Sequestration of Human Muscarinic Acetylcholine Receptor hm1—hm5 Subtypes: Effect of G Protein-Coupled Receptor Kinases GRK2, GRK4, GRK5 and GRK6

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ABSTRACT

Sequestration of porcine muscarinic acetylcholine receptor m2 subtypes (m2 receptors) expressed in COS-7 cells is facilitated by coexpression of G protein-coupled receptor kinases 2 (GRK2). We examined the effect of coexpression of GRK2, GRK4, GRK5 and GRK6 on sequestration of human m1—m5 receptors expressed in COS-7 cells, which was measured as loss of [3H]N-methylscopolamine binding activity from the cell surface. Sequestration of m4 receptors as well as m2 receptors was facilitated by coexpression of GRK2 and attenuated by coexpression of the dominant negative form of GRK2 (DN-GRK2). Sequestration of m3 and m5 receptors also was facilitated by coexpression of GRK2 but not affected by coexpression of DN-GRK2. On the other hand, proportions of sequestered m1 receptors were not significantly different with coexpression of GRK2 and DN-GRK2. GRK4δ, GRK5 and GRK6 did not facilitate sequestration of m1—m5 receptors in COS-7 cells, except that the sequestration of m2 receptors was facilitated by coexpression of GRK4δ, GRK5 and GRK6. However, coexpression of GRK4δ, GRK5, but not GRK6, in BHK-21 cells facilitated sequestration of m2, but not m3, receptors. These results indicate that the effect of GRK2 to facilitate receptor sequestration is not restricted to m2 receptors but is generalized to other muscarinic receptors except m1 receptors and that other kinases, including GRK4δ, GRK5 and endogenous kinase(s) in COS-7 cells, also contribute to sequestration of m2 and m4 receptors.

Many kinds of G protein-coupled receptors are known to be phosphorylated by GRKs in an agonist-dependent manner (for a review, see Haga et al., 1994; Premont et al., 1995, Böhnl et al., 1997). Muscarinic receptors are also known to be phosphorylated by GRKs. Muscarinic acetylcholine receptor m2 subtypes (m2 receptors) are phosphorylated by GRK2 (beta adrenergic receptor kinase 1) (Kameyama et al., 1993; Richardson et al., 1993), GRK3 (Richardson et al., 1993), GRK5 (Kunapuli et al., 1994), GRK6 (Loudon and Benovic, 1994) and a muscarinic receptor kinase that is the same as or closely related to GRK2 (Haga and Haga, 1990, 1992). The number of phosphorylation sites is reported to be 10 or 11 for muscarinic receptor kinase (Nakata et al., 1994), 4 to 10 for GRK2 and GRK3 (Kunapuli et al., 1994), 1 to 1.5 for GRK5 (Kunapuli et al., 1994) and 0.6 for GRK6 (Loudon and Benovic, 1994). Muscarinic acetylcholine receptor m3 subtypes (m3 receptors) are also phosphorylated by GRK2 and GRK3 (four phosphorylation sites per receptor) but not by GRK5 and GRK6 (Debburman et al., 1995). Recently, m1 receptors have been reported to be phosphorylated in an agonist-dependent manner by the other kinase that is distinguishable from GRK2 or GRK3 (Tobin et al., 1996). Muscarinic acetylcholine receptor m1 subtypes (m1 receptors) had been reported to be not phosphorylated by GRK2 or endogenous kinase in Sf9 cells under the conditions in which m2 receptors were phosphorylated in an agonist-dependent manner by these kinases (Richardson and Hoesy, 1992; Haga et al., 1993), but recently m1 receptors were found to be phosphorylated by GRK2 after removal of unknown factor or factors that copurify with m1 receptors (four or five phosphorylation sites) (Haga et al., 1996). No direct evidence is available for phosphorylation of muscarinic acetylcholine receptor m4 and m5 subtypes (m4 and m5 receptors), as far as we know.

The phosphorylation of G protein-coupled receptors by GRKs is generally thought to be involved in homologous desensitization of receptors. Desensitization of G protein-coupled receptors occurs with three phases: uncoupling from...
G proteins, sequestration/internalization and down-regulation of receptors. Previously, we have shown that the 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 a kinase activity (Tsuga et al., 1994). When we found the relation between sequestration of m2 receptors and their phosphorylation by GRK2, various lines of evidence indicated that phosphorylation by GRK2 and sequestration of beta-adrenergic receptors are independent phenomena (Strader et al., 1987; Bouvier et al., 1988; Lohse et al., 1990; Kong et al., 1994). Therefore, we suggested previously that the phosphorylation by GRK2 might have different consequences depending on the species of receptors.

Recently, Ferguson et al. (1995) have shown that overexpression of GRK2 facilitates the sequestration of a beta adrenergic receptor mutant and suggested that phosphorylation by GRKs may play a broader role in agonist-promoted G protein-coupled receptor sequestration than envisaged above. Sequestration of the beta adrenergic receptor mutant was also shown to be facilitated by overexpression of beta-arrestin (Ferguson et al., 1996), and furthermore, beta-arrestin/ arrestin3 was found to interact with clathrin (Goodman et al., 1996). These results suggest that the sequestration of the beta adrenergic receptor mutant may occur through the following series of events: agonist-dependent phosphorylation of the receptor, binding of beta-arrestin to the phosphorylated receptors and binding of clathrin to beta-arrestin. Very recently, Pals-Rylaarsdam and Hosey (1997) also showed that phosphorylation of hm2 receptors facilitated its sequestration as well as desensitization. It is an open question of whether this scheme is also applicable to other G protein-coupled receptors. For Gαi-coupled receptors, the relationship between desensitization and phosphorylation by GRKs of alpha-1B adrenergic receptors has been reported (Diviani et al., 1996), but the relation between sequestration and phosphorylation by GRKs has not been reported to our best knowledge. The present article is concerned with the question of the generality of the relation between phosphorylation and sequestration of G protein-coupled receptors, and we specifically attempted to determine whether the sequestration of Gαi-coupled receptors is facilitated by phosphorylation with GRKs.

The results of this study provide evidence that coexpression of GRK2 facilitates the sequestration of m2, m3, m4 and m5 receptors and that GRK4δ and GRK5 may also facilitate sequestration of m2 receptors.

Methods

Materials. [3H]NMS (specific activity, 80.4 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Mammalian expression vector pSVL was from Pharmacia Biotech (Uppsala, Sweden). Restriction enzymes were from Toyobo (Osaka, Japan). cDNA of GRK2 was kindly donated by Dr. R. J. Lefkowitz (Duke University, Durham, NC). Expression vectors of GRK5 (pCMV5-GRK5) and GRK6 (pCMV5-GRK6) were provided by Drs. J. L. Benovic (Thomas Jefferson University, Philadelphia, PA) and H. Kurose (University of Tokyo, Tokyo, Japan). cDNA of GRK4δ was from Dr. R. T. Premont (Duke University, Durham, NC). cDNA of hm2 receptors was provided by Dr. W. Sádeé (University of California San Francisco, San Francisco, CA). cDNA of hm1 (Hm1pCD), hm4 (Hm4pCD) and hm5 (Hm5pCDp2) receptors was provided by Dr. T. I. Bonner (National Institutes of Health, Bethesda, MD). cDNA of hm3 receptors was from Dr. J. S. Gutz (National Institutes of Health, Bethesda, MD). Mammalian expression vector pEF-BOS was from Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan).

Cell culture. Syrian hamster kidney BHK-21(C-13) cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). African green monkey kidney COS-7 cells were from Dr. T. Shimizu (University of Tokyo, Tokyo, Japan). Both cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Cansera International, Rexdale, Ontario, Canada), 40 units/ml penicillin G (Meiji Seika Kaisha, Tokyo, Japan) and 40 mg/ml streptomycin sulfate (Meiji Seika Kaisha) at 37°C in 5% air/5% CO2.

Construction of mammalian expression vectors. Mammalian expression vectors for hm1—hm5 receptors were constructed as follows. An NsiI/BamHI fragment of cDNA of hm1 receptors was inserted in the Xbal site of pSVL after the conversion of the end of both fragments to blunt ends (pSVL-hm1). An XbaI fragment of Hm2pSG5 (Moro et al., 1993) was inserted in the XbaI site of pSVL (pSVL-hm2). A BamHI fragment of pEF-hm3 (Inoue et al., 1995) was inserted in the XbaI site of pSVL after the conversion of the end of both fragments to blunt ends (pSVL-hm3). A BamHI/BgII fragment of cDNA of hm4 receptor was inserted into BamHI site of pluescripts SK+ (pBluescript-hm4). The 363-bp SpeI/EcoRI fragment that includes hm4 cDNA between positions 1 and 363 was generated with the polymerase chain reaction using the primers 5’-GTTAGATTCGATGGCCAACCTACAGCTTT-3’ and 5’-GTTAGCAACGGCTCCGCTCGATAGATTCG-3’ and Hm4pCD as the template. An SpeI/BglII fragment of the generated fragment and PstI/EcoRV (1.4 kb) fragment of pBluescript-hm4 was inserted into SpeI/EcoRV site of pBluescript-Myc7 (Tsuga et al., 1994). Subsequently an SpeI/EcoRI fragment of this plasmid was inserted in the XbaI site of pSVL after the conversion of the end of both fragments to blunt ends (pSVL-hm4). An EcoRI/PstI fragment (1.7 kb) of cDNA of hm5 receptor was inserted in the XbaI site of pSVL after the conversion of the end of both fragments to blunt ends (pSVL-hm5). The construction of mammalian expression vectors for hm2 (pEF-hm2) and hm3 receptors (pEF-hm3) was described previously (Tsuga et al., 1994; Inoue et al., 1995).

The construction of mammalian expression vectors for GRK2 (pEF-GRK2) and DN-GRK2 (pEF-GRK2-K220W) was described previously (Tsuga et al., 1994). A mammalian expression vector for GRK4δ (pEF-GRK4δ) was constructed by inserting a KpnISacII fragment derived from pBluescript KS+ in the XbaI site of pEF-BOS (Mizushima and Nagata, 1990) after the conversion of the end of both fragments to blunt ends. The BamHI/NotI fragment of this vector was replaced with the BamHINotI fragment of pCDNA1-GRK4δ (Premont et al., 1996), yielding the vector pEF-GRK4δ.

Transfection of mammalian expression vectors and [3H]NMS binding assays. COS-7 and BHK-21 cells were transfected with use of the calcium phosphate method as described previously (Tsuga et al., 1994). COS-7 cells were transfected with 5 µg of expression vectors for receptors (pSVL-hm1 to pSVL-hm5) and 5 µg of expression vectors for GRKs (pEF-GRK2, pEF-GRK2-K220W, pEF-GRK4δ, pCMV5-GRK5, pCMV5-GRK6) per 107 cells on a 10-cm-diameter dish. BHK-21 cells were transfected with 5 µg of pEF-hm2 or pEF-hm3 and 5 µg of expression vectors for GRKs per 107 cells on 10-cm-diameter dish. In control cells, 5 µg of pEF-BOS was added instead of expression vectors of GRKs. At 3 to 5 hr after the transfection, cells were replated onto 12-well (COS-7) or 6-well (BHK-21) culture dishes. At 40 to 48 hr after transfection, various concentrations of carbachol were added to culture media. The [3H]NMS binding activity of intact cells was measured as described previously (Tsuga et al., 1994). After incubation with carbachol for various times, cells were washed with 1 ml of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.5) per well for three times and incubated with 1.2 to 1.6 nM [3H]NMS.
Total cellular RNA was isolated from 10^6 COS-7 or BHK-21 cells and 1005-1024 and 1219-1238 of human GRK4 (GRK 5) cDNA of GRK2 (GRK 3) were roughly estimated by visual comparison of the immunostained bands of samples and purified GRK2 obtained from Sf9 cells as described previously (Tsuga et al., 1994). Supernatant fractions of the cells were subjected to SDS-PAGE followed by electrophoretic transfer onto Immobilon transfer membranes (Millipore, Bedford, MA). The membranes were subsequently incubated with a blocking buffer [5% skim milk and 0.1% Tween-20 (w/v) in PBS] for 1 hr at room temperature and then with 1:2000 diluted antisera for 2 hr at room temperature. After removal of the secondary antiserum, the membranes were washed five times with PBS and then incubated with a staining solution [0.8 mg/ml 3,3'-diaminobenzidine • 4 HCl (Dojindo Laboratories, Mabiki-Machi, Kumamoto, Japan), 0.4 mg/ml NiCl_2, 0.0025% H_2O_2, 50 mM Tris • HCl, pH 7.5]. The amounts of GRK2 were roughly estimated by visual comparison of the immunostained bands of samples and purified GRK2 obtained from S9 cells as described previously (Tsuga et al., 1994).

RNA isolation and amplification of GRK cDNA fragment. Total cellular RNA was isolated from 10^6 COS-7 or BHK-21 cells using GlassMAX RNA Microisolation Spin Cartridge System (Life Technologies) and then treated with RNase-free DNase I (supplied with the Spin Cartridge System) for 15 min at room temperature. GRK cDNA segments were amplified from 1 μg of COS-7 or BHK-21 total RNA using RT-PCR high (Toyobo) and the specific primers for cDNA of GRK2 (5'-ATTCGACGGCGGTCTTCTCATCTT-3' and 5'-ATTCGAGGTGGCAGAGACTGTCTTGG-3'), complementary to residues 1558-1580 and 1987-2010 of human GRK2 cDNA, respectively), GRK4 (5'-ATTCGAGGTGGCAGAGACTGTCTTGG-3' and 5'-GCTGGTACGGGAGCTCTTCCGAG-3'), GRK5 (5'-ATTCGAGGTGGCAGAGACTGTCTTGG-3' and 5'-ATTCGAGGTGGCAGAGACTGTCTTGG-3'), and GRK6 (5'-ATTCGAGGTGGCAGAGACTGTCTTGG-3' and 5'-ATTCGAGGTGGCAGAGACTGTCTTGG-3'). These primers amplified product fragments of 465, 249, 336 and 405 bp for GRK2, GRK4, GRK5 and GRK6, respectively.
receptors was increased by coexpression of GRK2 and decreased by coexpression of DN-GRK2: \( t_{1/2} \) values for hm2 receptors treated with 10\(^{-5} \) M carbamylcholine were estimated to be 37, 14 and 46 min for control and GRK2- and DN-GRK2-expressing cells, respectively.

Figure 2b shows the sequestration of hm2 receptors treated with different concentrations of carbamylcholine for 120 min. Sequestration was apparent at \( 10^{-7} \) to \( 10^{-6} \) M carbamylcholine for cells expressing hm2 receptors with GRK2, whereas \( \geq 10^{-5} \) M concentrations of carbamylcholine were necessary for the sequestration to be clearly observed in cells expressing hm2 receptors alone or hm2 receptors with DN-GRK2. The \( EC_{50} \) value for carbamylcholine was 0.34 \( \mu M \) for cells expressing GRK2, which was markedly lower than values for control or DN-GRK2-expressing cells (2.1 and 4.6 \( \mu M \), respectively) (table 1). Proportions of sequestered hm2 receptors in cells treated with \( 10^{-5} \) M carbamylcholine were 47 \( \pm \) 5\% (average \( \pm \) standard deviation), 56 \( \pm \) 8\% or 39 \( \pm \) 5\% for cells expressing hm2 receptors alone or with GRK2 or DN-GRK2. The last value is significantly lower than the former two values. These results confirm previous findings that the sequestration of porcine m2 receptors was facilitated by coexpression of GRK2 and attenuated by coexpression of DN-GRK2, except that proportions of sequestered receptors are greater for hm2 receptors than for porcine m2 receptors and that the effect of DN-GRK2 for hm2 receptors was significant only at \( 10^{-5} \) M and not at \( 10^{-4} \) and \( 10^{-3} \) M carbamylcholine.

Sequestration of hm4 receptors was also facilitated by coexpression of GRK2 and attenuated by coexpression of DN-GRK2, and the effects of GRK2 and DN-GRK2 were greater for hm4 than for hm2 receptors (fig. 2, c and d, table 1). The half-time (\( t_{1/2} \)) of sequestration for hm4 receptors treated with \( 10^{-4} \) M carbamylcholine was estimated to be 49, 26 and 103 min for control and GRK2-, and DN-GRK2-expressing cells, respectively (fig. 2c). Proportions of sequestered hm4 receptors were significantly higher for cells expressing GRK2 and lower for cells expressing DN-GRK2 than for control cells in a wide range of carbamylcholine concentration from \( 10^{-7} \) to \( 10^{-3} \) M. \( EC_{50} \) values for carbamylcholine were decreased or increased by coexpression of GRK2 or DN-GRK2, respectively (fig. 2d, table 1). These results indicate that the sequestration of both hm2 and hm4 receptors as well as that of porcine m2 receptors is facilitated by an endogenous kinase and coexpression of GRK2.

**Sequestration of hm3, hm5 and hm1 receptors in COS-7 cells.** Sequestration of hm1, hm3 and hm5 receptors expressed in COS-7 cells is summarized in figure 3 and table 2. The rates of sequestration of hm3 and hm5 receptors were increased by coexpression of GRK2 and decreased by coexpression of DN-GRK2: \( t_{1/2} \) values for hm2 receptors treated with 10\(^{-5} \) M carbamylcholine were estimated to be 37, 14 and 46 min for control and GRK2- and DN-GRK2-expressing cells, respectively. Significant differences compared with control cells were \( *P < .05 \) or \( **P < .01 \).

**TABLE 1**

Sequestration of hm2 and hm4 receptors coexpressed with GRKs in COS-7 cells

<table>
<thead>
<tr>
<th></th>
<th>Proportions of sequestered receptors</th>
<th>( EC_{50} )</th>
<th>Proportions of sequestered receptors</th>
<th>( EC_{50} )</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>( \mu M )</td>
<td>%</td>
<td>( \mu M )</td>
</tr>
<tr>
<td>Control</td>
<td>48 ( \pm ) 4 (7) 2.1</td>
<td></td>
<td>45 ( \pm ) 4 (3) 9.0</td>
<td></td>
</tr>
<tr>
<td>GRK2</td>
<td>58 ( \pm ) 5 (6)* 0.34</td>
<td></td>
<td>60 ( \pm ) 5 (6)* 1.6</td>
<td></td>
</tr>
<tr>
<td>DN-GRK2</td>
<td>43 ( \pm ) 4 (4) 4.6</td>
<td></td>
<td>25 ( \pm ) 3 (3)* 16</td>
<td></td>
</tr>
<tr>
<td>GRK46</td>
<td>55 ( \pm ) 3 (3) 1.0</td>
<td>41 ( \pm ) 6 (3) 4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRK5</td>
<td>50 ( \pm ) 2 (3) 1.3</td>
<td>44 ( \pm ) 5 (3) 9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRK6</td>
<td>54 ( \pm ) 3 (3) 1.8</td>
<td>45 ( \pm ) 7 (3) 7.3</td>
<td></td>
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</tr>
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</table>

* \( p < .01 \) compared with control cells.
not appreciably affected by coexpression of GRK2 or DN-GRK2, whereas the proportion of sequestered receptors increased in GRK2-expressing cells but not in DN-GRK2-expressing cells (fig. 3, a and c). Proportions of sequestered hm3 and hm5 receptors in control cells were 20% to 25% (fig. 3, a and c). Proportions of sequestered receptors in GRK2-expressing cells were much greater for hm5 (51% at maximum) than for hm3 (34% at maximum) (fig. 3, a and c, table 2). Coexpression of DN-GRK2 did not affect the sequestration of hm3 and hm5 receptors, indicating that the sequestration of hm3 and hm5 receptors is not facilitated by the endogenous kinase. The rate of sequestration of hm1 receptors (\( t_{1/2} = 43 \text{ min} \) in the presence of \( 10^{-3} \text{ M carbamylcholine} \)) was lower compared with hm3 and hm5 receptors (\( t_{1/2} = 11 \text{ and 12 min, respectively} \)), and the proportion of sequestered hm1 receptors (10 ± 5%) was also much lower compared with other muscarinic receptors (fig. 3, e and f). The rate of sequestration and proportion of sequestered hm1 receptors were increased by coexpression of GRK2. Unexpectedly, both the rate and the proportion were also increased by coexpression of DN-GRK2. Differences between cells expressing GRK2 and DN-GRK2 were not statistically significant.

**Effects of coexpression of GRK4, GRK5 and GRK6 on sequestration of muscarinic receptors in COS-7 cells.**

Tables 1 and 2 summarize the effect of coexpression of GRK4, GRK5 and GRK6 as well as GRK2 and DN-GRK2 on sequestration of hm1—hm5 receptors expressed in COS-7 cells. The sequestration was not affected by coexpression of GRK4, GRK5 and GRK6 except that the sequestration of hm1 and hm3 receptors was significantly decreased by coexpression of GRK5 and GRK4, respectively, and the sequestration of hm2 receptors tended to be facilitated by coexpression of GRK4, GRK5 and GRK6. The reason is not known why sequestration of hm1 and hm3 was decreased by coexpression of GRK5 and GRK4, respectively.

**Effects of coexpression of GRKs on sequestration of muscarinic receptors in BHK-21 cells.** In previous experiments, we observed that proportions of sequestered m2 receptors increased in GRK2-expressing cells but not in DN-GRK2-expressing cells (fig. 3, a and c). Proportions of sequestered receptors in GRK2-expressing cells were much greater for hm5 (51% at maximum) than for hm3 (34% at maximum) (fig. 3, a and c, table 2). Coexpression of DN-GRK2 did not affect the sequestration of hm3 and hm5 receptors, indicating that the sequestration of hm3 and hm5 receptors is not facilitated by the endogenous kinase. The rate of sequestration of hm1 receptors (\( t_{1/2} = 43 \text{ min} \) in the presence of \( 10^{-3} \text{ M carbamylcholine} \)) was lower compared with hm3 and hm5 receptors (\( t_{1/2} = 11 \text{ and 12 min, respectively} \)), and the proportion of sequestered hm1 receptors (10 ± 5%) was also much lower compared with other muscarinic receptors (fig. 3, e and f). The rate of sequestration and proportion of sequestered hm1 receptors were increased by coexpression of GRK2. Unexpectedly, both the rate and the proportion were also increased by coexpression of DN-GRK2. Differences between cells expressing GRK2 and DN-GRK2 were not statistically significant.

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ceptors were much less for BHK-21 cells compared with for COS-7 cells and that coexpression of DN-GRK2 did not attenuate the sequestration of m2 receptors in BHK-21 cells. These results indicate that BHK-21 cells do not have the endogenous kinase present in COS-7 cells and facilitate the sequestration of m2 receptors. Actually, GRK4δ and possibly GRK6 were found to be expressed in COS-7 but not in BHK-21 cells (fig. 1, a—d). There is a possibility that the coexpression of GRK4δ, GRK5 or GRK6 in COS-7 cells does not affect the sequestration of muscarinic receptors because the effect of exogenous kinases overlaps the effect of GRK4δ, GRK6 or other endogenous kinase in COS-7 cells. Thus, we expressed GRK2, GRK4δ, GRK5 or GRK6 in BHK-21 cells and examined their effects on sequestration of hm2 and hm3 receptors (fig. 4). Sequestration of hm2 and hm3 receptors expressed in BHK-21 cells was not affected by coexpression of GRK6. On the other hand, coexpression of GRK4δ and GRK5 apparently facilitated sequestration of hm2 receptors: the proportions of sequestered hm2 receptors were estimated to be 32% to 34% in cells coexpressing GRK4δ or GRK5 in contrast to 25% in control cells, although EC50 values for carbamylcholine did not change (fig. 4, b and c, and table 3). The effect of coexpression of GRK4δ and GRK5, however, was smaller than that of coexpression GRK2; 40% of hm2 receptors were sequestered, and EC50 values for carbamylcholine decreased by a factor of 16 with coexpression of GRK2. In contrast to hm2 receptors, the sequestration of hm3 receptors was not affected by coexpression of GRK4δ and GRK5 (fig. 4e).

Discussion

Previously, we have shown that the sequestration of porcine m2 receptors expressed in COS-7 cells is facilitated by coexpression of GRK2 (Tsuga et al., 1994). In the present study, we extended this observation and showed that sequestration of hm2, hm3, hm4 and hm5 receptors is also facilitated by coexpression of GRK2. Because the facilitation is not observed by coexpression of DN-GRK2 lacking catalytic activity, it is reasonable to assume that the sequestration is facilitated by phosphorylation of these muscarinic receptors with GRK2. In fact, m2 receptors have been shown to be phosphorylated in vivo (Tsuga et al., 1994; Pals-Rylaarsdam et al., 1995) and in vitro (Richardson et al., 1993; Kameyama et al., 1993; Nakata et al., 1994) by GRK2, and m3 receptors have also been shown to be phosphorylated in vitro by GRK2 (Debburman et al., 1995).

Phosphorylation sites in m2 receptors have been located in

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Sequestration of [3H]NMS binding sites on BHK-21 cells expressing hm2 and hm3 receptors. BHK-21 cells expressing hm2 receptors with or without GRK2 (a), GRK4δ (b), GRK5 (c) or GRK6 (d) and BHK-21 cells expressing hm3 receptors with or without GRK2, GRK4δ, GRK5 or GRK6 (e) were incubated with indicated concentrations of carbamylcholine for 120 min and then subjected to [3H]NMS binding assays at 4°C for 4 hr. Results are shown as mean ± S.D. from three independent experiments. Significant differences compared with control cells were *P < .05 or **P < .01.
the central part of the third intracellular loop (Nakata et al., 1994). Displacement by alanine of serine and threonine residues in the putative phosphorylation sites in hm2 and hm3 receptors has been shown to impair the sequestration of mutated receptors (Moro et al., 1993). These results are consistent with the idea that phosphorylation by GRK2 of serine/threonine residues in the third intracellular loop of m2 and m3 receptors somehow facilitates their sequestration. No direct evidence is available for phosphorylation of m4 and m5 receptors by GRK2. It is likely, however, that m4 and m5 receptors are also phosphorylated by GRK2 at the third intracellular loops, and thereby their sequestration is facilitated because the third intracellular loops of m4 and m5 receptors contain serine and threonine residues, which are flanked by acidic amino acid residues and thought to be phosphorylation sites of GRK2.

Pals-Rylaarsdam et al. (1995) reported that the phosphorylation of hm2 receptors by GRK2 is related to receptor desensitization but not to sequestration, based on findings that the level of sequestration of hm2 receptors expressed in HEK293 cells was not affected by coexpression of GRK2 or DN-GRK2 and that the agonist-dependent sequestration was observed for m2 receptor mutants without GRK-phosphorylation sites. It should be noted, however, that coexpression of GRK2 facilitates the sequestration of hm2 receptors mainly by reducing the effective concentrations of carbamylcholine rather than by increasing the proportions of sequestered receptors, and hence the effect of coexpression of GRK2 is difficult to observe with 1 mM carbamylcholine only, at which concentration the sequestration was observed by Pals-Rylaarsdam et al.

Furthermore, it should be pointed out that the sequestration of m2 and other muscarinic receptors may involve both GRK-dependent and -independent components. The presence of significant sequestration of hm2—hm5 receptors in cells coexpressing DN-GRK2 is consistent with this hypothesis. The difference in the sequestration of hm1 and hm2 receptors in cells coexpressing DN-GRK2 may be due to the difference in the GRK-independent sequestration, as in the study by Goldman et al. (1996). Zhang et al. (1996) reported that the sequestration of G protein-coupled receptors may occur by at least two distinct pathways: one mediated by dynamin and the other not mediated by these factors.

In contrast with hm2—hm5 receptors, it is not clear whether there is a relation between phosphorylation by GRK2 and sequestration of hm1 receptors because sequestration of hm1 receptors was apparently facilitated by coexpression of either GRK2 or DN-GRK2. Recently, hm1 receptors were shown to be phosphorylated in vitro by GRK2 (Haga et al., 1996), and phosphorylation sites were located in the central part of the third intracellular loop and include serine and threonine residues, which were shown to be critical for sequestration of m1 receptors (Moro et al., 1993). Agonist-dependent phosphorylation of hm1 receptors by GRK2 could be observed after the removal of unknown factor or factors that copurify with hm1 receptors (Haga et al., 1996). We can speculate that the phosphorylation by endogenous kinase of hm1 receptors and their sequestration are suppressed by a putative inhibitory factor and that the inhibition is relieved by interaction with either GRK2 or DN-GRK2. Another possible explanation is that sequestration of hm1 receptors is facilitated by forming a complex with either GRK2 or DN-GRK2. Both GRK2 and DN-GRK2 are expected to interact with beta gamma subunits. It is interesting to know if whether beta gamma subunits are involved in the internalization of m1 receptors. Regardless of the mechanism, the sequestration of hm1 receptors is different from that of other muscarinic receptors.

The finding that sequestration of hm2 and hm4 receptors is attenuated by coexpression of DN-GRK2 suggests that there is at least an endogenous kinase that phosphorylates hm2 and hm4 receptors and facilitates their sequestration. In fact, hm2 receptors were phosphorylated by the endogenous kinase in an agonist-dependent manner (Tsuga et al., 1994). The relevant kinase is not likely to be GRK2 because (1) GRK2 could not be detected with immunostaining or RT-PCR and (2) the sequestration of hm3 and hm5 receptors was facilitated by expression of GRK2 but not attenuated by DN-GRK2. GRK5 is also not likely to be the relevant kinase because GRK5 also could not be detected by immunostaining or RT-PCR. On the other hand, we could detect expression of GRK4 in COS-7 cells, but not in BHK-21 cells, with RT-PCR. Coexpression of GRK4 facilitated the sequestration of hm2 receptors expressed in BHK-21 cells but only slightly facilitated the sequestration of hm2 receptors in COS-7 cells. In contrast, the sequestration of hm3 receptors, which were not affected by DN-GRK2, was not facilitated by coexpression of GRK4 in either COS-7 or BHK-21 cells. GRK6 was also not detected in COS-7 cells with RT-PCR. However, coexpression of GRK6 in BHK-21 cells did not facilitate sequestration of hm2 receptors, suggesting that as previously reported, a GRK receptor may not be a good substrate for GRK6 (Loudon and Benovic, 1994). These results are consistent with the idea that GRK4 is an endogenous kinase in COS-7 cells that phosphorylates hm2 and hm4 receptors and facilitates their sequestration, although we cannot exclude the possibility that there are additional endogenous kinases, including GRK5.

Sequestration of hm2, but not hm3, receptors expressed in BHK-21 cells was found to be facilitated by coexpression of GRK5 but not by GRK6. These results have some correlation with the extent of in vitro phosphorylation; the number of phosphorylation sites in hm2 receptors is estimated to be 4 to 10 for GRK2 (Richardson et al., 1993; Nakata et al., 1994), 1.5 for GRK5 (Kunapuli et al., 1994) and 0.5 for GRK6 (Loudon and Benovic, 1994), and those for hm3 receptor are estimated to be 2 to 4 for GRK2 and none for GRK5 or GRK6 (Debburman et al., 1995). GRK5-phosphorylation sites in hm2 receptors have not been determined, but they are likely to reside in the third intracellular loop, including many serine and threonine residues, because substrate specificity of GRK2 and GRK5 appears to be very similar (Fredericks et al., 1996).

Recently, an agonist-dependent sequestration of a beta adrenergic receptor mutant expressed in HEK293 cells was shown to be facilitated by overexpression of GRK2 (Ferguson et al., 1995), GRK3 or GRK5 (Menard et al., 1996). Although the sequestration of wild-type beta adrenergic receptors has been reported to not be linked with their phosphorylation by GRKs, the recent report indicated that the mutation of GRK-phosphorylation sites partially attenuated their agonist-dependent sequestration. The sequestration of beta adrenergic receptors is likely to be composed of GRK-dependent and -independent components. Furthermore, dopamine D2 receptors expressed in COS-7 cells were found to become seques-
tered in an agonist-dependent manner when GRK2 or GRK5 was coexpressed, whereas virtually no sequestration was observed in the absence of coexpression of GRKs (Ito and Haga, 1996). The sequestration of dopamine D2 receptors in this system appears to be composed of only GRK-dependent process. These results, together with the present results, indicate that (1) the agonist-dependent sequestration of G protein-coupled receptors may be composed of GRK-dependent and -independent components, (2) the relevant GRK species may be different depending on receptor species and (3) GRK2 is involved in the sequestration of receptors linked to either Gαs (beta adrenergic), Gαi/Gai (muscarinic m2, m4 and dopamine D2) or Gαq (muscarinic m3 and m5). Recently, it was proposed that the phosphorylation by GRK2 facilitates the sequestration of phosphorylated receptors by increasing their affinity for beta-arrestin, and the expression level of GRKs and beta-arrestin determines kinetics of sequestration of beta adrenergic receptors (Ferguson et al., 1996; Ménard et al., 1997). Furthermore, Goodman et al. (1996) demonstrated that beta-arrestin/arrestin3 interacts with clathrin, a major protein of coated vesicles. These results suggest that the phosphorylation by GRK2 of m2 muscarinic and beta adrenergic receptors may be involved in both internalization and uncoupling through facilitation of their interaction with beta-arrestin/arrestin3. In fact, beta-arrestin has been shown to interact with the phosphorylated form of m2 receptors (Gurevich et al., 1993). Very recently, Schlador and Nathanson (1997) showed that desensitization and sequestration of m2 receptors were synergistically enhanced by GRK2 and beta-arrestin-1. This result suggests that the sequestration of muscarinic as well as beta adrenergic receptors is also dependent on the interaction of beta-arrestin with phosphorylated receptors. We propose that the relation between the agonist-dependent phosphorylation and facilitation of receptor sequestration is not limited to m2 receptors but is generalized to include at least four muscarinic receptors, beta adrenergic receptors and dopamine D2 receptors, and that GRK4 and GRK5 in addition to GRK2 may be involved in this relation.

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