Phosphorylation-independent Regulation of Metabotropic Glutamate Receptor 1 Signaling Requires G Protein-coupled Receptor Kinase 2 Binding to the Second Intracellular Loop*

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Gurpreet K. Dhami†‡, Andy V. Babwah‡, Rachel Sterne-Marr**, and Stephen S. G. Ferguson†§‡‡

From the †Cell Biology Research Group, Robarts Research Laboratory and §Department of Physiology and Pharmacology, The University of Western Ontario, 100 Perth Drive, London, Ontario N6A 5K8, Canada and **Biology Department, Siena College, Loudonville, New York 12211

Metabotropic glutamate receptors (mGluRs) are members of a unique class of G protein-coupled receptors (class III) that include the calcium-sensing and γ-aminobutyric acid type B receptors. The activity of mGluRs is regulated by second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs). The attenuation of both mGluR1a and mGluR1b signaling by GRK2 is phosphorylation- and β-arrestin-independent and requires the concomitant association of GRK2 with both the receptor and Gαq11. G protein interactions are mediated, in part, by the mGluR1a intracellular second loop, but the domains required for GRK2 binding are unknown. In the present study, we showed that GRK2 binds to the second intracellular loop of mGluR1a and mGluR1b and also to the mGluR1a carboxy-terminal tail. Alanine scanning mutagenesis revealed a discrete domain within loop 2 that contributes to GRK2 binding, and the mutation of either lysine 691 or 692 to an alanine within this domain resulted in a loss of GRK2 binding to both mGluR1a and mGluR1b. Mutation of either Lys691 or Lys692 prevented GRK2-mediated attenuation of mGluR1b signaling, whereas the mutation of only Lys692 prevented GRK2-mediated inhibition of mGluR1a signaling. Thus, the mGluR1a carboxy-terminal tail may also be involved in regulating the signaling of the mGluR1a splice variant. Taken together, our findings indicated that kinase binding to an mGluR1 domain involved in G protein-coupling is essential for the phosphorylation-independent attenuation of signaling by GRK2.

Metabotropic glutamate receptors (mGluRs)† comprise a family of eight G protein-coupled receptors (GPCRs) that are activated by the excitatory amino acid glutamate and play an important role in regulating neuronal development and synaptic plasticity (1–3). Group I mGluRs (mGluR1 and mGluR5) are coupled via the heterotrimeric G protein Gαq11 to the activation of phospholipase C, resulting in the formation of both inositol 1,4,5-trisphosphate and diacylglycerol, as well as increases in intracellular Ca2+ concentrations. The alternative gene splicing of mGluR1 generates five carboxy-terminal-domain mGluR1 splice variants (1a, 1b, 1c, 1d, and 1e) (1–4). The G protein-coupling domain for these slice variants is conserved, and it is mediated by amino acid residues localized within the second and third intracellular loops and the membrane proximal region of the mGluR1 carboxy-terminal tail (4).

Similar to most GPCRs, mGluR activity is regulated by serine/threonine protein kinases (5–9). In general, the desensitization of GPCRs involves either phosphorylation by second messenger-dependent protein kinase or phosphorylation by G protein-coupled receptor kinases (GRKs) to promote the binding of arrestin proteins (10, 11). GRK2, GRK4, and GRK5 have each been demonstrated to contribute to the desensitization and internalization of mGluR1 in both heterologous cell cultures and Purkinje cells (5–7, 12–15). Although GRK2 and GRK4 promote the β-arrestin-dependent internalization of mGluR1 in response to agonist activation (12–15), GRK2-mediated desensitization of mGluR1a and mGluR1b appears to be independent of both GRK2-mediated phosphorylation and β-arrestin binding (12, 15).

The crystal structure for GRK2 reveals that the kinase is composed of three functional domains: a regulator of G protein signaling homology (RH) domain, a protein kinase domain, and a carboxy-terminal Gβγ binding pleckstrin homology domain (16). Several recent studies have demonstrated that the expression of the GRK2 RH domain alone is sufficient to mediate the desensitization of a number of GPCRs (15, 17–22). The GRK2 and GRK3 RH domains specifically interact with Gαq/11 family proteins in an AlF4−-dependent manner and may also function as weak GTPase-activating proteins for Gαq/11 (23). Based on the crystal structure, it appears that the three vertices of GRK2 (amino-terminal RH domain, central catalytic domain, and carboxy-terminal pleckstrin homology domain) are ordered such that they may interact simultaneously with the receptor, Gαq/11, and Gβγ to attenuate receptor activity (16). In agreement with this, GRK2 mutants impaired in Gαq/11 binding (R106A, D110A, and M114A) interact with mGluR1 but do not mediate mGluR1 desensitization (24). Previously, we identified a GRK2-D527A mutant that is impaired in mGluR1 binding and homology; GST, glutathione S-transferase; IL, intracellular loop; PBS, phosphate-buffered saline.

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§§ Holds a Canada Research Chair in Molecular Neuroscience, recipient of the Premier’s Research Excellence Award, and a Career Investigator of the Heart and Stroke Foundation of Ontario. To whom correspondence should be addressed: Robarts Research Institute, 100 Perth Drive, P. O. Box 5015, London, Ontario N6A 5K8, Canada. Tel.: 519-663-3822; Fax: 519-663-3314; E-mail: ferguson@roberts.ca.

† The abbreviations used are: mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IP, inositol phosphate; RH, regulator of G protein signaling.
did not attenuate mGluR1 signaling but retained the ability to interact with Goq/11 (24). Thus, the mechanism underlying GRK2 phosphorylation-independent attenuation of mGluR1a signaling appears to be RH domain-dependent, requiring the binding of GRK2 to both Goq/11 and mGluR1.

Because GRK2 binding to mGluR1 appears to be essential to allow the phosphorylation-independent antagonism of mGluR1a signaling, we tested the hypothesis that GRK2 might bind to overlapping regions of intracellular mGluR1 domains that are essential for G protein coupling. We found that GRK2 binding to mGluR1a and mGluR1b is primarily mediated by residues localized within intracellular loop 2 and that the mutation of either Lys691 or Lys692 to alanine results in a loss of GRK2 binding to both receptors. Mutation of either Lys691 or Lys692 prevented GRK2-mediated inhibition of mGluR1a signaling. Thus, our studies revealed that the second intracellular loop is the primary GRK2 binding site and indicated that GRK2 binding to an additional site on the mGluR1a carboxyl-terminal tail reveals subtle differences in the regulation of mGluR1a and mGluR1b signaling by GRK2.

**EXPERIMENTAL PROCEDURES**

**Materials—** HEK293 human embryonic kidney cells were obtained from American Type Culture Collection (Manassas, VA). Inositol-free Dulbecco’s modified Eagle’s medium, minimal essential medium, fetal bovine serum, trypsin, and gentamicin were from Invitrogen. The anti-GRK2 antibody was raised against the peptide sequence DREARKKANQKLGH, corresponding to rat GRK2. Anti-GST antibodies, donkey anti-rabbit IgG conjugated to horseradish peroxidase, ECL Western blotting detection reagents, and the protein G-Sepharose beads were from GE Healthcare (Oakville, Ontario, Canada). Quisqualate was purchased from Tocris Cookson, Inc. (Ellisville, MO).[3H]Inositol was obtained from PerkinElmer Life Sciences. The Dowex 1-X8 (formate form) resin with 200–400 mesh was purchased from Bio-Rad. All other biochemical reagents were purchased from Sigma.

**Plasmid Construction—** The FLAG-mGluR1a and GRK2 constructs have been described previously (7, 15, 24). Point mutations were introduced into the second intracellular loop domain of both mGluR1a and mGluR1b using the QuikChange site-directed mutagenesis kit (Stratagene). For GST fusion proteins, mGluR1a PCR-generated sequences were cloned as GST-tagged EcoRI-XhoI products into the GST-prokaryotic expression vector pGEX-4T (GE Healthcare). The PCR-generated sequences contain mGluR1a intracellular loop (IL) 1 (GST-IL1, amino acids 589–650), loop 2 (GST-IL2, amino acids 657–727), loop 3 (GST-IL3, amino acids 735–807), the membrane-proximal portion of the carboxyl tail (amino acids 816–860), and the rest of the carboxyl tail (amino acids 861–1199) (Fig. 1A). The IL2 (amino acids 673–710) alanine scanning mutants were generated by PCR and cloned as GST-tagged EcoRI-XhoI products into the GST-prokaryotic expression vector pGEX-4T. pGEX-4T-based constructs were transformed into the Escherichia coli BL21. The sequence integrity of each of the mutants was confirmed by automated DNA sequencing.

**Cell Culture and Transfection—** HEK293 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin. Cells were transfected with plasmid cDNAs using a modified calcium phosphate method (25). Following transfection (18 h), the cells were incubated with fresh medium, allowed to recover for 6–8 h, and re-seeded into either 6- or 24-well dishes and then grown for an additional 18 h prior to experimentation.

**Inositol Phosphate Formation—** Inositol lipids were radiolabeled by incubating the cells overnight with 1 μCi/ml myo-[3H]inositol in Dulbecco’s modified Eagle’s medium. Unincorporated myo-[3H]inositol was removed by washing the cells with Hanks’ balanced salt solution (116 mm NaCl, 20 mm Hepes, 11 mm glucose, 5 mm NaHCO3, 4.7 mm KCl, 2.5 mm CaCl2, 1.2 mm KH2PO4, 0.5 mm MgCl2, pH 7.4). The cells were then pre-incubated for 1 h in Hanks’ balanced salt solution 37°C and then pre-incubated in 500 μl of the same buffer containing 10 μM LiCl for an additional 10 min at 37°C. The cells were then incubated in either the absence or presence of increasing concentrations (0–30 μM) of quisqualate for 30 min at 37°C. The reaction was stopped by ice on the addition of 500 μl of perchloric acid and then neutralized with 400 μl of 0.72 M KOH, 0.6 M KHCO3. The total [3H]inositol incorporated into the cells was determined by counting the radioactivity present in 50 μl of the cell lysate. Total inositol phosphate was purified from the cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form) 200–400 mesh exchange resin. [3H]Inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system.

**GST-mGluR1a Fusion Protein Purification and Pull-down Assays—** GST-mGluR1a peptides were generated by growing recombinant BL21 bacteria at 21°C to an A600 of 0.6–1.0. Cultures were induced for 3 h with 1 mm isopropyl 1-thio-α-D-galactopyranoside, pelleted, resuspended in phosphate-buffered saline (PBS) containing protease inhibitors (described under “Co-immunoprecipitation”), and lysed by mild sonication. The bacterial lysates were cleared of cellular debris by centrifugation and then applied to glutathione-Sepharose 4B (Sigma) overnight at 4°C. GST-mGluR1a peptides bound to the matrix were washed extensively in PBS containing 0.3% Triton X-100. 1 μg of mGluR1a GST peptide was used in each pull-down assay. HEK293 cell lysates overexpressing GRK2 were prepared and cleared of cellular debris by centrifugation; 500 μg of total protein was used in each pull-down assay. Matrix-bound mGluR1a peptides and HEK293 cell lysates were incubated together and mixed overnight at 4°C. The matrix-bound protein complexes were washed extensively in PBS containing 0.3% Triton X-100 and then eluted off the matrix in SDS loading buffer by boiling for 3–5 min at 100 °C. Eluted samples were analyzed by SDS-PAGE and Western blotting with anti-GRK2 and anti-GST antibodies.

**Co-immunoprecipitation—** The cells from 100-mm dishes were washed twice with ice-cold PBS and lysed in 400 μl of cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100 containing protease inhibitors, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 20 μg/ml phenylmethylsulfonyl fluoride). The particulate fraction was re-suspended in centrifugation buffer, and 500 μg of cell lysate was incubated with 5 μl of anti-FLAG monoclonal antibody and 100 μl of 20% protein G-Sepharose beads or 40 μl of FLAG affinity gel for 12–16 h at 4°C in a 1-mL volume. The beads were washed three times with lysis buffer, and proteins were eluted in 3× SDS sample buffer and then separated by SDS-PAGE. The proteins were transferred onto nitrocellulose membranes by semi-dry electrophotography. The membranes were blocked with 10% milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) and then incubated with rabbit GRK2-specific and FLAG-specific (Sigma) antibodies diluted 1:1000 in wash buffer containing 3% skim milk. The membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) diluted 1:2500 in wash buffer containing 3% skim milk. Membranes were rinsed twice with wash buffer and twice with Tris-buffered saline and incubated with ECL Western blotting detection reagents.

**Receptor Expression—** Cells expressing FLAG-tagged mGluR1a were labeled on ice with an anti-FLAG antibody (1:500) for 45 min. The cells were washed with cold PBS and subsequently labeled with a goat anti-mouse IgG antibody conjugated to fluorescein isothiocyanate (1:500) for 45 min on ice. The cells were harvested, and cell surface immunofluorescence was assessed by flow cytometry as described previously (15).

**Data Analysis—** The means ± S.D. or S.E. are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data for statistical significance and to analyze data and fit curves for quisqualate dose-response. Statistical significance was determined by one-way analysis of variance with Tukey’s post hoc multiple comparison test.

**RESULTS**

**Identification of IL2 as a mGluR1 GRK2 Binding Domain—** To determine the intracellular domain of mGluR1a that binds GRK2, we constructed GST peptides corresponding to the mGluR1 intracellular loops and their adjacent transmembrane domains (IL1, IL2, and IL3) as well as the mGluR1a carboxyl-terminal tail GST-C1841–860 and GST-C1861–1199 (Fig. 1A). These GST peptides were tested for their capacity to co-purify GRK2 from HEK293 cell lysates transfected to express GRK2. GST-IL2 and GST-C1861–1199 were found to effectively co-purify GRK2, whereas GST-IL1, GST-IL3, and GST-C1841–860 did not bind to GRK2 (Fig. 1B). Because GRK2 retains the capacity to attenuate the signaling of a carboxyl-terminal tail-truncated mGluR1a mutant (mGluR1a–1–866)
Identification of the mGluR1 Binding Site for GRK2

A schematic representation of mGluR1a illustrating the regions of mGluR1a that were prepared as GST fusion proteins. B, a representative immunoblot of GRK2 co-precipitated with 1 μg of GST, GST-IL1, GST-IL2, GST-IL3, GST-C1–841–1199, and GST-C1661–1199 following incubation with 500 μg of HEK293 cell lysates expressing GRK2. HEK293 cells were transfected with 5 μg of pcDNA3 plasmid cDNA for GRK2. Data are representative of five independent experiments. C, schematic representation of the second intracellular loop alanine scanning mutant GST peptides employed to localize the GRK2 binding site in IL2. D, a representative immunoblot of GRK2 co-precipitated with 1 μg of GST, GST-IL2, GST-Mut1, GST-Mut2, GST-Mut3, and GST-Mut4 following incubation with 500 μg of HEK293 cell lysates expressing GRK2; expression of the GST peptides is also shown. HEK293 cells were transfected with 5 μg of pcDNA3 plasmid cDNA for GRK2. Data are representative of five independent experiments.

mGluR1b IL2 Mutant Signaling and GRK2 Regulation—GST co-precipitation experiments revealed that GRK2 binds to the maximum relative response for IP formation was impaired for mGluR1b-K690A, mGluR1b-K691A, mGluR1b-K692A, and mGluR1b-K691/692A when compared with the wild-type mGluR1b (Fig. 3A and Table I). In contrast, the half-maximal effective concentrations (EC50) were variably affected by individual mutation of lysine residues 690, 691, and 692 (Table I). The maximum relative response for IP formation was reduced 40–47% for mGluR1b-K690A, mGluR1b-K691A, and mGluR1b-K692A, whereas mGluR1b-K691/692A was completely uncoupled (Fig. 3A and Table I). Thus, mGluR1b-K691/692A could not be used to evaluate the effects of GRK2 expression on the attenuation of mGluR1b mutant signaling. Nevertheless, the coupling efficiency of mGluR1b-K690A, mGluR1b-K691A, and mGluR1b-K692A was sufficient to examine whether GRK2 expression might attenuate the basal and agonist-stimulated signaling of these mGluR1b mutants.

Overexpression of GRK2 reduced basal mGluR1b IP formation to 38 ± 9% of control (Fig. 3B). Consistent with the observation that GRK2 co-precipitated with mGluR1b-K690A, GRK2 overexpression also reduced basal mGluR1b-K690A activity (Fig. 3B). However, overexpression of GRK2 had no effect on basal IP formation in HEK293 cells expressing either mGluR1b-K691A or mGluR1b-K692A (Fig. 3B). GRK2 overexpression reduced the maximum velocity (Vmax) for quisqualate-stimulated mGluR1b IP formation to 31 ± 8% of control (Fig. 4A) and the Vmax for mGluR1b-K690A to 26 ± 8% of control (Fig. 4B). In contrast, overexpression of GRK2 had absolutely no effect on Vmax for either mGluR1b-K691A or mGluR1b-K692A-stimulated IP formation (Fig. 4, C and D). Previously, we demonstrated that mGluR1b is not phosphorylated by GRK2 and that β-arrestin binding is not required for mGluR1 desensitization (12, 15). Therefore, these data indicated that GRK2 binding to amino acid residues Lys691 and Lys692 in the second intracellular loop domain of mGluR1b was essential for the phosphorylation-independent regulation of mGluR1b signaling.

mGluR1a IL2 Mutant Signaling and GRK2 Regulation—GST co-precipitation experiments revealed that GRK2 binds to the second intracellular loop (15, 26–28), we further characterized GRK2 interactions with the second intracellular loop domain of mGluR1 by creating a series of four alanine scanning mutant GST fusion peptides that comprised the mGluR1 s intracellular loop flanked on each side by 5 amino acid residues from transmembrane domains 4 and 5 (Fig. 1C). Alanine scanning mutagenesis of the mGluR1 s intracellular loop revealed that GRK2 binding was lost when amino acid residues 688–692 (GSKKK) were mutated to alanine residues (Fig. 1D).

Because we had previously identified amino acid residue D527 in the GRK2 RH domain as critical for mediating GRK2 interactions with mGluR1b (24), we hypothesized that this acidic amino acid residue might associate with the three basic lysine residues localized within the second intracellular GSKKK motif. Therefore, we mutated lysine residues 690, 691, and 692 to alanine residues in mGluR1b, either individually or in combination (K691/692A), and examined whether the resulting mGluR1b mutants were impaired in their capacity to co-precipitate GRK2. When tested, we found that all of the mGluR1b lysine mutants were impaired in their capacity to co-precipitate GRK2 from HEK293 cells (Fig. 2A and B). However, only mGluR1b-K690A exhibited residual GRK2 binding (58 ± 16% of control) (Fig. 2B). The inability of any of the mGluR1b lysine mutants to co-precipitate GRK2 was not the consequence of differences in cell surface expression (Fig. 2C). Consequently, lysine residues 691 and 692 appeared to be the primary sites of interaction within the second intracellular loop of mGluR1b.

mGluR1b IL2 Mutant Signaling and GRK2 Regulation—Residues required for coupling to Gαq11 are localized to the second and third intracellular loops and proximal region of the carboxyl-terminal tail of mGluR1 (26–28). Therefore, we examined the relative ability of mGluR1b and the intracellular loop 2 mutants mGluR1b-K690A, mGluR1b-K691A, mGluR1b-K692A, and mGluR1b-K691/692A to stimulate inositol phosphate (IP) formation when expressed in HEK293 cells in response to increasing concentrations of quisqualate. We found that the maximum relative response for IP formation was impaired for mGluR1b-K690A, mGluR1b-K691A, mGluR1b-K692A, and mGluR1b-K691/692A when compared with the wild-type mGluR1b (Fig. 3A and Table I). In contrast, the half-maximal effective concentrations (EC50) were variably affected by individual mutation of lysine residues 690, 691, and 692 (Table I). The maximum relative response for IP formation was reduced 40–47% for mGluR1b-K690A, mGluR1b-K691A, and mGluR1b-K692A, whereas mGluR1b-K691/692A was completely uncoupled (Fig. 3A and Table I). Thus, mGluR1b-K691/692A could not be used to evaluate the effects of GRK2 expression on the attenuation of mGluR1b mutant signaling. Nevertheless, the coupling efficiency of mGluR1b-K690A, mGluR1b-K691A, and mGluR1b-K692A was sufficient to examine whether GRK2 expression might attenuate the basal and agonist-stimulated signaling of these mGluR1b mutants.

Overexpression of GRK2 reduced basal mGluR1b IP formation to 38 ± 9% of control (Fig. 3B). Consistent with the observation that GRK2 co-precipitated with mGluR1b-K690A, GRK2 overexpression also reduced basal mGluR1b-K690A activity (Fig. 3B). However, overexpression of GRK2 had no effect on basal IP formation in HEK293 cells expressing either mGluR1b-K691A or mGluR1b-K692A (Fig. 3B). GRK2 overexpression reduced the maximum velocity (Vmax) for quisqualate-stimulated mGluR1b IP formation to 31 ± 8% of control (Fig. 4A) and the Vmax for mGluR1b-K690A to 26 ± 8% of control (Fig. 4B). In contrast, overexpression of GRK2 had absolutely no effect on Vmax for either mGluR1b-K691A or mGluR1b-K692A-stimulated IP formation (Fig. 4, C and D). Previously, we demonstrated that mGluR1b is not phosphorylated by GRK2 and that β-arrestin binding is not required for mGluR1 desensitization (12, 15). Therefore, these data indicated that GRK2 binding to amino acid residues Lys691 and Lys692 in the second intracellular loop domain of mGluR1b was essential for the phosphorylation-independent regulation of mGluR1b signaling.

mGluR1a IL2 Mutant Signaling and GRK2 Regulation—GST co-precipitation experiments revealed that GRK2 binds to the second intracellular loop (15, 26–28), we further characterized GRK2 interactions with the second intracellular loop domain of mGluR1 by creating a series of four alanine scanning mutant GST fusion peptides that comprised the mGluR1 s intracellular loop flanked on each side by 5 amino acid residues from transmembrane domains 4 and 5 (Fig. 1C). Alanine scanning mutagenesis of the mGluR1 s intracellular loop revealed that GRK2 binding was lost when amino acid residues 688–692 (GSKKK) were mutated to alanine residues (Fig. 1D).
mGluR1a via both the second intracellular loop and the carboxyl-terminal tail domain (Fig. 1B), which we previously demonstrated was not required for the regulation of mGluR1a desensitization by GRK2 (15). Nevertheless, we examined whether the mGluR1a carboxyl-terminal tail might influence mGluR1a/GRK2 interactions and mGluR1a signaling following the mutation of lysine residues in the second intracellular loop that also contribute to GRK2 binding. Similar to what we observed for mGluR1b, mGluR1a-K690A retained residual GRK2 binding (59 ± 6% of control) (Fig. 5, A and B), whereas GRK2 binding to either mGluR1a-K691A or mGluR1a-K692A was significantly impaired (Fig. 5, A and B).

**TABLE I**

| Receptor        | EC<sub>50</sub> (nM) | Maximum response |
|-----------------|----------------------|------------------|
| mGluR1b         | 458                  | 100              |
| mGluR1b-K690A   | 66                   | 53               |
| mGluR1b-K691A   | 618                  | 56               |
| mGluR1b-K692A   | 275                  | 60               |
| mGluR1b-K691/692A | ND<sup>a</sup>   | 4                |
| mGluR1a         | 110                  | 100              |
| mGluR1a-K690A   | 85                   | 71               |
| mGluR1a-K691A   | 24                   | 55               |
| mGluR1a-K692A   | 73                   | 76               |

<sup>a</sup> ND, not determined.
We also found that the maximum relative response for IP formation for mGluR1a-K690A, mGluR1a-K691A, and mGluR1a-K692A was impaired when compared with wild-type mGluR1a (Fig. 6A and Table I). The maximum relative response for IP formation was reduced 24–45% for mGluR1a-K690A, mGluR1a-K691A, and mGluR1a-K692A (Table I). However, unlike what we observed for mGluR1b, overexpression of GRK2 reduced the basal activity of all of the mGluR1a constructs. This suggested that, despite a loss of ability of GRK2 to co-precipitate with the full-length receptor, GRK2 retained the capacity to regulate basal mGluR1a activity, perhaps through its association with $G_O$ (Fig. 6B).

GRK2 overexpression reduced the $V_{\text{max}}$ for quisqualate-stimulated IP formation in mGluR1a-, mGluR1a-K690A-, and mGluR1a-K691A-expressing HEK293 cells to 26 ± 7%, 47 ± 7%, and 44 ± 8% of control, respectively (Fig. 7, A–C). In contrast, overexpression of GRK2 had little effect on the $V_{\text{max}}$ for quisqualate-stimulated IP formation in mGluR1a-K692A-expressing cells ($V_{\text{max}} = 93 ± 7%$ of control), but it did cause a 2.5-fold rightward shift of the $EC_{50}$ for quisqualate-stimulated IP formation ($EC_{50} = 58$ versus 145 nM) (Fig. 7D). Consequently, although it appeared that stable mGluR1a-GRK2 complexes involved the second intracellular loop, association of GRK2 with the mGluR1a tail appears to contribute in part to GRK2-mediated regulation of mGluR1a signaling.

To determine whether the apparent desensitization of mGluR1a-K691A following GRK2 overexpression might be related to GRK2-mediated phosphorylation of the mGluR1a carboxyl-terminal tail, we examined whether the expression of a
catalytically inactive GRK2-K220R might mediate equivalent mGluR1a desensitization. We found that expression of either wild-type GRK2 or GRK2-K220R led to equivalent attenuation of agonist-stimulated basal activity with wild-type mGluR1a (Fig. 8A). To further investigate whether GRK2−Goq11 interactions are involved in the GRK2-mediated attenuation of mGluR1a-K691A signaling, we examined mGluR1a-K691A signaling in the presence of a GRK2 mutant that is both catalytically inactive and impaired in Goq11 binding (GRK2-K220R/D110A) (24). Similar to what we have reported previously for the wild-type mGluR1a (24), mGluR1a-K691A signaling is partially (but not completely) inhibited in the presence of GRK2-K220R/D110A (Fig. 8A). Basal IP formation in cells expressing mGluR1a-K691A is also reduced in cells expressing GRK2, GRK2-K220R, and GRK2-K220R/D110A (Fig. 8B). However, the GRK2-K220R/D110A mutant was less effective than either GRK2 or GRK2-K220R in reducing basal mGluR1a-K691A activity. Taken together, these results strengthen the conclusion that the GRK2 binding site localized to the mGluR1a carboxyl-terminal tail also contributes to GRK2 interactions and GRK2 phosphorylation-independent antagonism of mGluR1a signaling.

**Comparison of mGluR1a mutant-stimulated IP formation and GRK2 regulation of basal activity with wild-type mGluR1a.** A, quisqualate dose-responses for FLAG-mGluR1a, FLAG-mGluR1a-K690A, FLAG-mGluR1a-K699A, and FLAG-mGluR1a-K692A-stimulated IP formation in HEK293 cells. Data are normalized to the maximal response for FLAG-mGluR1a-stimulated IP formation in HEK293 cells. Data are normalized to the maximal response for FLAG-mGluR1a-stimulated IP formation in HEK293 cells. Data are normalized to the maximal response for FLAG-mGluR1a-stimulated IP formation. HEK293 cells were transfected with 5 μg of pcDNA3 plasmid cDNA for each of the mGluR1a constructs. The data represent the mean ± S.E. for four independent experiments. B, the effect of GRK2 overexpression on basal FLAG-mGluR1a, FLAG-mGluR1a-K690A, FLAG-mGluR1a-K699A, and FLAG-mGluR1a-K692A IP formation in HEK293 cells. HEK293 cells were transfected with 5 μg of pcDNA3 plasmid cDNA for each of the mGluR1a constructs. The data represent the mean ± S.E. for four independent experiments. *, p < 0.05 versus basal IP formation in the absence of GRK2.

**FIG. 6.**

**DISCUSSION**

In the present study, we provide evidence that the primary site for GRK2 binding to mGluR1 is localized to the second intracellular loop domain. In particular, GRK2 binding is mediated by two adjacent lysine residues localized to the central region of the mGluR1 s intracellular loop, Lys691 and Lys692. Mutation of these residues results in a loss of GRK2 binding to both mGluR1a and mGluR1b. Consistent with this observation, mGluR1b-K691A, mGluR1b-K692A, and mGluR1a-K692A mutants are also resistant to GRK2 expression-dependent attenuation of signaling. Thus, we conclude that GRK2 binding to the second intracellular loop is essential for the phosphorylation-independent regulation of mGluR1 signaling by GRK2. However, GRK2 is also observed to bind to a GST fusion protein corresponding to amino acid residues 861–1199 of the mGluR1a carboxyl-terminal tail, and this likely explains why GRK2 overexpression attenuates the activity of a mGluR1α-K691A mutant, despite the fact that this mutant does not effectively form a complex with GRK2 that can be co-precipitated. Taken together, our studies not only identify the second intracellular loop as the primary GRK2 binding site but also reveal subtle differences in the regulation of mGluR1 splice variant activity by GRK2.

Several residues within the second intracellular loop domain of mGluR1 involved in mGluR1/G protein-coupling surrounding the GSKKK motif that we have determined contributes to GRK2 association with mGluR1 (26–28). Our previous studies have demonstrated that the phosphorylation-independent attenuation of mGluR1 signaling requires concomitant binding of GRK2 to both the receptor and Goq11 via the α11 and α5 helices of the GRK2 RH domain, respectively (24). Given that the attenuation of mGluR1 signaling does not require β-arrestin as a co-factor for desensitization, it is probable that the role of GRK2 in attenuating mGluR1 signaling is analogous to the role played by β-arrestin for other GPCRs. Because the GRK2 structure appears to accommodate simultaneous association with the receptor, Goq11 and Gβγ (16), it seems reasonable that GRK2 interactions might be regulated by amino acid residues that are either in close proximity to or overlap with amino acid residues that are essential for G protein coupling. Consistent with this conclusion, we find that the mutation of lysine residues 690, 691, and 692 to alanine results in a partial attenuation of mGluR1α/b G protein coupling and that the mutation of both lysine 691 and lysine 692 results in a complete attenuation of mGluR1α/b signaling.

We observe that basic amino acid residues (lysine 691 and 692) localized to the second intracellular loop are essential for GRK2 binding. In addition, an acidic amino acid residue (aspartic acid 527) localized to the α11 helices of the GRK2 RH domain is also essential for GRK2 binding to mGluR1. This suggests that mGluR1/GRK2 interactions may be ionic in nature. Similarly, Pao and Benovic (29) recently reported that the several basic residues localized within IL2 and IL3 of α2-adrenergic receptor interact with acidic residues in the nucleotide gate of GRK2. The presence of ionic interactions between GRK2 and its substrate is also supported by the observation that GRK2-mediated receptor phosphorylation is inhibited by salt (30). An additional region that has been implicated in GRK2-GPCR interaction is a proline-rich motif just amino-terminal to the nucleotide gate (31). Point mutations in this region not only disrupted the interaction of GRK2 with rhodopsin but also abolished the ability of GRK2 to phosphorylate rhodopsin. This suggests that ionic interactions may represent a common mechanism by which GRKs associate with GPCRs to either mediate their phosphorylation or directly attenuate their signaling. The fact that we and others have identified...
distinct GRK2 domains that are important for receptor-GRK2 interactions highlights the point that receptor-GRK2 interactions may not be regulated by a conserved GRK2 motif and/or domain. Rather, the GRK2 domain required for GPCR binding may differ for each GPCR examined. This possibility will require future studies of the binding of GRK2 to additional GPCRs.

Previous studies examining differences in the activity of various mGluR1 splice variants have revealed that the mGluR1a carboxyl-terminal tail contributes to higher agonist potency, differences in phorbol ester-stimulated endocytosis, and increased spontaneous mGluR1 activity and regulates the localization of the receptor to dendrites (32–34). We have made an interesting and unexpected observation that there may also be subtle differences in the regulation of mGluR1a and mGluR1b alternative splice variants by GRK2. We find that GRK2 binds to both GST peptides corresponding to the second intracellular loop and carboxyl-terminal tail of mGluR1a. The binding of GRK2 to the mGluR1a carboxyl-terminal tail does not seem to be sufficient to facilitate the co-immunoprecipitation of a mGluR1a/GRK2 complex following the mutation of Lys691 or Lys692. However, carboxyl-terminal tail interactions allow phosphorylation-independent regulation of mGluR1a-K691A and mGluR1a-K692A mutant basal activity by GRK2. Moreover, mGluR1a-K691A differs from mGluR1b-K691A in that the activity of the mGluR1a-K691A mutant retains its regulation by GRK2 following agonist activation. However, GRK2-dependent regulation of mGluR1a remains phosphorylation-independent because the expression of a catalytically inactive GRK2 mutant also antagonizes mGluR1a-K691A signaling. Thus, it appears that the mGluR1a carboxyl-terminal tail may cooperate with the second intracellular loop to regulate GRK2 binding in a cellular environment.

In conclusion, we find that the phosphorylation-independent attenuation of mGluR1a signaling in response to GRK2 overexpression is not simply the consequence of the nonspecific sequestration of Goq by the GRK2 RH domain. Several lines of evidence now support this conclusion. First, mGluR1a signaling is effectively attenuated by a kinase-deficient GRK2-K220R

**Fig. 7.** Ability of GRK2 to attenuate mGluR1a and mGluR1a mutant-stimulated IP formation. Quisqualate dose-responses for (A) FLAG-mGluR1a, (B) FLAG-mGluR1a-K690A, (C) FLAG-mGluR1a-K691A, and (D) FLAG-mGluR1a-K692A-stimulated IP formation in HEK293 cells in either the absence or presence of GRK2. Data are normalized to the maximal response for wild-type and mutant FLAG-mGluR1a-stimulated IP formation in the absence of GRK2. HEK293 cells were transfected with 5 μg of pcDNA3 plasmid cDNA for GRK2 and 10 μg of pcDNA3 plasmid cDNA for each of the mGluR1a constructs. The data points represent the means ± S.E. for four independent experiments.
mutant (15). Second, we have identified a GRK2-D527A mutant that binds to Goαq11 normally but is unable to either bind mGLUR1α or attenuate mGLUR1a signaling (24). Third, we have now identified mGLUR1a and mGLUR1b point mutants that do not bind GRK2 and are not sensitive to GRK2 overexpression. Finally, the GRK phosphorylation-independent regulation of GPCR activity may not be unique to mGLUR1 because other Goαq-, Goαs-, and Goαq11-coupled GPCRs have been reported to associate with GRKs and desensitize in the absence of GRK kinase activity (17, 18, 35, 36). Taken together, these observations indicate that formation of receptor-GRK complexes may lead to the phosphorylation- and arrestin-independent inhibition of GPCR signaling. We suggest that this may be a physiologically relevant regulatory mechanism for many GPCRs.

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Phosphorylation-independent Regulation of Metabotropic Glutamate Receptor 1 Signaling Requires G Protein-coupled Receptor Kinase 2 Binding to the Second Intracellular Loop

Gurpreet K. Dhami, Andy V. Babwah, Rachel Sterne-Marr and Stephen S. G. Ferguson

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