We have previously shown that overexpression of G protein-coupled receptor kinase 6 (GRK6) enhanced the phosphorylation and desensitization of the endogenously expressed M₃ muscarinic acetylcholine (mACH) receptor in human SH-SY5Y neuroblastoma cells. In this study we have examined the potential role of endogenous GRK6 in the regulation of M₃ mACH receptor by blocking its action through the introduction of a kinase-dead, dominant-negative GRK6 (K²¹⁵RGRK6). K²¹⁵RGRK6 expression inhibited methacholine-stimulated M₃ mACH receptor phosphorylation by 50% compared with plasmid-transfected control cells. Guanosine-5′-O-[(3-O-methyl)thio]triphosphate and immunoprecipitation studies, conducted after agonist pretreatment (3 min), indicated that M₃ mACH receptor-Gq/11 uncoupling was attenuated by 50% in cells expressing K²¹⁵RGRK6 when compared with control cells. In contrast, expression of the related dominant-negative kinase K²¹⁵RGRK5 had no effect on M₃ mACH receptor phosphorylation or uncoupling. Time course studies also showed that agonist-stimulated [³²P]inositol phosphate accumulations were more sustained in cells expressing K²¹⁵RGRK6 compared with control and K²¹⁵RGRK5-expressing cells, whereas K²¹⁵RGRK6 expression had no effect on the phospholipase C response to direct stimulation of G proteins with AlF₄⁻. The ability of K²¹⁵RGRK6 to inhibit agonist-mediated M₃ mACH receptor phosphorylation and G protein uncoupling suggests that endogenous GRK6 mediates, at least in part, M₃ mACH receptor desensitization in the SH-SY5Y cell line. The majority of G protein-coupled receptors desensitize when stimulated continuously or repetitively. This process can be initiated via phosphorylation of the receptor, which leads to receptor-G protein uncoupling (1–3) and reduced receptor signaling to downstream pathways. Second messenger-regulated kinases, such as PKC (4) and cAMP-dependent protein kinase (5, 6), and specific G protein-coupled receptor kinases (GRKs) (7, 8) have been implicated in G protein-coupled receptor desensitization through phosphorylation of serine and threonine residues within the third intracellular loop or C-terminal tail of G protein-coupled receptors. Upon agonist stimulation, the human M₃ muscarinic acetylcholine (mACH) receptor is rapidly phosphorylated (9), and a number of different kinases have been implicated in this process, including PKC (10, 11) and casein kinase 1α (CK1α) (12). However, agonist-stimulated receptor phosphorylation by either CK1α or PKC does not appear to mediate rapid desensitization of the M₃ mACH receptor (10–12).

More recently, we have examined the potential involvement of GRKs in the desensitization of the M₃ mACH receptor endogenously expressed in the SH-SY5Y cell line (13). This human neuroblastoma expresses GRKs 3 and 6, and overexpression of GRK3 and GRK6 leads to enhanced M₃ mACH receptor phosphorylation and reduced activation of phospholipase C (PLC). However, only GRK6 overexpression enhanced uncoupling of the receptor from Gq/11 assessed by a [³⁵S]GTPγS binding/immunoprecipitation protocol. In contrast, only GRK3 overexpression suppressed AlF₄⁻ activation of PLC (13). These data strongly support an action of GRK3 independent of phosphorylation, possibly via direct binding to activated Gq/11 and/or free Gq/11 subunits, but highlight a potential role for GRK6 via receptor phosphorylation and uncoupling of Gq/11.

Although GRK6 overexpression can be shown to enhance the M₃ mACH receptor desensitization process in SH-SY5Y cells, this experimental approach is unable to resolve whether endogenous GRK6 contributes to M₃ mACH receptor regulation. Therefore, in an attempt to block the actions of endogenous GRK6, we have introduced a kinase-dead, dominant-negative mutant form of GRK6, created by introducing a K²¹⁵R point mutation into the ATP-binding domain, into SH-SY5Y cells to create cell lines stably expressing this construct. Using this approach we have shown that K²¹⁵RGRK6 expression inhibits the phosphorylation and subsequent desensitization of the M₃ mACH receptor. Our data suggest that GRK6 plays a significant role in the desensitization of the endogenously expressed M₃ mACH receptor in human SH-SY5Y neuroblastoma cells.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Creation of Stably Transfected Dominant-negative GRK Cell Lines—SH-SY5Y human neuroblastoma cells were cultured in minimal essential medium containing 5% fetal and 5% newborn calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and fungizone (2.5 µg/ml) (Invitrogen). All of the cells were maintained at 37 °C in humidified conditions under 5% CO₂. Wild-type SH-SY5Y cells were transfected with either pcDNA3 alone or human dominant-negative GRK5 or GRK6, both with a single point mutation at K²¹⁵R, cloned into pcDNA3 at BamHI and XhoI, or EcoRI for GRKs 5 (14) and 6, respectively (both kindly provided by E. Kelly, University of Bristol, Bristol, UK), using FuGENE 6 according to the manufacturer’s instructions.
GRK6 Regulates M₃ mACh Receptor Desensitization

After 48 h, genetin (300 µg/ml) was added to the cells. The surviving colonies were selected and expanded into cell lines.

Western Blotting—The cells were lysed and subjected to electrophoretic separation exactly as described previously (15, 16). The separated protein was transferred to nitrocellulose, and GRK expression was determined using anti-rabbit polyclonal IgG antibodies (1:5000 dilution) specific for GRK2, GRK3, GRK5, or GRK6 (Santa Cruz Biotechnology). Goαs2 expression was detected using an anti-rabbit polyclonal IgG (Santa Cruz Biotechnology). CK1α was detected using an anti-rabbit polyclonal as described previously (12). Protein expression was determined by the addition of ECL reagent (Amersham Biosciences), according to the manufacturer’s instructions and exposure to Hyperfilm (Amersham Biosciences).

Determination of mACh Receptor Number and Receptor Internalization—The Bmax and Kd values were determined by N-methyl-i[125]Hscopolamine ([125]HJMS) saturation binding analysis of SH-SY5Y cell monolayers grown to confluence in 24-well plates as described previously (13).

M₃ mACh receptor internalization was assessed after treatment with either vehicle or methacholine (100 µM) for 0–60 min at 37 °C, prior to intensive washing (four times with 1 ml of ice-cold Krebs buffer, pH 7.4). [3H]NMBS-binding sites were determined as above using a single saturating concentration (5 nM) of [3H]NMBS for 18 h at 4 °C. Nonspecific binding was determined by the addition of excess atropine (20 µM).

Receptor internalization was determined as the percentage of loss of specific [3H]NMBS-binding sites after methacholine treatment when compared with vehicle-treated controls.

M₃ mACh Receptor Phosphorylation—The effect of K215RGRK6 and K215RGRK5 expression on the phosphorylation of endogenously expressed M₃ muscarinic receptors was assessed by the method of Tobin and Nahorski (9). Briefly, either plasmid control or cells expressing either K215RGRK6 or K215RGRK5 were seeded into 6-well culture plates. Confluent cells were loaded with [32P]orthophosphate (5 µCi/ml; Amersham Biosciences) in phosphate-free Krebs buffer, pH 7.4, for 1 h prior to agonist stimulation (methacholine, 100 µM). After 3 min, the agonist was removed, and the cells were solubilized, immunoprecipitated, and electrophoretically resolved as described previously (13). Autoradiograms were documented and quantified using the GeneGenius system and software (Syngene, Cambridge, UK).

Casein Phosphorylation—Any potential kinase activity of dominant-negative GRK5 and GRK6 was assessed by measuring their ability to phosphorylate the substrate α-casein as described previously (13).

Measurement of Total [3H]Inositol Phosphate Accumulation—Either plasmid control or cells expressing K215RGRK6 or K215RGRK6 were seeded into 24-well culture plates at ~50% confluency. After 24 h, the cells were incubated in the presence of [3H]inositol (1 µCi/ml) in genetic-free medium for a further 24 h. Confluent cell monolayers were then washed twice with Krebs buffer (118.6 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 4.2 mM NaHCO3, 10 mM HEPES, 11.7 mM glucose, and 1.3 mM CaCl2, pH 7.4) and incubated for 15 min at 37°C. LiCl (final concentration, 10 mM) was added to each well for 10 min prior to the addition of methacholine. Reaction termination, sample neutralization, and separation of the [3H]inositol phosphate fraction (containing inositol mono-, bis-, and tris-phosphates) were performed as described previously (17).

Assessment of M₃ mACh receptor Goαs111 Coupling by [35S]GTPγS Binding—Plasmid control or dominant-negative GRK-expressing cells were grown in 80-cm² cell culture flasks until confluent. The cells were then harvested in 10 mM HEPES, pH 7.4, 0.2% (v/v) EDTA, 0.9% (v/v) NaCl. After centrifugation the cells were resuspended in Krebs buffer, pH 7.4, at 37 °C for 15 min prior to the addition of either vehicle or MCh (100 µM). After 3 min, excess ice-cold Krebs buffer (50 ml) was added, and the cells were pelleted at 1000 × g for 5 min. The cell pellets were then resuspended in 30 ml of 20 mM HEPES, pH 7.4, 10 mM EDTA and homogenized at maximum speed for 30 s using a PT210 Polytron. The resulting suspension was centrifuged at 20,000 × g for 15 min. The pellet was resuspended in 30 ml of 20 mM HEPES, pH 7.4, 0.1 mM EDTA and centrifuged for a further 15 min at 20,000 × g. The resulting pellet was resuspended at 1 mg/ml of protein and stored at −80 °C until required.

[35S]GTPγS binding and immunoprecipitation were performed as described previously (18, 19). Briefly, 50 µg of membranes were added to 1 µM [35S]GTPγS (PerkinElmer Life Sciences), 1 µM GDP, 100 µM methacholine in assay buffer (100 mM HEPES, 100 mM NaCl, 10 mM MgCl2, pH 7.4) and incubated for 2 min at 30 °C. Nonspecific binding was determined by inclusion of 10 µM GTPγS. Termination, solubilization, and Goαs11-specific immunoprecipitation were performed exactly as described previously (13). Desensitization was determined as a reduction in [35S]GTPγS binding after pretreatment with methacholine and expressed as a percentage of the response found when compared with a nonpretreated matched control. In addition to desensitization experiments, M₃ mACh receptor activation of Goαs111 was assessed in nonpretreated cell membranes via generation of methacholine (300 nM to 1 mM) concentration-response curves.

RESULTS

Creation of Stable Dominant-negative GRK Cell Lines—Transfection of wild-type SH-SY5Y neuroblastoma cells with either K215RGRK6 or K215RGRK5 and selection with genetin (300 µg/ml) yielded several surviving colonies, which were expanded into cell lines. Two clones that expressed K215RGRK6 and that were matched with plasmid controls for receptor number based on [3H]NMBS binding were selected for further study (named D1 and D5; Fig. 1A). Estimation of the level of K215RGRK6 expression was determined by serial dilution of cell lysates and subsequent Western blotting, indicating ~50-fold greater levels than endogenous GRK6 expression (data not shown). In an attempt to examine the specificity of the effects of K215RGRK6, a single clone expressing the closely related K215RGRK5 (D2, Fig. 1B) was chosen for some experiments. The level of overexpression of K215RGRK5 was difficult to assess because there was no detectable endogenous GRK5 in SH-SY5Y cells. To determine whether K215RGRK6 lacked kinase activity (i.e., was kinase-dead), HEK293 cells were transiently transfected with empty vector, GFP-tagged wt-GRK6, or GFP-tagged K215RGRK6. After 48 h the kinases were immunoprecipitated with either GFP polyclonal or wt-GRK6 antibodies. Kinase activity was assessed by addition of dephosphorylated
α-casein in the presence of [γ-32P]ATP (as described under “Experimental Procedures”). No phosphorylation of α-casein was detected after transfection with empty vector, followed by immunoprecipitation with GFP antibody (Fig. 1C). As expected, enhanced α-casein phosphorylation was detected after transfection of GFP-tagged GRK6. Furthermore, a slight phosphorylation of α-casein was detected after immunoprecipitation of endogenously expressed GRK6 from nontransfected HEK293 cells. In contrast, α-casein phosphorylation was not evident in the presence of K215RGRK6.

Effects of K215RGRK5 or K215RGRK6 on Expression of Endogenous GRKs or CK1α—In an attempt to determine whether overexpression of K215RGRK5 or K215RGRK6 altered the expression of other endogenously expressed kinases, we first blotted all the clones used in this study for GRKs 2, 3, 5, and 6, as well as CK1α (Fig. 2). Overexpression of K215RGRK5 had no effect on the expression of any of the kinases studied. Furthermore, K215RGRK6 overexpression had no effect on GRK2, GRK3, GRK5, or CK1α expression in any of the cell lines. However, it was not possible to assess whether K215RGRK6 altered the expression of endogenous GRK6 because both K215RGRK6 and wild-type GRK6 are not distinguishable with the antibody used (Fig. 2D).

Determination of M3 Receptor Number—Whole cell binding studies were undertaken using [3H]NMS to determine whether expression of dominant-negative constructs affected M3 mACh receptor expression. The data obtained for Bmax and Kp indicated that expression of either K215RGRK6 or K215RGRK5 had no significant effect on M3 mACh receptor expression (Table I). Moreover, receptor expression levels did not alter with passage (data not shown).

M3 mACh Receptor Phosphorylation—The effects of K215RGRK6 expression on methacholine-stimulated (100 μM) M3 mACh receptor phosphorylation was examined in clones D1 and D5. As shown in Fig. 3 (A and B), after 3 min of agonist exposure a robust phosphorylation was observed in the plasmid control cell lines (P1 and P3). In contrast, M3 mACh receptor phosphorylation was reduced by ∼50% in cells expressing K215RGRK6. Furthermore, expression of K215RGRK5 had no effect on agonist-stimulated M3 mACh receptor phosphorylation (Fig. 3C). Densitometric analysis confirmed that K215RGRK6 had no effect on basal M3 mACh receptor phosphorylation. However, K215RGRK6 significantly (p < 0.01) reduced agonist-stimulated M3 mACh receptor phosphorylation by 50 and 43% for D1 compared with P3 and for D5 compared with P1 clones, respectively (Fig. 4). Furthermore, expression of K215RGRK6 did not affect PKC-mediated M3 mACh receptor phosphorylation stimulated by phorbol 12,13-dibutyrate (PDBu) (1 μM, 3 min; Fig. 3D).

Assessment of M3 mACh Receptor Desensitization by [35S]GTPγS Binding—To determine whether the inhibition of agonist-stimulated M3 mACh receptor phosphorylation observed with K215RGRK6 expression had a subsequent effect on receptor function, we examined the direct interaction of the M3 mACh receptor and Goq/11 at the point of receptor catalyzed GTP/GDP exchange. Thus, we have used agonist-stimulated [35S]GTPγS binding followed by immunoprecipitation of Goq/11 in a membrane preparation (see “Experimental Procedures”).

First, we assessed whether expression of K215RGRK6 had any effects on acute M3 mACh receptor-Goq/11 interaction. Concentration-response curves in membranes from nonpretreated K215RGRK6-expressing clones, undertaken at 2 min, the optimal time point for M3 mACh receptor-Goq/11 activation (13, 19),

### Table I

| Clone | Bmax (fmol/mg protein) | Kp (nM) |
|-------|------------------------|---------|
| P1    | 138 ± 15               | 0.16 ± 0.02 |
| P3    | 137 ± 9                | 0.22 ± 0.05 |
| D1    | 144 ± 9                | 0.16 ± 0.02 |
| D5    | 127 ± 6                | 0.22 ± 0.03 |
| D2    | 150 ± 11               | 0.21 ± 0.03 |

**FIG. 2.** Overexpression of K215RGRK5 or K215RGRK6 does not effect the expression of endogenously expressed GRKs or CK1α.

Whole cell [3H]NMS saturation binding to determine M3 mACh receptor expression in plasmid control, K215RGRK6, and K215RGRK5 expressing clones.

The Bmax and Kp values were determined using nonlinear regression analysis and curve-fitting programs of GraphPad Prism 3. The data are expressed as the means of specific binding ± S.E. for five or six separate experiments.
showed binding identical to that seen in controls (Fig. 5A). However, as described previously (11, 13), pretreatment of intact cells with MCh (100 μM) for 3 min leads to reduced agonist-stimulated [35S]GTPγS binding to immunoprecipitated Goq11 in the subsequent membrane assay (Fig. 5B). Plasmid control cells (P1 and P3) showed 59 (D1 versus P3) and 46% (D5 versus P1) less M3 mACh receptor-Goq11 coupling when compared with nonpretreated controls. In contrast, the degree of receptor-Goq11 uncoupling was inhibited by 50% with K215RGRK6 expression (Fig. 5B). Interestingly, despite agonist pretreatment, expression of K215RGRK5 had no effect on M3 mACh receptor-Goq11 uncoupling (Fig. 5B). These effects were not due to a loss of membrane-associated Goq11, because total Goq11 immunoreactivity in the membrane fraction was unaltered after 3 min of pretreatment (Fig. 5C).

Assessment of Phospholipase C Activity—To further study the effects of GRK6 on M3 mACh receptor regulation, we examined the effects of K215RGRK6 on PLC activity using total [3H]inositol phosphate accumulation. Time course studies indicated that clones expressing K215RGRK6 produced significantly (p < 0.01) greater [3H]inositol phosphate accumulations than plasmid controls matched for receptor number (Fig. 6, A and B). In addition, concentration-response curves to MCh produced after 3 min indicate that expression of K215RGRK6 enhanced [3H]inositol phosphate accumulation when compared with plasmid control cells (Fig. 7). Interestingly, expression of the structurally similar dominant-negative kinase K215RGRK5

**Fig. 4.** Analysis of the effects of K215RGRK6 expression on MCh-stimulated M3 mACh receptor phosphorylation in two clones (D1 and D5). The samples prepared as described under “Experimental Procedures” were subjected to densitometric analysis using the GeneGenius image analysis system and software (Syngene). M3 mACh receptor phosphorylation is shown as basal (open bars) and after methacholine (100 μM) stimulation (3 min, black bars). The data are expressed as the means ± S.E. of four separate experiments. Expression of K215RGRK6 significantly inhibited methacholine-stimulated M3 mACh receptor phosphorylation when compared with plasmid controls. *, p < 0.01; **, p < 0.001.

**Fig. 5.** Effects of K215RGRK6 expression on M3 mACh receptor-Goq11 coupling in SH-SY5Y cells. A, Concentration-response curves for methacholine-stimulated (300 nM to 1 mM) M3 mACh receptor-Goq11 recruitment in plasmid control (P3, □) and cells expressing K215RGRK6 (D1, □; D5, ○), as measured by [35S]GTPγS binding and Goq11 immunoprecipitation from nonpretreated cell membranes. [35S]GTPγS binding was conducted at 30°C and at the peak of Goq11 recruitment, 2 min. The data are expressed as the means ± S.E. (in cpm) and are representative of three separate experiments. Basal values were as follows: P3, 561 ± 86 cpm; D1, 554 ± 57 cpm; D5, 511 ± 81 cpm (n = 3). B, effect of K215RGRK6 and K215RGRK5 (D2) expression on methacholine-stimulated M3 mACh receptor uncoupling. Intact plasmid control (P1 and P3), K215RGRK6 (D1 and D5), or K215RGRK5-expressing cells were pre-treated with methacholine (100 μM) for 3 min at 37°C. After pretreatment, the cells were washed and converted into membranes, prior to a second methacholine (100 μM) challenge, for 2 min at 30°C, in the presence of [35S]GTPγS. Methacholine-stimulated [35S]GTPγS binding to Goq11 after pretreatment was determined as described under “Experimental Procedures,” and the data were expressed as fold over basal. M3 mACh receptor uncoupling was determined as the decrease in [35S]GTPγS binding to Goq11 after methacholine pretreatment when compared with the value obtained in a nonpretreated control (i.e., 0 = no uncoupling, whereas 100% = total receptor uncoupling). The data shown are the means ± S.E. of three separate experiments. K215RGRK6 expression significantly inhibited methacholine-stimulated M3 mACh receptor uncoupling when compared with plasmid control and K215RGRK5 overexpressing cells. **, p < 0.001. Basal values were as follows: P3, 812 ± 35 cpm; D1, 755 ± 90 cpm; D5, 602 ± 33 cpm; D2, 805 ± 58 cpm (for six to eight separate experiments). C, effects of methacholine (100 μM) pretreatment on Goq11 membrane association. The data shown are a representative immunoblot indicating the levels of membrane-bound Goq11 in plasmid control (P3) or K215RGRK6-expressing (D1) cells, either in the absence or presence of MCh pretreatment. Lane 1, P3 cells, no methacholine pretreatment; lane 2, MCh-pretreated P3 cells (100 μM, 3 min, 37°C); lane 3, D1 cells, no MCh pretreatment; lane 4, MCh-pretreated D1 cells (100 μM, 3 min, 37°C).
M3 mACh Receptor Internalization—To assess whether GRK6 mediated M3 mACh receptor internalization plasmid control, GRK6-overexpressing (clone 24) or K215RGRK6-expressing (D1) cells were exposed to MCh (100 μM) for up to 1 h. Subsequent [3H]NMS binding revealed that overexpression of GRK6 had no effect on M3 mACh receptor internalization. Furthermore, inhibition of endogenous GRK6 with K215RGRK6 failed to inhibit M3 mACh receptor internalization (Fig. 8).

DISCUSSION

We have recently shown that recombinant GRK6 can enhance both M3 mACh receptor phosphorylation and receptor/Gαq/11 uncoupling, subsequently increasing desensitization of the endogenously expressed M3 mACh receptor in the SH-SY5Y neuroblastoma cell line (13). These data are suggestive of a role for endogenous GRK6 in the regulation of M3 mACh receptor desensitization. Therefore, in an attempt to block the action of endogenously expressed GRK6 in SH-SY5Y cells, we have introduced a kinase-dead, dominant-negative mutant, K215RGRK6.

Our data show for the first time the potential role of endogenous GRK6 in the regulation of an endogenously expressed Gαq/11-coupled receptor. Despite the many studies indicating that GRKs are able to enhance the phosphorylation of overexpressed receptors (reviewed in Ref. 1), relatively few have examined the role of endogenous GRK6 activity in the regulation of endogenously expressed receptors. Introduction of antisense GRK6 oligonucleotides inhibited desensitization of the calcitonin gene-related peptide receptor stably expressed in HEK293 cells (20). However, several recent studies have reported the use of differing versions of dominant-negative GRK6 and have provided conflicting evidence as to their effectiveness in inhibiting endogenous GRK6. Lazari et al. (21) were able to inhibit endogenous GRK6 phosphorylation of follitrophin receptors in HEK293 cells by introducing a double point-mutated GRK6 (K215M/K216M). Zhou et al. (22) introduced a triple point-mutated (R215K/D484S/D485S) dominant-negative GRK6 that reduced agonist-stimulated thromboxane A2 receptor phosphorylation when compared with equivalent overexpression of GRK6 (22). However, unlike the present data, where we show a 50% reduction in agonist-stimulated M3 mACh receptor phosphorylation with expression of K215RGRK6, the action of endogenous GRK6 appeared not to be inhibited by this dominant-negative (R215K/D484S/D485SGRK6) (22). In view of these findings we chose to determine the catalytic activity of our dominant-negative GRK6 using immunoprecipitation of GFP-tagged GRK6 or K215RGRK6 and α-casein as substrate. These studies
Interestingly, despite the close structural similarity, we introduced the dominant-negative version of GRK5/GRK6 (13) or K215RGRK6. Nevertheless, to further examine the ability of GRK3 overexpression, was unaffected by either wt-GRK3 sensitization, it is interesting to speculate which phosphoac-

the ability of GRK2 and GRK3 to directly bind to activated, GTP-bound Gαq/11 via their N-terminal RGS domain is not seen with GRK5 and GRK6 (23, 24). In addition, direct stimulation of PLC via activation of G proteins using AlF4−, which is inhibited by GRK3 overexpression, was unaffected by either wt-

The finding that certain GRKs can mediate inhibition of PLC activity via nonreceptor phosphorylation mechanisms (23, 24) cautions against the use of dominant-negative GRKs. However, the ability of GRK2 and GRK3 to directly bind to activated, GTP-bound Gαq/11, via their N-terminal RGS domain is not seen with GRK5 and GRK6 (23, 24). In addition, direct stimulation of PLC via activation of G proteins using AlF4−, which is inhibited by GRK3 overexpression, was unaffected by either wt-

the specific recruitment of GRK6 by the receptor after agonist activation. Because GRK6 appears to regulate M3 mACH receptor desensitization, it is interesting to speculate which phosphoac-

Because GRK6 appears to regulate M3 mACH receptor desensitization, it is interesting to speculate which phosphoac-

ceptor sites GRK6 may phosphorylate. Despite the wealth of studies implicating GRK involvement in receptor desensitization, no consensus sequence has been agreed for GRK phosphorylation (3, 28, 29). Several studies have identified serine or threonine clusters as favored targets for GRK-mediated receptor phosphorylation (30, 31). Wu et al. (32) have mapped the phosphorylation sites for GRK2 to two such regions (K315S/S33 and 343/345S/S51) in the third intracellular loop of the M3 mACH receptor. Recent evidence examining the phosphorylation profile of the B2 bradykinin receptor has indicated that GRK (2, 3, 5 or 6) phosphorylation is limited to three distinct serines situated in the C-terminal tail (33). Closer examination of the individual serine moieties highlighted distinct phosphorylation patterns between GRKs, raising the possibility that different GRKs may regulate different receptor signaling mechanisms. These data suggest that GRK6 and GRK2 M3 mACH receptor phosphorylation sites may not be mutually exclusive but that phosphorylation patterns may differ both temporally and positionally. However, it is also noteworthy that unlike for the bradykinin B2 receptor, determination of M3 mACH receptor phosphorylation patterns is potentially much more complex because of the presence of 36 serine and 16 threonine residues within the third intracellular loop alone. Nevertheless, we have previously shown that although GRK3 and GRK6 enhance M3 mACH receptor phosphorylation, only GRK6 mediates uncoupling of the receptor from Gq/11 (13). Furthermore, inhibition of endogenous GRK6 with a 30-fold excess of K215RGRK6 only produced a 50% decrease in M3 receptor phosphorylation and Gq/11 uncoupling. This finding could suggest either that increasing K215RGRK6 overexpression may further inhibit M3 mACH receptor phosphorylation and Gq/11 uncoupling or more likely that other kinases such as GRK2 or 3 may contribute to M3 mACH receptor desensitization in SH-SY5Y cells.

Many studies have reported that GRK overexpression not only leads to enhanced desensitization but also accelerated internalization of receptors (reviewed in Refs. 1 and 3). The present data indicate that although GRK6 is capable of desensitizing the M3 mACH receptor, it appears not to play a significant role in receptor internalization. This finding may appear surprising, but for some receptors kinase-mediated phosphorylation does not always enhance receptor internalization. Indeed, in agreement with our data, Lazari et al. (21) found that although dominant-negative GRK2 or GRK6 are equally capable of inhibiting follitropin receptor phosphorylation, only inhibition of GRK2-mediated phosphorylation prevents receptor internalization. Moreover, our data suggest that the potential pattern of phosphoacceptor sites that mediate M3 mACH desensitization and internalization may be different. The presence of GRK2, GRK3, CK1α, and PKCs in SH-SY5Y cells and their ability to phosphorylate the M3 mACH receptor (10–13, 32) raise the possibility that one or more of these kinases may be responsible for regulating M3 mACH receptor internalization. Indeed, recent preliminary studies have shown that dominant-negative CK1α inhibits M3 receptor internalization when concurrently overexpressed in HEK293 cells (34). Furthermore, we have shown that overexpression of GRK3 in SH-SY5Y cells (13), and others have reported that GRK2 in Chinese hamster ovary cells (35) enhanced M3 mACH receptor internalization. It will be interesting to examine which of these kinases plays a role in the internalization of the endogenously expressed M3 mACH receptor in SH-SY5Y cells and whether this is crucial for recruitment of adaptor proteins and initiating alternative signaling cascades.

In conclusion we have examined the potential role of endogenously expressed GRK6 in the desensitization of M3 mACH receptors in the human SH-SY5Y cell line. Introduction of a
dominant-negative, catalytically inactive GRK6 (K215RGRK6) inhibited both agonist-stimulated M3 mACh receptor phosphorylation and receptor/Goαq11 uncoupling. Furthermore, K215RGRK6 expression partially reversed the time-related decay of agonist activation of PLC. In contrast, the closely related K215RGRK5 was unable to affect M3 mACh signaling in any way. Despite the obvious effects of GRK6 on M3 mACh receptor PLC-coupled signaling, manipulation of GRK6 activity had no effect on receptor internalization. Overall these data suggest that endogenous GRK6 regulates, at least in part, M3 mACh receptor desensitization in human SH-SY5Y neuroblastoma cells.

Acknowledgments—We thank Dr. Eamonn Kelly (Department of Pharmacology, University of Bristol, Bristol, UK) for kindly donating the K215RGRK5 and K215RGRK6 constructs. We gratefully acknowledge the contribution of Dr. J. Parkinson, who made the GFP-tagged GRK6 and K215RGRK6 constructs.

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J. Biol. Chem. 2002, 277:15523-15529.
doi: 10.1074/jbc.M111217200 originally published online February 20, 2002

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