (GRK2) AND ATTENUATION BY A DOMINANT-NEGATIVE MUTANT OF GRK2*

Hirofumi Tsuga†, Kimihiko Kameyama, Tatsuya Haga, Hitoshi Kurose, and Taku Nagao

From the Department of Biochemistry, Institute for Brain Research, Faculty of Medicine and the Department of Toxicology and Pharmacology, Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

(Received for publication, July 26, 1994, and in revised form, September 8, 1994)

Sequestration of m2 receptors (muscarinic acetylcholine receptor m2 subtypes), which was assessed as loss of N-[3H]methylscopolamine ([3H]NMS) binding activity from the cell surface, was examined in COS 7 and BHK-21 cells that had been transfected with expression vectors encoding the m2 receptor and, independently, vectors encoding a G protein-coupled receptor kinase (GRK2) (β-adrenergic receptor kinase 1) or a GRK2 dominant-negative mutant (DN-GRK2). The sequestration of m2 receptors became apparent when the cells were treated with 10-4 M or higher concentrations of carbamylcholine. In this case, approximately 40% or 20-25% of the [3H]NMS binding sites on COS 7 or BHK-21 cells, respectively, were sequestered with a half-life of 15-25 min. In cells in which GRK2 was also expressed, the sequestration became apparent in the presence of 10-7 M carbamylcholine. Approximately 40% of the [3H]NMS binding sites on both COS 7 and BHK-21 cells were sequestered in the presence of 10-4 M or higher concentrations of carbamylcholine. When DN-GRK2 was expressed in COS 7 cells, the proportion of [3H]NMS binding sites sequestered in the presence of 10-4 M or higher concentrations of carbamylcholine was reduced to 20-30%. These results indicate that the phosphorylation of m2 receptors by GRK2 facilitates their sequestration. These results are in contrast with the absence of a correlation between sequestration and the phosphorylation of β-adrenergic receptors by the GRK2 and suggests that the consequences of phosphorylation by GRK2 are different for different receptors.

Muscarinic acetylcholine receptor (mACHR) m2 subtypes are phosphorylated in an agonist-dependent manner by G protein-coupled receptor kinases, including GRK2 (β-adrenergic receptor kinase 1) (1-3), GRK3 (3), GRK5 (4) and a muscarinic receptor kinase that is the same or closely related to GRK2 (5, 6). Agonist-dependent phosphorylation of mACHRs in vivo has also been reported, although the relevant kinases have not been identified (7, 8). It is generally thought that the phosphorylation of mACHRs is involved in their desensitization, but it is not known how mACHRs are desensitized following phosphorylation by a specific kinase. The present study focuses on the effect of receptor phosphorylation by GRK2 on the sequestration of the receptors.

A hydrophilic ligand N-[3H]methylscopolamine ([3H]NMS) containing a quaternary nitrogen is known to bind exclusively to mACHRs displayed on the cell surface, whereas a lipophilic ligand, [3H]quinuclidinyl benzilate ([3H]QNB), containing a tertiary nitrogen can label the total mACHR population (9). Exposure of neuroblastoma cells to agonists has been shown to cause the rapid disappearance of a certain portion of [3H]NMS binding sites from cell surfaces without changing the amounts of [3H]QNB binding sites (10-12). This phenomenon is referred to as sequestration or internalization of receptors and has been observed for a variety of receptors, including G protein-coupled receptors.

To our knowledge, the relationship between the phosphorylation of mACHRs by GRK2 and their sequestration has not yet been reported. In the case of β-adrenergic receptors, a correlation between receptor phosphorylation by GRK2 and sequestration has been contradicted by different lines of evidence, including studies using β-adrenergic receptor mutants lacking GRK2 phosphorylation sites (13-15). The specific GRK2 inhibitor heparin (16), and a dominant-negative mutant of GRK2 (17). It should be noted, however, that the GRK2 phosphorylation sites are different for mACHR and β-adrenergic receptors in the carboxyl-terminal domains in β-adrenergic receptors (18, 19) and in the central part of the third intracellular loop in m2 receptors (mACHR m2 subtypes) (20). Sadée and his groups (21-23) have shown that the deletion of third intracellular loop or substitution of serine and threonine residues in the central part of the third intracellular loop by alanine residues attenuates the sequestration of m1, m2, and m3 receptors. These serine and threonine residues in m2 receptors are included in putative phosphorylation sites, which were determined by chemical analysis (20, 23). These results are consistent with the hypothesis that the phosphorylation of m2 receptors by GRK2 is involved in their sequestration.

In the present report, we provide evidence that the sequestration of m2 receptors is facilitated by expression of GRK2 and is attenuated by expression of a dominant-negative mutant of GRK2. This finding indicates that the phosphorylation by GRK2 may have different consequences depending on the species of receptors.

EXPERIMENTAL PROCEDURES

Materials—[3H]NMS (specific activity of 71.3 or 80.4 Ci/mmol) and [3H]QNB (specific activity of 36.4 Ci/mmol) were purchased from DuPont NEN, [3H]phosphatase was purchased from American Corp., atropine methyl nitrate was purchased from Sigma, mammalian expression vector pSVL was purchased from Pharmacia Biotech Inc., restriction enzymes were purchased from Toyobo Corp., and the Transformer™ site-directed mutagenesis kit was purchased from Clontech.
cDNA of GRK2 was kindly donated by Dr. R. J. Lefkowitz, cDNA of porcine mACHr m2 subtype (pKPM2) was donated by the late Dr. S. Numa, cDNA of human mACHr m2 subtype (Hm2p5SGS) was donated by Dr. W. Sadee, and mammalian expression vector pEF-BOS was donated by Drs. S. Nagata and T. Shimizu. Hybridoma cells expressing hE10 were obtained from the American Type Culture Collection.

Construction of Plasmid Vectors—Muscarinic receptor kinase 2 (GRK2) peptide (569G-688L) linked to the carboxyl terminus of glutathione S-transferase. This antiserum recognized both GRK2 and DN-GRK2. After incubation with antiserum, the membranes were washed five times with blocking buffer and incubated with secondary antiserum (goat anti-rabbit IgG coupled to horseradish peroxidase purchased from Jackson ImmunoResearch Laboratories, Inc.) in the blocking buffer for 1 h at room temperature. After removal of the secondary antiserum, the membranes were washed with PBS supplemented with 0.1% Tween 20 (w/v) for 1 h at room temperature and then with 1/2,000 diluted antiserum for 2 h at room temperature. The antibody was raised against a fusion protein consisting of a GRK2 (β-adrenergic receptor kinase 2) peptide (569G-688L) linked to the carboxyl terminus of glutathione S-transferase. This antiserum recognized both GRK2 and GRK3. After incubation with antiserum, the membranes were washed five times with blocking buffer and incubated with secondary antiserum (goat anti-rabbit IgG coupled to horseradish peroxidase purchased from Jackson ImmunoResearch Laboratories, Inc.) in the blocking buffer for 1 h at room temperature. After removal of the secondary antiserum, the membranes were washed with PBS supplemented with 0.1% Tween 20 (w/v) for 1 h at room temperature and then with 1/2,000 diluted antiserum for 2 h at room temperature. The antibody was raised against a fusion protein consisting of a GRK2 (β-adrenergic receptor kinase 2) peptide (569G-688L) linked to the carboxyl terminus of glutathione S-transferase. This antiserum recognized both GRK2 and GRK3. After incubation with antiserum, the membranes were washed five times with blocking buffer and incubated with secondary antiserum (goat anti-rabbit IgG coupled to horseradish peroxidase purchased from Jackson ImmunoResearch Laboratories, Inc.) in the blocking buffer for 1 h at room temperature. After removal of the secondary antiserum, the membranes were washed with PBS supplemented with 0.1% Tween 20 (w/v) for 1 h at room temperature and then with 1/2,000 diluted antiserum for 2 h at room temperature.

Facilitation of Muscarinic Receptor Sequestration by pARK

RESULTS

Expression of m2 Receptors—GRK2, or DN-GRK2 in COS 7 and BHK-21 Cells—Human or porcine m2 receptors were transiently expressed in COS 7 and BHK-21 cells alone or with GRK2 or GRK2 mutant with a tryptophan residue substituted for lysine 220 (DN-GRK2). A vector containing the elongation factor gene promotor (pEF-BOS) was used for expression of

2,5-diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and then radioactivity was measured. Triplicate (BH2-1K) or quadruplicate (COS 7) samples were assayed for each point.

Detection of GRK2 and DN-GRK2 by Immunostaining and of GRK2 by Phosphorylation of m2 Receptors—Immunoreactivity of GRK2 and GRK2 was detected as follows. 40–48 h after transfection, cells were fixed and incubated with a 1/2,000 diluted antiserum for 2 h at room temperature. The antibody was raised against a fusion protein consisting of a GRK2 (β-adrenergic receptor kinase 2) peptide (569G-688L) linked to the carboxyl terminus of glutathione S-transferase. This antiserum recognized both GRK2 and GRK3. After incubation with antiserum, the membranes were washed five times with blocking buffer and incubated with secondary antiserum (goat anti-rabbit IgG coupled to horseradish peroxidase purchased from Jackson ImmunoResearch Laboratories, Inc.) in the blocking buffer for 1 h at room temperature. After removal of the secondary antiserum, the membranes were washed with PBS supplemented with 0.1% Tween 20 (w/v) for 1 h at room temperature and then with 1/2,000 diluted antiserum for 2 h at room temperature.

Facilitation of Muscarinic Receptor Sequestration by pARK

RESULTS

Expression of m2 Receptors—GRK2, or DN-GRK2 in COS 7 and BHK-21 Cells—Human or porcine m2 receptors were transiently expressed in COS 7 and BHK-21 cells alone or with GRK2 or GRK2 mutant with a tryptophan residue substituted for lysine 220 (DN-GRK2). A vector containing the elongation factor gene promotor (pEF-BOS) was used for expression of
these proteins in BHK-21 cells and COS 7 cells, with the exception of m2 receptor, which was expressed in COS 7 cells using a vector containing an SV40 promoter (pSVL). Levels of expression were much higher in COS 7 cells compared with BHK-21 cells, and the promoter for elongation factor was more efficient than SV40 promoter. The [3H]NMS binding sites on COS 7 cells transfected with pSVL-pm2, in combination with pEF-BOS, pEF-GRK2, or pEF-GRK2-K220W, were estimated to be 250–600, 500–1,400, and 500–1,300 fmol/mg protein of total homogenate, respectively. The [3H]NMS binding sites on BHK-21 cells transfected with pEF-hm2, in combination with pEF-BOS, pEF-GRK2, or pEF-GRK2-K220W, were estimated to be 140–230, 130–200, and 200–300 fmol/mg protein, respectively.

Expression of GRK2 or DN-GRK2 in COS 7 and BHK-21 cells was confirmed by immunostaining with anti-GRK2 antibodies (Fig. 1a). Expressed amounts of GRK2 and DN-GRK2 were estimated to be 12–25 and 3–6 pmol/mg of supernatant protein for COS 7 cells and 0.8–1.6 and 0.4–0.8 pmol/mg of supernatant protein for BHK-21 cells, respectively. Endogenous GRK2 was not detected in the supernatant of either COS 7 or BHK-21 cells. Kinase activity capable of phosphorylating m2 receptors in an agonist-dependent manner was detected in the supernatant fractions of COS 7 or BHK-21 cells transfected with pEF-GRK2, but the activity was not detected in the supernatant fraction of cells transfected with pEF-BOS or pEF-GRK2-K220W (Fig. 1b). The lack of kinase activity in cells expressing pEF-GRK2-K220W is consistent with previous results that the kinase activity was lost by substituting a lysine residue in the homologous position of Raf-1 kinase (32) or in the same position of GRK2 (17) by tryptophan or arginine residue, respectively.

To examine the phosphorylation of m2 receptors in vivo, we expressed Myc epitope-tagged m2 receptors in COS 7 cells with or without coexpression of GRK2 or DN-GRK2 and precipitated phosphorylated m2 receptors with the anti-Myc epitope antibody 9E10 (24). The m2 receptors were found to be phosphorylated in an agonist-dependent manner in vivo, and the phosphorylation was markedly increased or attenuated by the coexpression of GRK2 or DN-GRK2, respectively (Fig. 1c). This result indicates that DN-GRK2 functions as a dominant-negative mutant for GRK2 or related kinases.

Assessment of Sequestration—Sequestration of m2 receptors was assessed as the loss of [3H]NMS binding sites from the surface of intact cells following exposure to carbamylcholine. When COS 7 or BHK-21 cells were exposed to 10 μM carbamylcholine, the [3H]NMS binding to cells expressing m2 receptors did not significantly decrease following incubation for up to 120 min, whereas approximately 30% of the [3H]NMS binding disappeared from cells expressing both m2 receptors and GRK2 in less than 60 min. No such decrease was detected in cells expressing m2 receptors and DN-GRK2 (Fig. 2). The effect of carbamylcholine was suppressed by 10−6 M atropine methyl nitrate, and atropine methyl nitrate alone did not cause a decrease in [3H]NMS binding. In contrast with the [3H]NMS binding, the [3H]QNB binding did not significantly decrease upon incubation with carbamylcholine for up to 60 min, irrespective of the expression of GRK2 (Fig. 3). These results indicate that the agonist-dependent decrease of [3H]NMS binding, which is induced by expression of GRK2, is not due to down-regulation of m2 receptors but is due to sequestration of m2 receptors. An
approximately 15% decrease in the [3H]QNB binding activity after incubation of 120 min was observed in COS 7 cells, irrespective of the expression of GRK2. This may be due to the down-regulation of m2 receptors.

Sequestration of m2 Receptors in COS 7 Cells—Fig. 4 shows the effects of incubation time and concentrations of carbamylcholine on the decrease in [3H]NMS binding to COS 7 cells. In the presence of 10−6 M carbamylcholine, the decrease of [3H]NMS binding was apparent only for cells expressing GRK2 but not for cells expressing m2 alone or m2 plus DN-GRK2 (Fig. 4a). Time courses of the decrease of [3H]NMS binding to cells expressing GRK2 were essentially the same for 10−6, 10−5, or 10−4 M carbamylcholine, and 33–36% of [3H]NMS binding sites decreased with half-life of 15–25 min. The decrease in [3H]NMS binding to cells expressing m2 alone or m2 plus DN-GRK2 was apparent in the presence of 10−5 M or higher concentrations of carbamylcholine (Fig. 4, b and c). In the presence of 10−4 M carbamylcholine, the time courses of the decrease in [3H]NMS binding were virtually the same for cells expressing m2 alone and those expressing m2 plus GRK2 (Fig. 4c). On the other hand, the extent of the decrease of [3H]NMS binding activity was significantly lower in cells expressing m2 plus DN-GRK2 compared with cells expressing m2 alone or m2 plus GRK2 (Fig. 4, b and c).

Fig. 5 shows the [3H]NMS binding activity of cells exposed to various concentrations of carbamylcholine for 120 min. The decrease in [3H]NMS binding sites was apparent at 10−7–10−4 M carbamylcholine for cells expressing m2 plus GRK2, whereas 10−5 M or higher concentrations of carbamylcholine were necessary for a significant decrease in [3H]NMS binding sites to be observed in cells expressing m2 alone or m2 plus DN-GRK2. Proportions of decreased [3H]NMS binding sites in the presence of 10−5 M or higher concentrations of carbamylcholine were 35–45% for cells expressing m2 alone or m2 plus GRK2 but was 20–30% for cells expressing m2 plus DN-GRK2. These results indicate that GRK2 stimulates the sequestration of [3H]NMS binding sites in the presence of low concentrations of carbamylcholine and that DN-GRK2 attenuates the sequestration in the presence of high concentrations of carbamylcholine.

Sequestration of m2 Receptors in BHK-21 Cells—The effect of expression of GRK2 on sequestration of [3H]NMS binding sites was also observed for BHK-21 cells as shown in Fig. 6, despite the fact that the expression level of GRK2 in BHK-21 cells was 10–20 times lower compared with in COS 7 cells. Approximately 40% of [3H]NMS binding sites on cells expressing m2 plus GRK2 decreased with a half-life of 20 min in the presence of carbamylcholine of 10−5 M or higher concentrations. On the other hand, [3H]NMS binding sites on cells expressing m2 alone or m2 plus DN-GRK2 did not appreciably decrease upon exposure to 10−4 M carbamylcholine, and 20–25% of [3H]NMS binding sites decreased in the presence of carbamylcholine at 10−3 M or higher concentrations. In contrast with the results obtained with COS 7 cells, no difference was observed in the decrease of the [3H]NMS binding activity between cells expressing m2 alone and those expressing m2 plus DN-GRK2.
Facilitation of Muscarinic Receptor Sequestration by βARK

DISCUSSION

In the present studies, we have provided evidence that the expression of GRK2 in both COS 7 and BHK-21 cells induces the sequestration of m2 receptors at low concentrations of carbamylcholine. It would be reasonable to assume that this sequestration was induced by agonist-dependent phosphorylation of m2 receptors by GRK2 because the expression of GRK2 increases the phosphorylation of m2 receptors in vivo, and the expression of a DN-GRK2 mutant lacking the kinase activity does not induce sequestration nor increase the phosphorylation of m2 receptors.

The sequestration of m2 receptors on COS 7 cells at high concentrations of carbamylcholine was attenuated by expression of DN-GRK2. DN-GRK2 is thought to function as a dominant-negative mutant, as was shown for a similar mutant of GRK2 containing an arginine residue substituted for a lysine residue (17). Indeed, the expression of DN-GRK2 was found to suppress the phosphorylation of m2 receptors in COS 7 cells. DN-GRK2 may compete with GRK2 for interaction with agonist-bound m2 receptors. Agonist-bound m2 receptors appear to serve as both activators and substrates of GRK2 by interacting with the kinase via domains adjacent to transmembrane segments and the central part of the third intracellular loop, respectively (33). DN-GRK2 may also compete with GRK2 for interaction with G protein βγ subunits since GRK2 is known to be activated by G protein βγ subunits (5, 6) by interacting with them via the carboxyl-terminal domain (2, 34). Whether the target of DN-GRK2 is m2 receptors or G protein βγ subunits or both, the present results indicate that an endogenous GRK2 or a similar kinase is involved in the process of the sequestration of m2 receptors in COS 7.

Contrera et al. (35) reported that antisense oligodeoxynucleotides against GRK2 or GRK3 attenuated the desensitization of m2 receptors in cerebellar granule cells, as assessed by a de-
crease in the inhibition of adenylate cyclase activity mediated by m2 receptors. It was suggested, although not proven, that the desensitization was caused by the sequestration of m2 receptors.

The effect of expression of GRK2 on the sequestration of m2 receptors in COS 7 cells is largely on the effective concentration of carbacholamine rather than on the rate of sequestration or on the proportion of sequestered m2 receptors. Half-lives of sequestration of m2 receptors are 15–25 min, and the proportions of sequestered m2 receptors are 35–45%, with or without expression of GRK2, but the effective concentration of carbacholamine is more than 10-fold lower for cells expressing GRK2 compared with cells not expressing GRK2. Although the molecular mechanisms of sequestration are not known, the simplest assumption based on the present results may be that the sequestration is initiated by phosphorylation of m2 receptors by GRK2. The phosphorylation by GRK2 may cause different effects for different receptors.

In summary, we conclude that the phosphorylation of m2 receptors by GRK2 initiates their sequestration and suggests that consequences of phosphorylation by GRK2 may be different for different receptors.

Acknowledgments—We thank Dr. R. J. Lefkowitz for cDNA of GRK2, Dr. W. Sadée for cDNA of human m2 receptor and comments, Dr. S. Nagata for the mammalian expression vector pEF-BOS, Dr. T. Shimizu for COS 7 cells, the Japanese Cancer Research Resources Bank for BHK-21 cells, Drs. O. Moro and T. Ikegaya for advice and suggestions, and Dr. D. W. Saffen for comments and editing the manuscript.

REFERENCES