G Protein-coupled Receptor Kinase-mediated Desensitization of Metabotropic Glutamate Receptor 1A Protects against Cell Death*

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Metabotropic glutamate receptors (mGluRs) constitute a unique subclass of G protein-coupled receptors (GPCRs) that bear little sequence homology to other members of the GPCR superfamily. The mGluR subtypes that are coupled to the hydrolysis of phosphoinositide contribute to both synaptic plasticity and glutamate-mediated excitotoxicity in neurons. In the present study, the expression of mGluR1a in HEK 293 cells led to agonist-independent cell death. Since G protein-coupled receptor kinases (GRKs) desensitize a diverse variety of GPCRs, we explored whether GRKs contributed to the regulation of both constitutive and agonist-stimulated mGluR1a activity and thereby may prevent mGluR1a-mediated excitotoxicity associated with mGluR1a over-activation. We find that the co-expression of mGluR1a with GRK2 and GRK5, but not GRK4 and GRK6, reduced both constitutive and agonist-stimulated mGluR1a activity. Agonist-stimulated mGluR1a phosphorylation was enhanced by the co-expression of GRK2 and was blocked by two different GRK2 dominant-negative mutants. Furthermore, GRK2-dependent mGluR1a desensitization protected against mGluR1a-mediated cell death, at least in part by blocking mGluR1a-stimulated apoptosis. Our data indicate that as with other members of the GPCR superfamily, a member of the structurally distinct mGluR family (mGluR1a) serves as a substrate for GRK-mediated phosphorylation and that GRK-dependent “feedback” modulation of mGluR1a responsiveness protects against pathophysiological mGluR1a signaling.

Glutamate is the major excitatory neurotransmitter in the central nervous system and is essential in the regulation of brain functions and neural cell development (1, 2). Receptors that respond to glutamate are classified into two types, ionotropic and metabotropic. The ionotropic glutamate receptors are cation-specific ion channels that mediate fast excitatory glutamate responses and are subdivided into alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate and NMDA receptors (1, 2). In contrast, metabotropic glutamate receptors (mGluRs) are members of the G protein-coupled receptor (GPCR) superfamily and mediate slower glutamate responses by coupling to various second messenger cascades via heterotrimeric G proteins (1, 2). This property allows mGluRs to translate relatively short neuronal activation into long lasting changes in synaptic activity. As a consequence, mGluR signaling plays an important role in the processes underlying synaptic plasticity (e.g. memory and learning) (3).

The mGluR family of receptors constitute a unique subclass of GPCRs that bear no sequence or structural homology to prototypic Class I (rhodopsin, β2-adrenergic family) and Class 2 (secretin family) GPCRs other than the retention of the seven transmembrane spanning domain topology that is characteristic of a GPCR (4). Furthermore, unlike other GPCR family members, mGluRs couple to heterotrimeric G proteins via the second intracellular loop domain of the receptor rather than the third intracellular loop domain (5). The mGluR family consists of eight receptor subtypes that can be subdivided into three groups on the basis of sequence homology, pharmacology, and G protein-coupling specificity. Group 1 mGluRs (mGluR1 and mGluR5) are coupled via Gs to the stimulation of phospholipase Cβ (PLCβ) leading to increases in intracellular inositol 1,4,5-triphosphate and the release of intracellular Ca2+ stores. Group 2 (mGluR2 and mGluR3) and group 3 mGluRs (mGluR4, mGluR6, mGluR7, and mGluR8) are negatively coupled to adenylyl cyclase (1, 2). Unlike other members of the mGluR family, group 1 mGluRs are implicated in glutamate-stimulated excitotoxicity that leads to neuronal cell death associated with acute brain ischemia and neurotrauma (6, 7), as well as contribute to a variety of neurodegenerative diseases such as Huntington’s disease, amyotrophic lateral sclerosis, and Alzheimer’s disease (8, 9).

An important physiological “feedback” mechanism that protects against both acute and chronic GPCR overstimulation is the desensitization of receptor responsiveness to agonist. GPCR desensitization occurs as a consequence of covalent receptor modification in response to phosphorylation by intracellular kinases. At least two families of kinases contribute to the desensitization of prototypic GPCRs, second messenger-dependent protein kinases and GPCR-specific G protein-cou-
pled receptor kinases (GRKs). Within seconds of receptor stimulation these kinases phosphorylate serine and threonine residues within the intracellular domains of GPCRs, thereby uncoupling receptors from heterotrimeric G proteins (10–12).

Despite the fact that group 1 mGluRs desensitize in response to protein kinase C phosphorylation (13, 14), it is unknown whether group 1 mGluR activity is regulated by GRK-mediated phosphorylation. Therefore, in the present study we examined whether GRKs contribute to the phosphorylation and desensitization of mGluR1a. Consistent with the hypothesis that mGluR1a is a substrate for GRK-mediated phosphorylation, we found that GRK2 protein expression reduced both constitutive- and agonist-stimulated mGluR1 activity and protected against mGluR1a-mediated cell death. Taken together, these data propose that GRK2-dependent receptor regulatory mechanisms play an important role in limiting mGluR-mediated synaptic plasticity and excitotoxicity in vivo.

**Experimental Procedures**

**Materials**—Restriction enzymes were obtained from Promega and New England Biolabs Inc. The pcDNA1-amp expression vector was acquired from Invitrogen. Human embryonic kidney cells (HEK) 293 cells were from American Tissue Culture Collection. Fetal bovine serum was from HyClone Laboratories Inc. Gentamicin, minimal essential medium (MEM), and 0.05% trypsin containing 0.5 ml EDTA were purchased from Life Technologies, Inc. Rabbit polyclonal antibodies raised against mGluR1a and GRK4 were obtained from Upstate Biotech Inc. The anti-GRK5 and -GRK6 rabbit polyclonal antibodies were obtained from Dr. Robert J. Lefkowitz. The anti-GRK2 rabbit polyclonal antibody was raised against the peptide sequence, DREAARKKAK, corresponding to rat GRK2. The anti-PARP monoclonal antibody (clone C11-10) was the gift of Dr. James Kirkland. Donkey anti-rabbit IgG conjugated to horseradish peroxidase was from Amersham Pharmacia Biotech. ECL Western blotting Detection Reagents were also acquired from Amersham Pharmacia Biotech. Quisqualate, 4-carboxy-3-hydroxyphenylglycine, and 1-aminoadin-1,5-dicarboxylic acid were obtained from Tocris Cookson Inc. [32P]Orthophosphate was from ICN Biomedicals, Inc. Aminoethylisothiouronium chloride (AEIOH), 3-(N-Morpholino)propanesulfonic acid (MOPS), and other sodium salts were obtained from Sigma. [3H]Myo-inositol was from Research Diagnostics Inc., and the fluorescein isothiocyanate-rabbit IgG conjugated to horseradish peroxidase was from Amersham Pharmacia Biotech. The anti-FLAG monoclonal antibody was purchased from Research Diagnostics Inc., and the fluorescein isothiocyanate-conjugated goat anti-mouse IgG was from Sigma. [32P]Orthophosphate was from PerkinElmer Life Sciences. The Dowex 1-X8 (formate form) resin with 200–400 mesh was purchased from Bio-Rad. All other reagents were obtained from commercial sources.

**Plasmid Construction**—The cDNA for mGluR1a was subcloned into the XbaI site of the pcDNA1-amp expression vector. To engineer a FLAG-tagged mGluR1a construct, the mGluR1a cDNA in pcDNA1-amp was used as the template for the initial round of PCR using a 5′ oligonucleotide primer 5′-TTTCCTGGTTGGTGCTTCGCAGTATAAGCAGG- CATGTAAGCGCCAGGTCACTACACCTAGG-3′ and a 3′ oligonucleotide 5′-GACACCCCCAGAATTCATTTG-3′. The 5′ primer encoded the last 6 amino acids of the signal sequence followed by the ECL epitope tag (DYKDDDDK) and the first seven amino acids of the FLAG epitope (DYKDDDDK) on a plasmid for the initial round of PCR using a 5′ oligonucleotide primer 5′-ATTAGTTGAACCAGCTAGACAGG-3′ and a 3′ oligonucleotide primer 5′-AACCAATCCTCAAGAGATTTGGTGTGGTGC-3′. The resulting PCR product was digested with BamHI and XbaI and then cloned into the pcDNA1-amp mGluR1a vector digested with the same enzymes.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells were maintained in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum and 100 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5% CO2. The cells used in each of the experiments, except the cell death experiments, were transfected using a modified calcium phosphate method as described previously (15). Following transfection (~18 h), the cells were incubated with fresh medium and allowed to recover 8 h and allowed to grow an additional 18 h before any experimentation. The cells used in the cell death experiments were transfected with LipofectAMINE Plus (Life Technologies, Inc.) following the manufacturer’s instructions. The FLAG-mGluR5 constructs in HEK 293 cells was confirmed by Western blot. The protein content of HEK 293 cells transiently transfected with 10 μg of each mGluR construct was determined using Bio-Rad D_2 Protein Assay Kit. 100 μg of whole cell protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by semi-dry electrotransfer onto nitrocellulose membranes. The membranes were blocked with 10% milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, 0.05% Nonidet P-40, and 0.05% Tween 20) and then incubated with polyclonal anti-mGluR1 diluted 1:1000 in wash buffer containing 3% skimmed milk. The membranes were rinsed with wash buffer and then incubated with secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1:2500 in wash buffer containing 3% skimmed milk. The membranes were rinsed with wash buffer, incubated with ECL Western blotting detection reagents, and then exposed to K-Omat Blue XB-1 film. Expression of the various GRK constructs was assessed in the same manner using a polyclonal GRK2-specific antibody (1:1000), a polyclonal anti-GRK4 antibody (1:2000), and polyclonal anti-GRK5/6 antibody (1:2000).

**Results**

**GRK5-dependent mGluR1a Desensitization**

**Materials**—Restriction enzymes were obtained from Promega and New England Biolabs Inc. The pcDNA1-amp expression vector was acquired from Invitrogen. Human embryonic kidney cells (HEK) 293 cells were from American Tissue Culture Collection. Fetal bovine serum was from HyClone Laboratories Inc. Gentamicin, minimal essential medium (MEM), and 0.05% trypsin containing 0.5 ml EDTA were purchased from Life Technologies, Inc. Rabbit polyclonal antibodies raised against mGluR1a and GRK4 were obtained from Upstate Biotech Inc. The anti-GRK5 and -GRK6 rabbit polyclonal antibodies were obtained from Dr. Robert J. Lefkowitz. The anti-GRK2 rabbit polyclonal antibody was raised against the peptide sequence, DREAARKKAK, corresponding to rat GRK2. The anti-PARP monoclonal antibody (clone C11-10) was the gift of Dr. James Kirkland. Donkey anti-rabbit IgG conjugated to horseradish peroxidase was from Amersham Pharmacia Biotech. ECL Western blotting Detection Reagents were also acquired from Amersham Pharmacia Biotech. Quisqualate, 4-carboxy-3-hydroxyphenylglycine, and 1-aminoadin-1,5-dicarboxylic acid were obtained from Tocris Cookson Inc. [32P]Orthophosphate was from ICN Biomedicals, Inc. Aminoethylisothiouronium chloride (AEIOH), 3-(N-Morpholino)propanesulfonic acid (MOPS), and other sodium salts were obtained from Sigma. [3H]Myo-inositol was from Research Diagnostics Inc., and the fluorescein isothiocyanate-rabbit IgG conjugated to horseradish peroxidase was from Amersham Pharmacia Biotech. The anti-FLAG monoclonal antibody was purchased from Research Diagnostics Inc., and the fluorescein isothiocyanate-conjugated goat anti-mouse IgG was from Sigma. [32P]Orthophosphate was acquired from PerkinElmer Life Sciences. The Dowex 1-X8 (formate form) resin with 200–400 mesh was purchased from Bio-Rad. All other reagents were obtained from commercial sources.

**Plasmid Construction**—The cDNA for mGluR1a was subcloned into the XbaI site of the pcDNA1-amp expression vector. To engineer a FLAG-tagged mGluR1a construct, the mGluR1a cDNA in pcDNA1-amp was used as the template for the initial round of PCR using a 5′ oligonucleotide primer 5′-TTTCCTGGTTGGTGCTTCGCAGTATAAGCAGG-CATGTAAGCGCCAGGTCACTACACCTAGG-3′ and a 3′ oligonucleotide 5′-GACACCCCCAGAATTCATTTG-3′. The 5′ primer encoded the last 6 amino acids of the signal sequence followed by the ECL epitope tag (DYKDDDDK) and the first seven amino acids of the FLAG epitope (DYKDDDDK) on a plasmid for the initial round of PCR using a 5′ oligonucleotide primer 5′-ATTAGTTGAACCAGCTAGACAGG-3′ and a 3′ oligonucleotide primer 5′-AACCAATCCTCAAGAGATTTGGTGTGGTGC-3′. The resulting PCR product was digested with BamHI and XbaI and then cloned into the pcDNA1-amp mGluR1a vector digested with the same enzymes.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells were maintained in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum and 100 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5% CO2. The cells used in each of the experiments, except the cell death experiments, were transfected using a modified calcium phosphate method as described previou...
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anion-exchange chromatography using Dowex 1-X8 (formate form) anion-exchange resin with 200–400 mesh. [3H]Inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

**GRK2 Co-immunoprecipitation**—Transiently transfected HEK 293 cells were incubated for 1 h at 37 °C in HBSS followed by stimulation with 50 μM quisqualate. The cells were solubilized in lysis buffer containing protease inhibitors (25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotonin, 20 μg/ml leupeptin). The protein content of the lysates was determined using the Bio-Rad Protein Assay Kit. FLAG-mGluR1a was immunoprecipitated with a monoclonal anti-FLAG M2 antibody using protein G-Sepharose beads from cell lysates containing 500 μg of protein. The immunoprecipitated proteins were subjected to SDS-PAGE followed by electroblotting onto nitrocellulose membranes.

**DNA Fragmentation**—HEK 293 cells were seeded in 6-well plates at a density of 4 × 10⁵ cells/well and were transfected using LipofectAMINE Plus the next day. Adherent and non-adherent cells were collected, rinsed with PBS, and then fixed with 70% ethanol 24, 48, and 72 h post-transfection. Following fixation, the cells were washed with PBS and then incubated for 30 min at room temperature with 500 μl of PBS containing 20 μg/ml propidium iodide, 0.1% (v/v) Triton X-100, and 200 μg/ml RNase A. DNA content was determined by measuring propidium iodide fluorescence using flow cytometry (18).

**PARP Immunoblot Analysis**—HEK 293 cells transiently transfected using LipofectAMINE Plus were harvested 48 h post-transfection. The cells were washed with cold PBS, pelleted by centrifugation, and then stored at -20 °C until assayed. The cell pellet was resuspended in 1 ml of cold PBS, and 100 μg of whole cell protein was subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.05% Tween 20 and 10% milk and then incubated with an anti-mouse conjugated to horseradish peroxidase diluted 1:2500 in 3% milk TBS. The membranes were washed with TBS and then incubated with an anti-PARP monoclonal antibody (clone 72) diluted 1:10,000 in 3% milk. The membranes were washed with PBS and then incubated for 30 min at room temperature with 500 μl of PBS containing 20 μg/ml propidium iodide, 0.1% (v/v) Triton X-100, and 200 μg/ml RNase A. DNA content was determined by measuring propidium iodide fluorescence using flow cytometry (18).

**Data Analysis**—The mean and S.E are expressed for values obtained from the number of separate experiments indicated. Dose-response data were analyzed using GraphPad Prism. Statistical significance was determined using unpaired two-tailed t test.

**RESULTS**

**GRK2 Expression-dependent Inhibition of mGluR1a-mediated Cell Death**—Because the majority of studies investigating either mGluR1a structure-activity relationships or GRK2-mediated receptor desensitization mechanisms have utilized HEK 293 cells (e.g. Refs. 5, 19, and 20), HEK 293 cells were used as a model system to examine the contribution of GRKs to mGluR1a desensitization. We observed that the transfection of HEK 293 resulted in a substantial and consistent loss of mGluR1a-transfected cells when compared with mock-transfected cells at 24, 48, and 72 h post-transfection but peaked at 72 h post-transfection (Fig. 1, A and B). Surprisingly, the co-expression of GRK2 with the mGluR1a prevented the cell death associated with the expression of mGluR1a (Fig. 1C). Therefore, subsequent experiments tested the mechanism by which cell survival was mediated by the co-transfection of GRK2 with the mGluR1a.

**Specificity for GRK-mediated Phosphorylation and Desensitization of FLAG-mGluR1a**—In order to determine whether mGluR1a might serve as a substrate for GRK-mediated phosphorylation and desensitization, a C-terminally tagged FLAG epitope-tagged mGluR1a construct was tested for expression and functionality coupling to PLCβ-stimulated increases in intracellular Ca²⁺ concentrations in HEK 293 cells. Immunoblot analysis revealed that the expression of the FLAG-mGluR1a protein expression was equivalent to or better than that observed for the wild-type receptor (Fig. 2A). Furthermore, in response to stimulation with 50 μM quisqualate, the FLAG-mGluR1a-mediated increases in intracellular Ca²⁺ concentrations were indistinguishable from that of the wild-type receptor (Fig. 2, B and C). Quisqualate did not elicit a response in cells lacking transfected mGluR1a (Fig. 2D).

To determine whether GRKs are involved in the desensitization of mGluR1a responsiveness, we tested whether the co-expression of GRK2, GRK4, GRK5, and GRK6 resulted in a reduction in quisqualate-stimulated inositol phosphate formation. The overexpression of both GRK2 and GRK5 effectively inhibited mGluR1a-stimulated increases in inositol phosphate formation in response to increasing concentrations of quisqualate (Fig. 3). The maximal mGluR1a-mediated response to quisqualate was reduced 66 and 59% by GRK2 and GRK5, respectively. GRK4 and GRK6 expression had no effect on agonist-stimulated mGluR1a activity (Fig. 3). Taken together, these observations suggest that only GRK2 and GRK5 are involved in the desensitization of agonist-stimulated mGluR1a activity.

To establish a role for GRK phosphorylation in the desensitization of mGluR1a responsiveness, we sought to examine whether GRK2 contributed to the agonist-stimulated whole cell phosphorylation of mGluR1a. We focused on GRK2, because GRK2 protein is widely distributed throughout the central nervous system and is localized to mGluR1a-expressing brain regions (21, 22). In the absence of agonist, the FLAG-mGluR1a was substantially phosphorylated, and the phosphoprotein migrated with a molecular mass of approximately 140–170 kDa (Fig. 4A). This phosphoprotein was absent in immunoprecipitates from cells that were not transfected with FLAG-mGluR1a (Fig. 4A). Upon stimulation with either 10 μM quisqualate or 1 μM PMA, mGluR1a phosphorylation was increased 1.7 ± 0.2- and 1.9 ± 0.3-fold above control levels (control unstimulated), respectively (Fig. 4A). Overexpression of GRK2 resulted in a
significant increase in mGluR1a phosphorylation in both the absence (1.6 \pm 0.1-fold above control basal phosphorylation) and presence (2.5 \pm 0.4-fold above control basal phosphorylation) of 10 \mu M quisqualate (Fig. 4A).

Since GRK2 overexpression led to increased phosphorylation and desensitization of mGluR1a, we tested whether GRK2 protein endogenously expressed in HEK 293 cells contributed to the agonist-stimulated increase in mGluR1a phosphorylation. Therefore, we examined the effects of expressing two dominant-negative GRK2 mutants, GRK2-K220R and GRK2-CT, on both the agonist-stimulated phosphorylation and desensitization of mGluR1a. The GRK2-K220R mutant is catalytically inactive and is thought to function as a dominant-negative inhibitor of GPCR phosphorylation by competing with endogenous GRK2 for the receptor (23). In contrast, the GRK2-CT dominant-negative mutant comprises the GRK2 carboxyl-terminal domain that mediates GRK2 plasma membrane translocation via its interaction with the \( \beta \gamma \)-subunit of the heterotrimeric G protein (24, 25). Thus, the GRK2-CT mutant competes for \( \beta \gamma \)-binding sites, thereby preventing GRK2 membrane translocation (24). Overexpression of GRK2-K220R and GRK2-CT mutants effectively blocked agonist-stimulated mGluR1a phosphorylation (Fig. 4B). Consistent with the blockade of mGluR1a phosphorylation, the GRK2-CT mutant potentiated mGluR1a-stimulated inositol phosphate formation (Fig. 4B). Although less effective than wild-type GRK2, GRK2-K220R mutant expression resulted in the attenuation of mGluR1a-stimulated inositol phosphate formation (Fig. 4B).

Previously, wild-type GRK2 and GRK2-K220R were demonstrated to co-immunoprecipitate with the parathyroid hormone receptor (26). This interaction, rather than receptor phosphorylation, was proposed to underlie the GRK2-mediated desensitization of this receptor subtype (26). Therefore, we tested whether both the wild-type GRK2 and the GRK2-K220R mutant physically associate with the FLAG-mGluR1a in HEK 293 cells by co-immunoprecipitation. Although we were unable to detect endogenous GRK2 in FLAG-mGluR1a immunoprecipitates, following their overexpression, both the wild-type GRK2 and the GRK2-K220R mutant were co-immunoprecipitated with FLAG-mGluR1a in the absence of agonist stimulation (Fig. 5, A and B). However, the amount of GRK2 protein co-immunoprecipitated was unchanged following agonist stimulation for 5 and 45 min (Fig. 5, A and B). This was not due to either nonspecific immunoprecipitation or contamination of immunoprecipitates with GRK2, since no GRK2 was immunoprecipitated from HEK 293 cells lacking FLAG-mGluR1a (Fig. 5, A and B). Since most GPCRs are rapidly phosphorylated by GRKs, we examined whether agonist stimulation would increase GRK2 protein association at 15, 30, and 60 s. When tested, the amount of wild-type GRK2 and GRK2-K220R mu-
represent the mean ± S.E. of three independent experiments. In these experiments the data were normalized to FLAG-mGluR1a phosphorylation in the absence of agonist treatment. PMA, phorbol 12-myristate 13-acetate. B, the effect of wild-type GRK2, GRK2-K220R, and GRK2-CT expression on mGluR1a-stimulated IP formation in response to increasing concentrations of quisqualate for 30 min at 37 °C. The data represent the mean ± S.E. for three independent experiments. In all these experiments, HEK 293 cells were transfected with 5 µg of pcDNA1-Amp plasmid cDNA encoding FLAG-mGluR1a with and without 10 µg of pcDNA1-Amp plasmid cDNA encoding either wild-type GRK2, GRK2-K220R, or GRK2-CT constructs. *, p < 0.05 versus basal FLAG-mGluR1a phosphorylation.

![Co-immunoprecipitation of GRK2 and GRK2-K220R with FLAG-mGluR1a](image)

**FIG. 5.** Co-immunoprecipitation of GRK2 and GRK2-K220R with FLAG-mGluR1a. A, immunoblot of wild-type GRK2 protein co-immunoprecipitated (IP) with the FLAG-mGluR1a from lysates of HEK 293 transfected to express either FLAG-mGluR1a alone, FLAG-mGluR1a with wild-type GRK2, or wild-type GRK2 alone following 0, 5, and 45 min of treatment with 50 µM quisqualate. B, immunoblot of GRK2-K220R protein co-immunoprecipitated with the FLAG-mGluR1a from lysates of HEK 293 transfected to express either FLAG-mGluR1a alone, FLAG-mGluR1a with the GRK2-K220R mutant, or the GRK2-K220R mutant alone following 0.5, and 45 min of treatment with 50 µM quisqualate. C, immunoblot of GRK2 protein co-immunoprecipitated with the FLAG-mGluR1a from lysates of HEK 293 cells transfected with FLAG-mGluR1a with either the wild-type GRK2 or the GRK2-K220R mutant following 0, 15, 30, and 60 s of treatment with 50 µM quisqualate. The data are representative immunoblots from three independent experiments. In all experiments HEK 293 cells were transfected with 10 µg of pcDNA1-Amp plasmid DNAs encoding either mGluR1a, wild-type GRK2, or GRK2-K220R mutant.

important protein co-immunoprecipitated with the FLAG-mGluR1a was increased following 15–30 s of agonist stimulation and then returned to basal levels (Fig. 5C). These observations indicate that both the wild-type GRK2 and GRK2-K220R mutant can physically associate with the mGluR1a and suggest that mGluR1a phosphorylation is not absolutely required for GRK2-mediated desensitization. However, the observation that wild-type GRK2 is more effective at desensitizing mGluR1a activity indicates that receptor phosphorylation contributes to the GRK2-mediated mGluR1a desensitization phenomenon.

**GRK-mediated Regulation of the Basal mGluR1 Activity**— Similar to a previous report (19), mGluR1a exhibited substantial basal levels of inositol phosphate formation when expressed in HEK 293 cells (Fig. 6). This high basal receptor activity was not observed following the expression of another Gq-coupled receptor, the angiotensin II type 1A receptor, at similar receptor densities (Fig. 6). Constitutive mGluR1a activity was not reduced by the treatment of cell cultures with competitive group I antagonists, such as 4-carboxy-3-hydroxyphenylglycine and 1-aminoindan-1,5-dicarboxylic acid (data not shown) (19). Moreover, whereas glutamate has been observed to accumulate in HEK 293 cell cultures, the treatment of cultures with the glutamate-degrading enzyme glutamate/pyruvate transaminase did not reduce mGluR1a basal activity (data not shown) (19). However, the expression of GRK2 with mGluR1a significantly reduced basal mGluR1a activity to 12 ± 3% of control values (Fig. 6). In contrast, GRK2-CT mutant and GRK4 expression had no effect on basal mGluR1a activity (Fig. 6). Although GRK5 was as effective as GRK2 at attenuating agonist-stimulated mGluR1a activity (Fig. 3A), GRK5 was less effective than GRK2 at reducing basal mGluR1a-mediated inositol phosphate formation (Fig. 6). GRK6, which had no effect on agonist-stimulated mGluR1a responsiveness, had a small but statistically insignificant effect on basal mGluR1a-stimulated inositol phosphate formation. Thus, the GRK2 expression-dependent survival of mGluR1a-expressing cell cultures (Fig. 1) may be a consequence of the GRK-dependent inactivation of constitutive mGluR1a activity.

**Characterization of GRK2-mediated Inhibition of mGluR1a-mediated Cell Death**— Group 1 mGluR-mediated cell death has been demonstrated to involve both necrosis and apoptosis in vivo (7). Therefore, we examined the mechanism by which GRK2 overexpression specifically protected against mGluR1a-
or GRK6. Cells were labeled with 1 
HEK 293 cells were transfected with 5 
C

Fig. 6. Effect of GRK expression on basal mGluR1a activity. HEK 293 cells were transfected with 5 μg of plasmid cDNA encoding the angiotensin II type 1A receptor (AT1R), FLAG-mGluR1a alone or with 10 μg of wild-type GRK2, GRK2-K220R, GRK2-CT, GRK4, GRK5, or GRK6. Cells were labeled with 1 μCi/ml [3H]inositol overnight. Unincorporated [3H]inositol was washed away, and the cells were preincubated for 1 h at 37 °C in a physiological salt solution followed by a 10-min incubation in the same buffer supplemented with 10 mM LiCl. The basal activity of mGluR1a signaling was measured as the fraction of the incorporated [3H]inositol converted to [3H]inositol phosphates in the absence of agonist stimulation. The data represent the mean ± S.E. for 4–13 experiments. *, p < 0.05 versus control (mGluR1a alone).

stimulated loss of cell viability. By using propidium iodide staining and flow cytometry, the mGluR1a-mediated death of HEK 293 cells was quantified on the basis of reduced DNA content (sub-G0/G1) compared with cellular DNA content in control cells or cells transfected to express the mGluR1a with or without the GRK2-CT receptor. Similarly, mGluR1a expression with or without the GRK2-CT receptor was reduced to 11% of the population of mGluR1a-expressing cells was undergoing cell death as compared with 6% ± 1% of the control population (Fig. 7B). When GRK2 was co-expressed with mGluR1a, the percentage of dying cells was reduced to 11 ± 3%, which was not significantly different from cells co-transfected with GRK2 alone or control mock-transfected populations (Fig. 7B). The addition of the 10 μM quisqualate to the cultures did not effect cell viability (data not shown).

To determine whether the reduction in DNA content measured by propidium iodide represented an index of apoptosis, we examined the proportion of cleaved poly(ADP-ribose) polymerase (PARP) (27). As a positive control for apoptotic induced cell death, HEK 293 cells were treated with 1 μM staurosporine for 4, 8, and 14 h (Fig. 7C). In response to staurosporine treatment, intact 112-kDa PARP protein was cleaved to reveal an 86-kDa cleavage product that is indicative of apoptosis (Fig. 7C). Similarly, mGluR1a expression with or without the GRK2-CT resulted in the detection of the 86-kDa PARP cleavage product, whereas no PARP cleavage occurred in either mock-transfected control cells or cells transfected to express the mGluR1a with GRK2 (Fig. 7C). We conclude from these data that GRK2-dependent regulation of mGluR1a responsiveness plays a critical role in protecting excessive excitotoxic mGluR1a activation.

DISCUSSION

GPCR desensitization represents an important regulatory mechanism by which acute and/or chronic receptor overstimulation is avoided. Whereas these regulatory mechanisms are well characterized for prototypic GPCRs, such as the β2-adrenergic receptor, previous to this study only second messenger-dependent protein kinases have been reported to contribute to the desensitization of mGluR family members. In the present study, we demonstrate that in addition to serving as a substrate for second messenger-dependent kinases, mGluR1a is a target for GRK-mediated phosphorylation. Our study expands the NRK substrate specificity to include representative members of every GPCR subfamily. GRK phosphorylation of mGluR1a, specifically by GRK2 and GRK5, results in the desensitization of agonist-stimulated mGluR1a responses. In addition, GRK-mediated phosphorylation also contributes to the regulation of spontaneous mGluR1a activity. Moreover, we observe that GRK2-dependent mGluR1a desensitization protects against mGluR1a-mediated cell death, at least in part, by blocking mGluR1a-stimulated apoptosis. Taken together, these observations provide direct evidence supporting the assertion that feedback modulation of mGluR1a activity by GRK phosphorylation and desensitization is essential for regulated mGluR1a signal transduction. Furthermore, because mGluR5 play an important role in regulating synaptic plasticity and excitotoxicity, it is likely that GRK-mediated mGluR1a desensitization limits both neuronal excitability and neuronal injury under pathophysiological conditions.

To our knowledge, these experiments represent the first demonstration that mGluR1a activity is regulated by GRKs. Although the agonist-stimulated desensitization of mGluR1a responsiveness is relatively well documented, this desensitization has been presumed to be solely the consequence of phosphorylation by second messenger-dependent protein kinases, such as PKC (13, 28). Nonetheless, the extended carboxyl-terminal tails of both mGluR1a and mGluR5 contain more than 50 serine and threonine residues that conform to known consensus motifs for protein kinase phosphorylation. GRKs are considered to phosphorylate preferentially serine and threonine residues that are either clustered together or are flanked by acidic amino acid residues (29, 30). Consistent with the ability of mGluR1a to serve as a substrate for GRK phosphorylation, many of the serine and threonine residues within the carboxyl-terminal domain of mGluR1a are clustered and/or flanked by acidic amino acid residues. Maximal mGluR1a desensitization required receptor phosphorylation since a catalytically inactive GRK2 mutant (K220R) was only partially effective at uncoupling agonist-stimulated mGluR1a responses. Furthermore, the desensitization of mGluR1a responsiveness was GRK subtype-specific and was mediated by GRK2 and GRK5 but not GRK4 and GRK6.

Unlike many GPCRs, mGluR1a exhibits substantial spontaneous activity in the absence of agonist stimulation. In the present study, spontaneous mGluR1a activity was at least 10-fold higher than that observed for the angiotensin II type 1A receptor (Fig. 6). The mutation of residues within the transmembrane spanning and intracellular loop domains of many GPCRs results in the generation of constitutively activated mutant receptors (31–34). These constitutively activating mutations in GPCRs are linked to genetic diseases such as retinitis pigmentosa, precocious puberty, and thyroid adenomas (32–34). Constitutive mGluR1a activity appears to be an intrinsic property of the mGluR1a that is mediated by the carboxyl-terminal tail domain of mGluR1a, since mGluR1 alternative splice variants (mGluR1b and mGluR1c) that have short carboxyl-terminal tails do not exhibit constitutive receptor activity (19). It is possible that the size of the mGluR1a carboxyl-terminal tail allows it to act as a modular receptor subdomain that regulates interactions with heterotrimeric G proteins in
the absence of agonist stimulation. Furthermore, although known competitive mGlur1a antagonists do not inhibit basal mGlur1a activity (19), we find that the co-expression of GRK proteins, in particular GRK2, significantly reduced constitutive mGlur1a activity in HEK 293 cells to levels equivalent to that observed for the Gq-coupled angiotensin II type 1A receptor. Thus, GRKs modulate intrinsic mGlur1a activity, in addition to agonist-stimulated responses. This novel GRK regulatory property extends to some but not all GPCRs. For example, constitutively active α2- and β2-adrenergic receptors are substrates for GRK2-mediated phosphorylation in the absence of agonist, whereas a constitutively active angiotensin II type 1A receptor does not exhibit increased basal phosphorylation (35–37).

The co-expression of GRK2 with the mGlur1a resulted in a profound increase in survival of mGlur1a-expressing cells. This protection appears to involve the desensitization of mGlur1a-mediated activation of PLCβ. In the absence of GRK2, constitutive mGlur1a activation of PLCβ leads to the sustained elevations in inositol phosphate formation that may lead increases in intracellular Ca2+ concentrations and/or PKC activation, a situation that is considered to induce cellular necrosis and/or apoptosis (38). However, measures of resting intracellular Ca2+ concentrations in mGlur1a-expressing HEK 293 cells did not reveal substantial increases in resting Ca2+ concentrations when compared with angiotensin II type 1A receptor-expressing cells. This observation might suggest that IP3 receptor adaptation to IP3 may occur in response to continued elevation of intracellular IP3 concentrations over the 48–72-h time frame HEK 293 cells express mGlur1a. A similar observation was reported previously for cardiac ryanodine receptor responses to maintained Ca2+ stimuli (39). Furthermore, the mGlur1a-mediated HEK 293 cell death was progressive over a 24–72-h period suggesting that relatively small but maintained increases in intracellular Ca2+ concentrations may be toxic to cells. mGlur1a-mediated cell death does not appear to be related to chronic PKC activation, since no cleavage of PKCδ was observed in HEK 293 cells expressing mGlur1a (data not shown). The constitutive mGlur1a activity may also be further exacerbated by low concentrations of contaminating glutamate released by cells into the cell culture medium.

In the present study, we provide evidence that sustained basal mGlur1a activation of PLCβ leads to increased apoptosis as measured by PARP cleavage. Thus GRK-mediated mGlur1a desensitization protects, at least in part, against mGlur1a-stimulated apoptosis. Nonetheless, we do not exclude the possibility that necrosis also contributes to the death of mGlur1a-expressing cells, since group 1 mGlurA-mediated excitotoxicity in vitro is considered to involve a combination of necrosis and apoptosis (7). However, regardless of the cellular process by which cell death was ultimately achieved, GRK-dependent mGlur1a desensitization clearly represents an important receptor regulatory mechanism protecting against mGlur-mediated excitotoxicity. A potential mechanism by which group 1 mGlurA activation is thought to contribute to neuronal cell death is through the PKC-dependent potentiation of NMDA currents (7). However, our data indicate that mGlur1a-mediated cell death can be initiated in an NMDA receptor-inde-
dependent manner, since HEK 293 cells do not express endogenous NMDA receptors.

The activation of group 1 mGluRs is associated with increased neuronal excitability and contributes to the regulation of normal brain functions such as memory and learning (3). However, group 1 mGluRs are also implicated in the progression of excitotoxic damage caused by acute brain trauma and ischemia, as well as the neuronal damage associated with neurodegenerative diseases including Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (8, 9). GRK2 protein is most abundantly expressed in the brain and primary blood leukocytes (20). GRK protein is widely expressed in brain regions that express group 1 mGluRs, such as Purkinje cells and the granular layer of the cerebellum as well as the various molecular layers of the hippocampus (20, 21). Moreover, GRK2 protein is localized to the cell bodies of neurons as well as dendritic spines and axon terminals (20). Therefore, the GRK-mediated regulation of mGluR1a activity documented in the present study is consistent with a potentially important role for GRKs in regulating mGluR-dependent neuronal excitability and the induction of synaptic plasticity in vivo. As a consequence, the GRK-mediated desensitization of group 1 mGluRs may limit excitotoxic cell damage in response to prolonged mGluR activation during pathological states such as epilepsy and stroke that result in increased glutamate concentrations in the brain.

In summary, we demonstrate that GRK2-mediated phosphorylation and desensitization is a GPCR regulatory property that is conserved for mGluR1a, a representative member of the distinct subclass of GPCRs. This result indicates that GRK-mediated receptor phosphorylation is a conserved regulatory mechanism that operates independently of receptor classification as determined by GPCR agonist-selectivity, G protein-coupling specificity, sequence homology, or molecular structure. Moreover, we provide clear evidence that GRK-mediated mGluR1a desensitization protects against cell death in vitro. Additional studies will be needed to determine if GRK-mediated desensitization of group 1 mGluRs plays a role in limiting injury in pathological conditions, development, and/or the regulation of memory and learning.

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G Protein-coupled Receptor Kinase-mediated Desensitization of Metabotropic Glutamate Receptor 1A Protects against Cell Death
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