

Role of G Protein-coupled Receptor Kinase 4 and β -Arrestin 1 in Agonist-stimulated Metabotropic Glutamate Receptor 1 Internalization and Activation of Mitogen-activated Protein Kinases*

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The metabotropic glutamate 1 (mGlu₁) receptor in cerebellar Purkinje cells plays a key role in motor learning and motor coordination. Here we show that the G protein-coupled receptor kinases (GRK) 2 and 4, which are expressed in these cells, regulate the mGlu₁ receptor by at least in part different mechanisms. Using kinase-dead mutants in HEK293 cells, we found that GRK4, but not GRK2, needs the intact kinase activity to desensitize the mGlu₁ receptor, whereas GRK2, but not GRK4, can interact with and regulate directly the activated G α_q . In cells transfected with GRK4 and exposed to agonist, β -arrestin was first recruited to plasma membranes, where it was co-localized with the mGlu₁ receptor, and then internalized in vesicles. The receptor was also internalized but in different vesicles. The expression of β -arrestin V53D dominant negative mutant, which did not affect the mGlu₁ receptor internalization, reduced by 70–80% the stimulation of mitogen-activated protein (MAP) kinase activation by the mGlu₁ receptor. The agonist-stimulated differential sorting of the mGlu₁ receptor and β -arrestin as well as the activation of MAP kinases by mGlu₁ agonist was confirmed in cultured cerebellar Purkinje cells. A major involvement of GRK4 and of β -arrestin in agonist-dependent receptor internalization and MAP kinase activation, respectively, was documented in cerebellar Purkinje cells using an antisense treatment to knock down GRK4 and expressing β -arrestin V53D dominant negative mutant by an adenovirus vector. We conclude that GRK2 and GRK4 regulate the mGlu₁ receptor by different mechanisms and that β -arrestin is directly involved in glutamate-stimulated MAP kinase activation by acting as a signaling molecule.

Metabotropic glutamate (mGlu)¹ receptors, which are activated by the excitatory amino acid glutamate, are part of an

original family of G protein-coupled receptors (GPCR) called the family 3 GPCRs (1–3). These include all the mGlu receptor subtypes, Ca²⁺-sensing and GABA_B receptors, and some putative olfactory, pheromone, and taste receptors. Eight subtypes of mGlu receptors have been identified, which are implicated in different aspects of physiology and pathology of the central nervous system. Group I mGlu receptors (mGlu₁ and mGlu₅), which stimulate polyphosphoinositide hydrolysis by coupling to G_q, are localized in the peripheral parts of postsynaptic dendrites and contribute to the regulation of synaptic plasticity. For example, mGlu₁ receptor present in cerebellar Purkinje cells plays a key role in motor learning and motor coordination. Similar to many other GPCRs, the signal transduction of the mGlu₁ receptor is strictly regulated by multiple mechanisms acting at different levels of signal propagation (1). After prolonged or repeated stimulation, receptors are profoundly desensitized. Protein kinase C is clearly involved in this process, although a protein kinase C-independent component of mGlu₁ receptor desensitization was also observed (4). The activated α subunit of the G_q (G α_q) can in turn be inhibited by RGS (for regulators of G protein signaling) proteins (5). These RGS proteins work by interacting with G α and by increasing the intrinsic GTPase activity of G α , acting as GTPase-activating proteins (6, 7). Recent studies from our and other laboratories have documented that G protein-coupled receptor kinases (GRKs) and arrestins are involved in the mechanism of agonist-stimulated mGlu₁ receptor phosphorylation, desensitization, and internalization. Using transfected HEK293 cells, it was shown that the mGlu₁ receptor is phosphorylated and desensitized by different GRK subtypes (8, 9) in an agonist-dependent manner. In these cells agonist treatment induced the internalization of the mGlu₁ receptor, and this mechanism was β -arrestin- and dynamin-dependent (10, 11). The mGlu₁ receptor is also internalized tonically (*i.e.* in an agonist-independent manner) by a mechanism that is β -arrestin- and dynamin-independent and likely involves a clathrin-mediated endocytic pathway (11). Based on these results and on studies with different receptor types, it was suggested that multiple endo-

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¹ The abbreviations used are: mGlu, metabotropic glutamate; GPCR, G protein-coupled receptor; G protein, GTP-binding protein; GRK, G protein-coupled receptor kinase; GRK-Nter, the N-terminal domain of

GRK; IP, inositol phosphate; GFP, green fluorescent protein; G α , the α subunit of G protein; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; RGS, regulators of G protein signaling; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAF, platelet-activating factor; PAFr, PAF receptor.

cytic pathways may contribute to the internalization of the same GPCR (11).

We have recently shown that GRK4, one GRK subtype originally identified in testis and sperm cells, may play a major role in the regulation of the $mGlu_1$ receptor. In HEK293 cells transfected with GRK4 and in cultured cerebellar Purkinje cells, which naturally express high levels of GRK4, we demonstrated that this kinase is important for the desensitization and for rapid internalization of the $mGlu_1$ receptor (8).

The present study shows that the stimulation of the $mGlu_1$ receptor induces the rapid redistribution of β -arrestin, which is first recruited to plasma membranes and then internalized in intracellular vesicles. Our results support the possibility that β -arrestin acts as a signaling protein that mediates the $mGlu_1$ receptor-mediated activation of MAP kinases (MAPK).

EXPERIMENTAL PROCEDURES

Materials—Polyclonal anti- $mGlu_1$ antibody was from Upstate Biotechnology; polyclonal anti-RGS4, anti-ERK1, and anti- $G\alpha_q$ were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-phospho-ERK1/2 was from Cell Signaling Technology; monoclonal anti-GRK2/3 was from Upstate Biotechnology; polyclonal anti-GRK4 was from Santa Cruz; monoclonal anti-FLAG M5 was from Eastman Kodak Co.; Alexa-594 protein labeling kit, Alexa-488 anti-mouse, and anti-rabbit IgGs were from Molecular Probes (Eugene, OR); monoclonal anti- β -arrestin antibody (F4C1) was kindly provided by Dr. L. A. Donoso; Cy3-conjugated anti-rabbit IgG was from Sigma.

Clones and Mutants—To generate a fusion protein between GST and the N-terminal domain of GRK2 (GST-GRK2-Nter) and of GRK4 (GST-GRK4-Nter), we used a PCR-based method as previously described (12). The GRK4-(K216M,K217M) was prepared as previously described (8). The following plasmids were generous gifts: GRK2-(K220R) from C. Scorer (Glaxo Wellcome, Stevenage, UK), $G\alpha_q$ from A. Gilman (University of Texas, Dallas, TX), $G\alpha_q$ (Q209L) from N. Dhanasekaran (Temple University, Philadelphia, PA), β -arrestin V53D from Federico Mayor (Universidad Autonoma de Madrid, Madrid, Spain), human $mGlu_1$ receptor in pCDNA 3 from M. Corsi (Glaxo Wellcome, Verona, Italy), the human EAAC1 from J. P. Pin (CNRS, Montpellier, France) and M. A. Hediger (Harvard Medical School, Boston, MA), and PAF receptor (PAFr) in pCDM8-FLAG plasmid from C. Gerard (Harvard Medical School, Boston, MA).

Cell Culture, Transfection, and IP Measurement—Cerebellar neurons were prepared from Wistar rats as previously described (8, 13), with minor modifications to obtain a Purkinje cell-rich culture. Seven-day-old pups were sacrificed by cervical dislocation and the cerebella excised and minced with a scalpel. The cerebellar cells were disgregated with 0.025% trypsin and 0.01% DNase I in Krebs Ringer plus 0.03% $MgSO_4$, 0.3% BSA for 15 min at 37 °C. The cells were washed with the same buffer containing 40 μ g/ml trypsin inhibitor and 0.01% DNase I and dissociated by repeated passage through a fine-tipped pipette. The cell suspension was centrifuged at 400 $\times g$ for 2 min, and cells resuspended carefully in 2 ml of the same buffer. After 30–45 min, the Purkinje cells are enriched from granules by gravity. The upper part of the suspension (granules) was removed very carefully, and the sediment (Purkinje cells) was rinsed with culture medium. Recovered cells were plated at a density of 20–25 $\times 10^4$ cells/cm² onto poly-L-lysine-coated chamber slides in serum-free defined medium: Eagle's medium supplemented with 1 mg/ml BSA, 10 μ g/ml insulin, 0.1 nM L-thyroxine, 0.1 mg/ml transferrin, 1 μ g/ml aprotinin, 30 nM selenium, 100 μ g/ml streptomycin, and 100 units/ml penicillin. The cultures were maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C. The cultures, which consisted of ~2–3% Purkinje cells (assessed by calbindin immunostaining), were used after 15–20 days *in vitro*.

HEK293 cells were transfected as described (8). One day after transfection, the cells were washed in PBS and incubated for 18 h with Dulbecco's modified Eagle's medium/Glutamax-1 (Invitrogen), then washed and incubated overnight with minimal essential medium/Glutamax-1 containing 3 μ Ci/well *myo*-[³H]inositol (Amersham Biosciences). On the third day, IP production was measured as described (8). Briefly, cells were washed twice and incubated for 1–2 h at 37 °C in 1 ml of HEPES-buffered saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 0.1% glucose, 20 mM HEPES, pH 7.4), washed again with HEPES-buffered saline, and pre-incubated for 15 min in the same buffer containing 10 mM LiCl, 1.8 units/ml glutamic pyruvic transaminase, 2 mM sodium pyruvate. The stimulus was carried out for 30 min

with 100 μ M quisqualate, unless otherwise indicated. The reaction was stopped by replacing the incubation medium with 1 ml of ice-cold perchloric acid (5%). Inositol phosphates were separated by an ion exchange chromatography column of Dowex AG1-X8 (formate form) (200–400-mesh, 350- μ l bed volume). Usually 1 $\times 10^6$ cells were co-transfected with 1 μ g of $mGlu_1$ plasmid along with 5 μ g of GRK cDNA, or empty vector. For $mGlu_{1a}$, 5 μ g of the plasmid encoding the glutamate transporter EAAC1 (8) was included.

Cerebellar Purkinje Cell GRK4 Antisense Oligonucleotide Treatment and Adenovirus Infection—For antisense oligonucleotide treatment, the experiments were performed as previously described (8). Cerebellar neurons were prepared as described (8) and maintained in culture for 2–3 weeks, at which time two-end phosphorothioate oligonucleotides at 1 μ M final concentration were added for 4–5 days. The sequence of the GRK4 antisense oligonucleotide and of the scrambled oligonucleotide (used as control) are as described in Ref. 8.

Recombinant adenovirus was prepared according to Ref. 14 with minor modifications. The β -arrestin V53D cDNA was subcloned into the multiple cloning site of the shuttle plasmid (pAd-CMV-TRK) by standard cloning procedures. The purified shuttle plasmid was digested with the restriction enzyme *PmeI* to obtain the "rescue fragment." The fragment was then purified on agarose gel, and 2 μ g of purified rescue fragment was used for homologous recombination. The adenoviral plasmid pAdEasy-1 (14) was then mixed with the rescue fragment, and the DNA mixture was transformed into the BJ5183 bacterial strain and incubated overnight. Colonies were screened by digesting the DNA with *BglII* and performing a Southern blot to confirm the presence of the cDNA insert. The DNA with the proper orientation was transformed into the DH5a bacterial strain. The recombinant construct, purified using Qiagen Maxi preparation kit, was digested overnight with *PacI* and transfected into HEK293 cells using LipofectAMINE (Invitrogen). Adenoviral plaques were purified twice by infecting HEK293 cells in agar. Virus was purified by CsCl gradient centrifugation, dialyzed, and titrated by plaque assay. The recombinant adenovirus obtained expresses both green fluorescent protein (GFP) and β -arrestin V53D under independent cytomegalovirus promoters. A virus expressing only GFP was used as a control. For infection cerebellar Purkinje cells were incubated with the recombinant adenovirus at a multiplicity of infection of 50 plaque-forming units/cell for 3 h at 37 °C in medium without serum. The virus-containing medium was then removed, and cells were incubated in standard medium plus serum. At 48 h after infection, >95% of the cultured cerebellar Purkinje cells were infected, as assessed by the expression of GFP. The expression of β -arrestin V53D was confirmed by immunocytochemistry in cerebellar Purkinje cells or by immunoblot in a U87MG glioblastoma cell line.

Binding of G Proteins to GRK N-terminal and Immunoprecipitation—These experiments were performed as described previously (12). Cytosolic proteins (150 μ g) from HEK293 cells transfected with the $G\alpha_q$ subunit were mixed with 40 μ l of slurry containing GST-GRK-Nter fusion proteins bound to glutathione agarose beads in a final volume of 400 μ l of binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol, 10 μ M GDP, 3 mM $MgCl_2$), in the presence or absence of 47 mM $MgCl_2$, 30 μ M $AlCl_3$, and 20 mM NaF. After 1 h at 4 °C the beads were washed three times with 1 ml of ice-cold binding buffer and the resins containing the eventual bound proteins were analyzed by immunoblotting, using anti- $G\alpha_q$ antibody (Santa Cruz Biotechnology). One fraction of starting material (30–40 μ g, ~25% of the total cytosolic proteins used for binding) was also included in the gel (indicated as S in Fig. 1).

Immunoprecipitation was done as follows. After treatments, cells were rapidly washed in ice-cold PBS and solubilized in Triton X-100 lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM β -glycerophosphate) for 15 min. The lysates were clarified by centrifugation (10,000 $\times g$ for 10 min). Protein concentration of supernatants was determined, and 600 μ g of total proteins were incubated with 5 μ g of anti- $G\alpha_q$ antibodies for 2 h at 4 °C followed by addition of 50 μ l of di-protein A-Sepharose pre-equilibrated in HNTG buffer (20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, 5% glycerol), and an additional 1-h incubation at 4 °C. Immunoprecipitates were washed four times in HNTG buffer, and the pellets were boiled in Laemmli buffer for 5 min before electrophoresis. Immunoprecipitates and starting materials were subjected to 10% SDS-polyacrylamide gel under reducing condition. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane and immunoblotted using anti-GRK2 (Upstate Biotechnology) and anti-GRK4 (Santa Cruz) antibodies.

Western Blotting—After treatments, cells were rapidly washed in

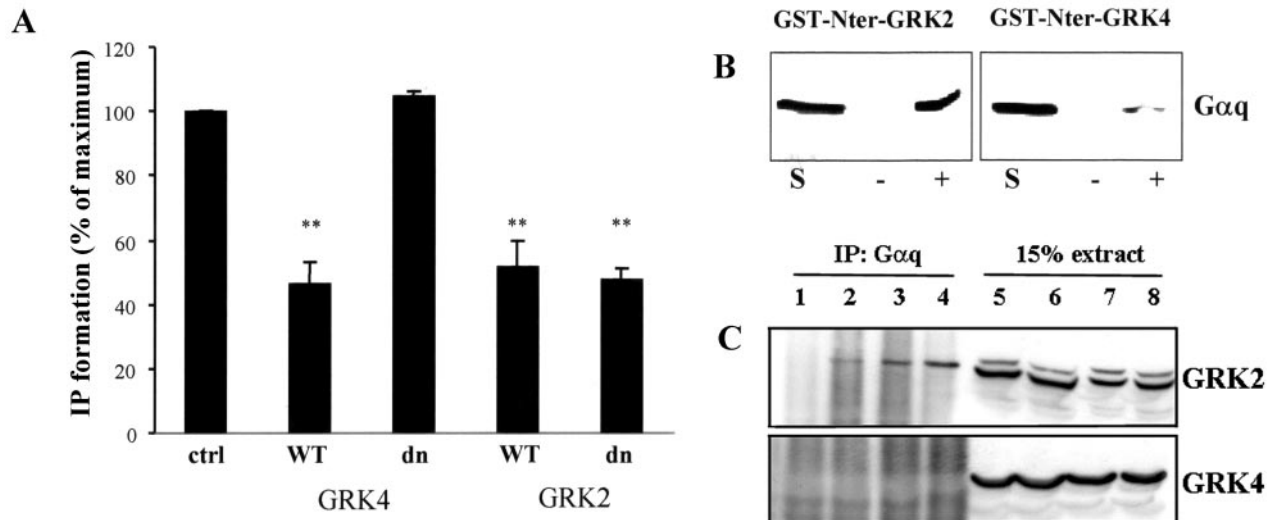


FIG. 1. Regulation of $mGlu_{1a}$ receptor by GRK2 and GRK4. *A*, HEK293 cells were transfected with the $mGlu_{1a}$ receptor and co-transfected with mock (*ctrl*), GRK2 or GRK4 wild type (*WT*), or kinase-dead mutant (*dn*) and (100 μ M) quisqualate-stimulated IP formation was measured (means \pm S.E. of 5 separate experiments; **, $p < 0.01$, Tukey's test). *B*, binding of GRK2- and GRK4-Nter to activated $G\alpha_q$. Recombinant purified GST-GRK-Nter proteins conjugated to glutathione-agarose beads were incubated with cytosolic proteins (150 μ g) from $G\alpha_q$ -transfected HEK293 cells in the absence (-) or presence (+) of 30 μ M $AlCl_3$ and 20 mM NaF to activate the $G\alpha_q$. Incubation (1 h at 4 $^\circ$ C) was stopped by centrifugation (300,000 $\times g$). After three extensive washings, $G\alpha_q$ bound to the column was detected by immunoblot using anti- $G\alpha_q$ antibody. Starting material (S) (30–40 μ g of cytosolic preparation, about one fourth of total cytosol used for binding) is also included in the immunoblot. The experiment shown was repeated three times with similar results. *C*, co-immunoprecipitation of $G\alpha_q$ and GRKs from HEK293 cells. HEK293 cells co-expressing $G\alpha_q$ (*lanes 1, 2, 5, and 6*) or $G\alpha_q(Q209L)$ (*lanes 3, 4, 7, and 8*) and either GRK2 or GRK4 (as indicated) were untreated (*lanes 1, 3, 5, and 7*) or exposed to 100 μ M quisqualate (*lanes 2, 4, 6, and 8*) for 5 min. Cells were then harvested and lysed, and IP (*lanes 1–4*) was performed using an anti- $G\alpha_q$ antibody as described under "Experimental Procedures." Initial cell extract (15%) was included for comparison (*lanes 5–8*). The experiment shown was repeated three times with similar results.

ice-cold PBS and solubilized in Triton X-100 lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM β -glycerophosphate) for 15 min. The lysates were clarified by centrifugation (10,000 $\times g$ for 10 min), and 80–100 μ g of proteins were separated by SDS-PAGE electrophoresis, blotted onto nitrocellulose, and probed using a commercial anti-phosphospecific antibody against phosphorylated ERK1/2. Monoclonal anti-phospho-ERK1/2 antibody was used at 1:2000 dilution. The membranes were stripped according to instructions from the manufacturer and reprobed with polyclonal anti-ERK1/2 antibody at 1:5000 dilution (Santa Cruz Biotechnology). Other Western blot analyses were performed as described (12). The immunoreactive bands were visualized either by enhanced chemiluminescence using horseradish peroxidase-linked secondary antibody.

Immunofluorescence Confocal Analysis—HEK293 cells transfected as above and 20-day-old Purkinje cell primary cultures were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The autofluorescence was quenched by incubation for 30 min in 50 mM NH_4Cl , 50 mM glycine in PBS, and nonspecific interactions were blocked by treatment with blocking solution (0.05% saponin, 0.5% BSA in PBS) for 30 min at room temperature. Cells were incubated (8) with anti- $mGlu_{1a}$ (0.3 μ g/ml, overnight at 4 $^\circ$ C), anti- β -arrestin (F4C1) (12.5 μ g/ml, 1 h, room temperature), anti-phospho-ERK1/2 (4 μ g/ml, 1 h, room temperature), anti-ERK1 (2 μ g/ml, 1 h, room temperature), and anti-RGS4 (2 μ g/ml, overnight at 4 $^\circ$ C) antibodies in blocking solution. The chamber slides were then incubated with blocking solution containing Alexa-488 anti-rabbit (1:400, Molecular Probes), Cy3-conjugated anti-rabbit (1:200, Sigma), or Alexa-488 anti-mouse IgG (1:400, Molecular Probes) for 1 h at room temperature. For PAFr localization, an anti-FLAG M5 monoclonal antibody directed at MDYKDDDDKEF amino acid sequence at the N-terminal Met-FLAG fusion protein of PAFr was used; this antibody was conjugated to the fluorochrome Alexa-594 and diluted at 1 μ g/ml in blocking buffer (1 h, room temperature). Each incubation step was carried out in the dark and followed by careful washes with PBS (six times/3 min each). After immunostaining the coverslips were mounted on slides with Mowiol 4–88 and analyzed by a Zeiss LSM 510 laser scanning microscope equipped with an Axiovert 100 M-BP and by the Confocal Imaging System Ultraview (PerkinElmer Life Sciences). The internalization of the $mGlu_1$ receptor and of PAF receptors was quantified as previously described (8). Co-localization was quantified as previously reported

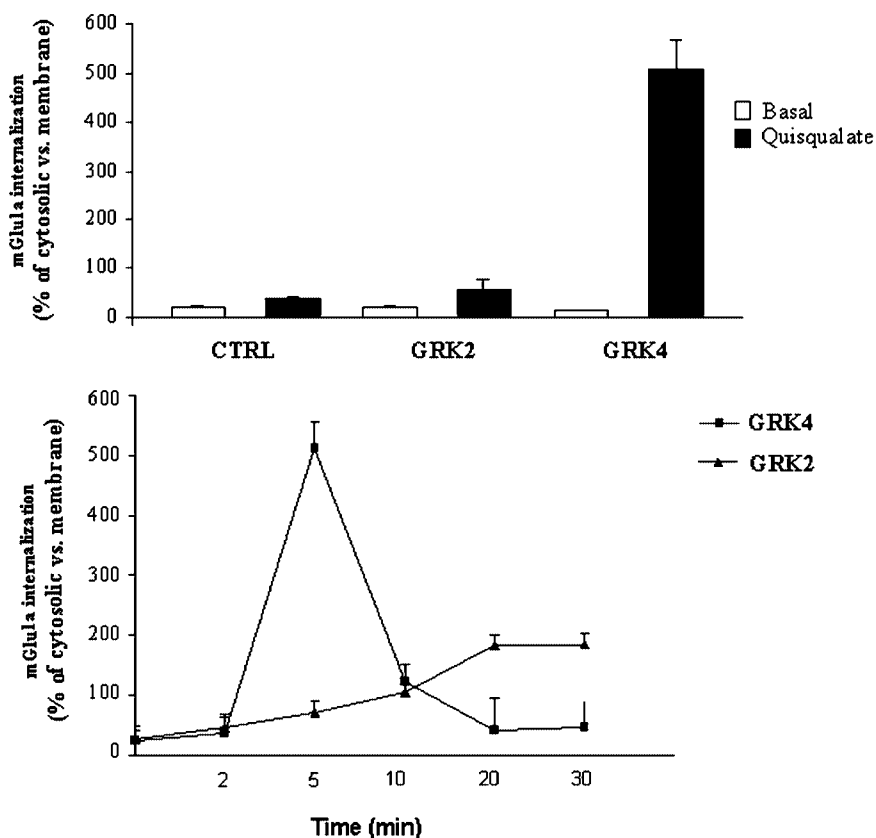
(8) or using the "co-localization and correlation" option of the Ultra-view software.

RESULTS

GRK4-dependent $mGlu_1$ Receptor Desensitization and Internalization—GRK2 and GRK4 are the two GRK subtypes expressed in cerebellar Purkinje cells (8). As these cells represent one relevant site of the $mGlu_1$ receptor expression and function, we investigated the role of GRK2 and GRK4 in the regulation of this receptor. Previous studies have shown that the agonist-dependent phosphorylation of the $mGlu_{1a}$ receptor expressed in HEK293 cells is significantly enhanced when GRK2 (9) or GRK4 (8) are co-transfected. To assess the role of GRK-dependent receptor phosphorylation in the homologous desensitization of the $mGlu_1$ receptor-stimulated IP production, we used GRK2 and GRK4 kinase-dead mutants in which the kinase activity was disrupted by site-directed mutagenesis of key amino acids located in the catalytic domain. Both mutants, which are named GRK2-(K220R) and GRK4-(K216M,K217M) for GRK2 and GRK4, respectively, lost their ability to phosphorylate receptor substrates (8, 12). We determined the agonist-stimulated IP production in HEK293 cells transfected with the $mGlu_1$ receptor and the effect of co-expression of different GRK mutants (Fig. 1A). According to our previous results (8), the co-transfection of either GRK2 or GRK4 resulted in a 35–40% reduction of the agonist-stimulated response. By contrast, the effect of the two kinase-dead mutants was substantially different; the GRK4-(K216M,K217M) mutant was ineffective, whereas the GRK2-(K220R) desensitized the $mGlu_1$ receptor-stimulated signaling to the same extent as the GRK2 wild type. These results indicated that the phosphorylation of the $mGlu_1$ receptor was necessary for GRK4-mediated receptor desensitization, whereas GRK2 utilized, at least in part, a phosphorylation-independent mechanism for receptor regulation.

The likely mechanism by which GRK2 could regulate $mGlu_1$ receptor signaling in a phosphorylation-independent manner is

FIG. 2. GRK4-dependent internalization of $mGlu_{1a}$ receptor. *Upper panel*, HEK293 cells transfected with the $mGlu_{1a}$ receptor and co-transfected with mock (*ctrl*), GRK2 or GRK4 were untreated (basal) or treated with quisqualate (100 μ M) for 5 min, and the receptor internalization was assessed by confocal microscopy analysis. *Lower panel*, time course of the $mGlu_{1a}$ receptor internalization in cells transfected with GRK4 (squares) or GRK2 (triangles) and treated with quisqualate for the indicated times. Data (means \pm S.E., $n = 15$) are the ratio of cytosolic versus membrane receptor immunofluorescence. Membrane immunofluorescence is taken as 100%.



provided by the RGS-like domain present in the N terminus of GRK2, because we and others have previously shown that this is a functionally active domain able to regulate the GPCR-stimulated G_q signaling by direct binding and inhibition of the activated G_{α_q} (12, 15). According to this hypothesis, our results suggest that, unlike GRK2, the GRK4 N terminus, which also contains an RGS homology domain, should be unable to interact with G_{α_q} and to regulate its signaling cascade at the G protein level. To test this possibility we prepared a GST-GRK4-Nter fusion protein and we measured the binding of this domain to G_{α_q} , using the GST-GRK2-Nter as a positive control. For binding experiments, the cytosolic proteins from HEK293 cells transfected with G_{α_q} were incubated with agarose-conjugated GST-GRK-Nter fusion proteins. Unbound proteins were removed by extensive washing, and G_{α_q} bound to GST-GRK-Nter proteins was revealed by immunoblot. According to previous findings (12), when the incubation was done in the presence of AlF_4^- (*i.e.* G_{α_q} was in the active state), a substantial fraction of G_{α_q} was bound to GST-GRK2-Nter, whereas in the absence of the AlF_4^- (with G_{α_q} in the inactive state), the G_{α_q} bound to GST-GRK2-Nter was undetectable (Fig. 1B). When similar experiments were done using GST-GRK4-Nter, the amount of G_{α_q} interacting with this domain was significantly lower even when G_{α_q} was in the active state (Fig. 1B). The amount of the G_{α_q} bound to GRK4-Nter (in the presence of AlF_4^-) was estimated to be \sim 3–5% of the starting material, whereas that bound to GRK2-Nter was estimated at \sim 25% of the starting material. To assess whether GRK2, but not GRK4, interacts with the activated G_{α_q} in cells, we investigated the interaction of G_{α_q} and GRKs by co-immunoprecipitation in transfected HEK293 cells. We used both the wild type G_{α_q} or the constitutively active mutant $G_{\alpha_q}(Q209L)$, which can bind *in vitro* to the GRK2-Nter even in the absence of AlF_4^- (12). G_{α_q} was immunoprecipitated from cells transfected with GRK2 or GRK4 plus G_{α_q} or $G_{\alpha_q}(Q209L)$, and the presence of GRK sub-

types in the immunoprecipitates was assessed by immunoblot (Fig. 1C). GRK2 was co-immunoprecipitated in an agonist-dependent manner from cells expressing G_{α_q} , showing that GRK2 and G_{α_q} interact in intact cells and that this binding depends on the active state of G_{α_q} . In cells expressing $G_{\alpha_q}(Q209L)$, GRK2 was co-immunoprecipitated even in the absence of agonist, although we consistently found that the amount of GRK2 co-immunoprecipitated with $G_{\alpha_q}(Q209L)$ was enhanced by quisqualate treatment. This indicates that the activation of the $mGlu_1$ receptor by agonist may favor the interaction between GRK2 and G_{α_q} in intact cells, perhaps by modulating the active state of GRK2, which, in turn, could govern the interaction with G_{α_q} . This hypothesis is consistent with our previous finding showing that the presence of an agonist-stimulated receptor increased the ability of GRK2-Nter to inhibit G_{α_q} -stimulated IP production (Fig. 4 of Ref. 12). By contrast, GRK4 was never co-immunoprecipitated with G_{α_q} , indicating that this kinase does not interact with G_{α_q} . The levels of expression of GRKs (Fig. 1C), G_{α_q} , and $G_{\alpha_q}(Q209L)$ (data not shown) were comparable in different samples.

In our experimental conditions β -arrestin was not co-immunoprecipitated with GRK2 or with GRK4 in either the presence or absence of agonist stimulation (not shown).

GRK4 is also primarily involved in $mGlu_1$ receptor internalization (Fig. 2). In HEK293 cells transiently expressing the $mGlu_1$ receptor, exposure to quisqualate for 5 min did not induce a significant level of receptor internalization and the co-expression of GRK2 resulted in a 2–3-fold increase of receptor internalization. In cells transfected with GRK2, the maximal internalization was observed after 20–30 min of agonist treatment (Fig. 2). The expression of GRK4 drastically enhanced the internalization of the $mGlu_1$ receptor induced by the quisqualate, and this effect was rapid with a maximal peak at 5 min of agonist stimulation and was reversible within 30 min (Fig. 2).

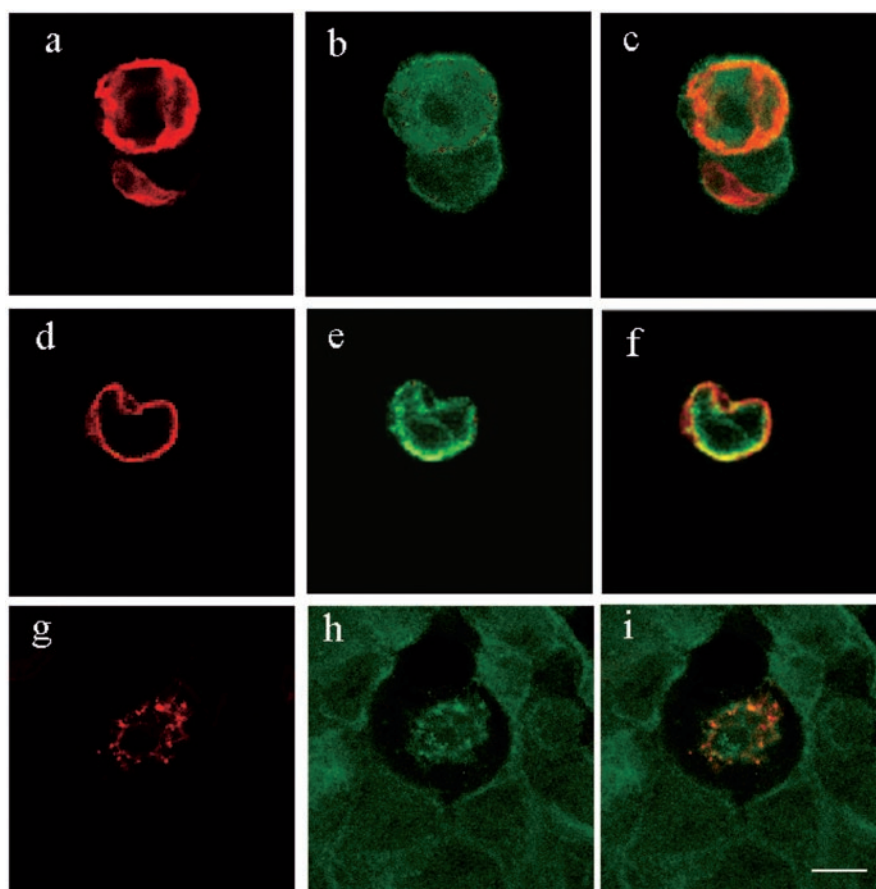


FIG. 3. Agonist-induced redistribution of the $mGlu_{1a}$ receptor and β -arrestin in HEK293 cells. HEK293 cells transfected with $mGlu_{1a}$ receptor and treated with vehicle (*a–c*) or with 100 μ M quisqualate for 2 min (*d–f*) or for 5 min (*g–i*) were double-stained with anti- $mGlu_1$ and anti- β -arrestin antibodies for immunofluorescence confocal microscopy analysis. The distributions of the $mGlu_{1a}$ receptor (in red; *a*, *d*, and *g*) and of β -arrestin (in green; *b*, *e*, and *h*) are shown in a single-channel image. The overlay is shown in *c*, *f*, and *i*, and co-localization is in yellow (*f*). Scale bar, 12 μ m.

Previous work from our laboratory documented a substantial co-localization of the $mGlu_1$ receptor and GRK4 both under basal conditions and after internalization. We investigated whether GRK2 could also be co-localized with the receptor. Agonist-induced receptor internalization was assessed in HEK293 cells co-transfected with the $mGlu_1$ receptor and GRK2, and the reciprocal co-localization of the receptor and the kinase was determined in cells untreated or exposed to agonist for 5, 20, and 40 min. By confocal microscopy analysis, we found that $12 \pm 2\%$, $24 \pm 6\%$, $6 \pm 2\%$, and $9 \pm 4\%$ of the $mGlu_1$ receptor and $8 \pm 1\%$, $18 \pm 2\%$, 5 ± 1 , and $18 \pm 4\%$ of GRK2 staining was reciprocally co-localized respectively after 0, 5, 20, and 40 min of exposure to agonist ($n = 20$). The limited amount of co-localization found in this time course indicates that the $mGlu_1$ receptor-GRK2 interaction is less persistent than that of GRK4 with the receptor.

Because GRK4 appeared to be a key regulator of the rapid agonist-promoted $mGlu_1$ receptor internalization both in HEK293 cells (Ref. 8 and present results) and in cerebellar Purkinje cells (8), this kinase was co-transfected with the $mGlu_1$ receptor in subsequent experiments, unless otherwise indicated.

Differential Sorting of $mGlu_1$ Receptor and β -Arrestin during Agonist-promoted Endocytosis—We analyzed the intracellular localization of the transfected $mGlu_1$ receptor and endogenous β -arrestin in HEK293 cells at various times after agonist treatment (Fig. 3). In unstimulated cells β -arrestin distribution is largely diffused in the cytosol, whereas after 2 min agonist stimulation we observed the redistribution of β -arrestin to the plasma membrane, where β -arrestin is co-localized, at least in part, with the $mGlu_1$ receptor (Fig. 3*f*). After 5 min of exposure to quisqualate, both $mGlu_1$ receptor and β -arrestin are mostly found in intracellular compartments, but they are localized in

distinct intracellular vesicles (Fig. 3*i*). After 5 min of quisqualate treatment, $26 \pm 5\%$ of the $mGlu_1$ receptor and $22 \pm 4\%$ of β -arrestin staining were reciprocally co-localized ($n = 20$). This finding indicates that $mGlu_1$ receptor and β -arrestin are not physically bound during agonist-promoted internalization, suggesting that the $mGlu_1$ receptor is internalized by a β -arrestin-independent mechanism. To test this possibility, we used the β -arrestin 1 dominant-negative mutant V53D (β arrV53D), which can block the β -arrestin-dependent internalization of several GPCRs (16). The agonist-stimulated internalization of the $mGlu_{1a}$ was not affected by the co-expression of β arrV53D, confirming that this receptor is internalized by a β -arrestin-independent mechanism (Fig. 4). After agonist treatment the amount of $mGlu_1$ receptor intracellular immunofluorescence (relative to that present on the plasma membranes) was $512 \pm 9\%$ in control cells and it was $430 \pm 7\%$ in cells expressing the β arrV53D ($n = 50$). The PAFr, which is internalized by a β -arrestin-dependent mechanism (17), was used as positive control in parallel experiments. As expected we found that, following agonist treatment, PAFr is internalized and is co-localized with β -arrestin (not shown) and that the agonist-induced PAFr internalization was prevented by the co-expression of β arrV53D (Fig. 4). After agonist treatment the amount of PAFr intracellular immunofluorescence (relative to that present on the plasma membranes) was $728 \pm 4\%$ in control cells and it was $125 \pm 6\%$ in cells expressing the β arrV53D ($n = 20$).

We investigated the effect of β arrV53D on the agonist-promoted redistribution of endogenous β -arrestin. Although the quisqualate treatment for 5 min induced the internalization of β -arrestin (Figs. 3 and 4), in cells transfected with β arrV53D after 5 min of agonist treatment, the β -arrestin immunofluorescence was predominantly localized to the plasma mem-

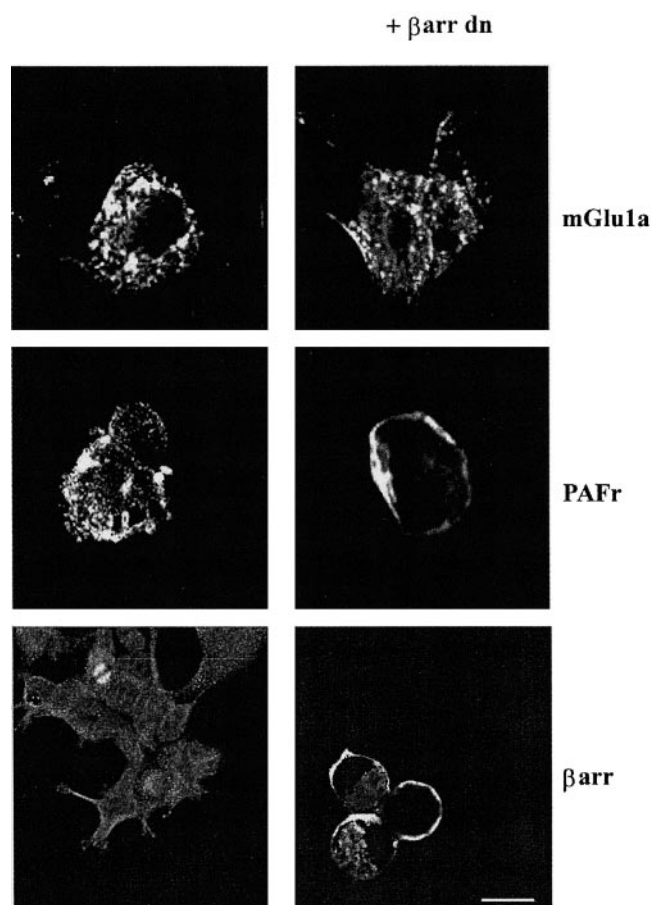


FIG. 4. Effect of β arrV53D on $mGlu_{1a}$ and PAF receptor internalization and β -arrestin redistribution. HEK293 cells transfected with plasmid DNA encoding $mGlu_{1a}$ or PAF receptor along with the empty vector or the β arrV53D (3 μ g/dish) (+ β arr dn) were treated with agonist. The distribution of $mGlu_{1a}$ and PAF receptor and β -arrestin was determined by immunofluorescence confocal microscopy. Scale bar, 7 μ m for $mGlu_{1a}$ and PAF receptor and 15 μ m for β -arrestin. These experiments are representative of three similar ones.

branes (Fig. 4). In cells transfected with β arrV53D, after agonist treatment the amount of β -arrestin intracellular immunofluorescence (relative to that present on the plasma membranes) was $77 \pm 14\%$ ($n = 29$). This suggests that the β arrV53D could prevent the internalization of endogenous β -arrestin.

$mGlu_{1a}$ Receptor-mediated MAPK Activation Is Blunted by β arrV53D—The experiments reported so far show that, in cells expressing the $mGlu_{1a}$ receptor, the exposure to agonist promotes the internalization of the receptor and induces the redistribution of β -arrestin in distinct intracellular compartments. We sought to examine whether the agonist-induced β -arrestin internalization could represent one signaling step for receptor-stimulated cellular response. We focused on MAPK activation because it was previously shown that many GPCRs can activate MAPK and that receptor and β -arrestin internalization is a key step toward this signaling cascade (18–20). In HEK293 cells expressing the $mGlu_{1a}$ receptor, exposure to quisqualate stimulated MAPK activation, as assessed by immunoblot using anti-phospho-ERK antibody, with a peak observed after 5 min of treatment (Fig. 5). After exposure to quisqualate for 5 min, the level of phospho-ERK1/2 was $349 \pm 33\%$ as compared with untreated cells ($n = 9$). When β arrV53D dominant negative mutant was co-expressed, the agonist-stimulated MAPK activation at 5 min of treatment was reduced by $78 \pm 4\%$ ($n = 4$). The immunoblot with the F4C1 monoclonal

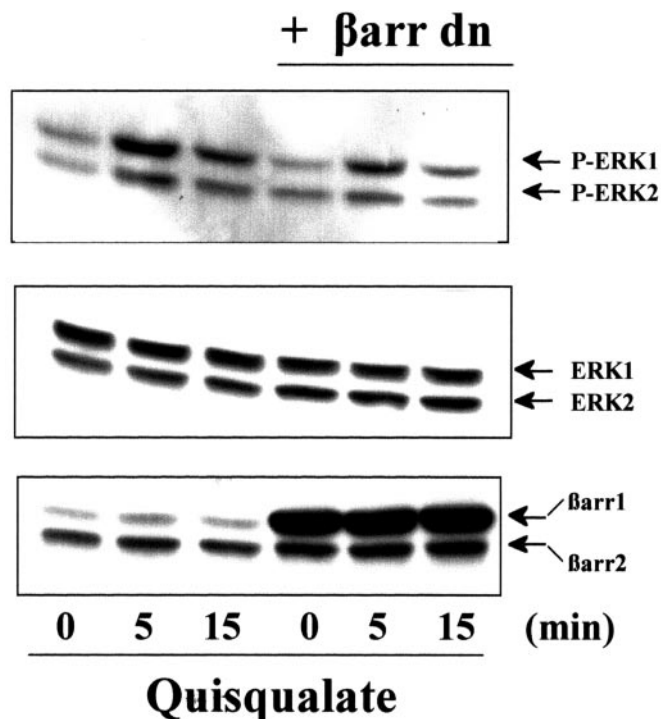


FIG. 5. Stimulation of ERK1/2 phosphorylation by $mGlu_{1a}$ receptor. Cells transfected with the $mGlu_{1a}$ receptor and co-transfected with empty vector or with β arrV53D dominant negative mutant (+ β arr dn) (3 μ g/dish), were treated with 100 μ M quisqualate for the indicated times. The experiment shown represents the immunoblot using anti-phospho-ERK1/2 (P-ERK1/2), anti-ERK1/2 or F4C1 anti-arrestin (β arr1/2) antibodies subsequently probed on the same membrane. This experiment is representative of three similar ones.

antibody, which recognizes an epitope common to all the arrestin subtypes, confirmed the overexpression of the β arrV53D and showed that β -arrestin 2, which is endogenously expressed in HEK293 cells, was not affected by the transfection (Fig. 5).

To see whether increasing the overexpression of β arrV53D could result in a complete blockade of quisqualate-induced MAPK activation, we performed experiments using 10 μ g of β arrV53D plasmid/dish to transfected HEK293 cells (instead of 3 μ g of plasmid/dish, used in the previous experiments). In cells transfected with 10 μ g of β arrV53D plasmid, agonist-stimulated MAPK activation was almost completely prevented (Fig. 6B). In these cells, after 5 min of quisqualate, the level of phospho-ERK1/2 was $113 \pm 14\%$ ($n = 4$) of that found in untreated cells. By contrast the higher levels of β arrV53D transfected did not inhibit the agonist-stimulated $mGlu_1$ receptor internalization (Fig. 6A). After agonist treatment the amount of $mGlu_1$ receptor intracellular immunofluorescence was $425 \pm 9\%$ in control cells and it was $448 \pm 15\%$ in cells transfected with 10 μ g of β arrV53D ($n = 10$). By confocal microscopy analysis, we could demonstrate in single cell that the overexpression of β arrV53D prevents the agonist-dependent ERK1/2 activation without affecting $mGlu_1$ receptor internalization (Fig. 6A).

In the experiments presented so far, we used HEK293 cells transiently expressing GRK4. To assess whether this kinase is necessary for $mGlu_1$ receptor-dependent MAPK activation, we examined this pathway in cells transfected with the $mGlu_1$ receptor without GRK4 co-transfected. GRK4 is not endogenously expressed in HEK293 cells. Exposure to quisqualate induced the activation of MAPK even in the absence of GRK4 (Fig. 6C), and the time course was similar to that of the earlier experiments (data not shown). This finding suggests that kinase(s) other than GRK4 could be involved in the $mGlu_1$ recep-

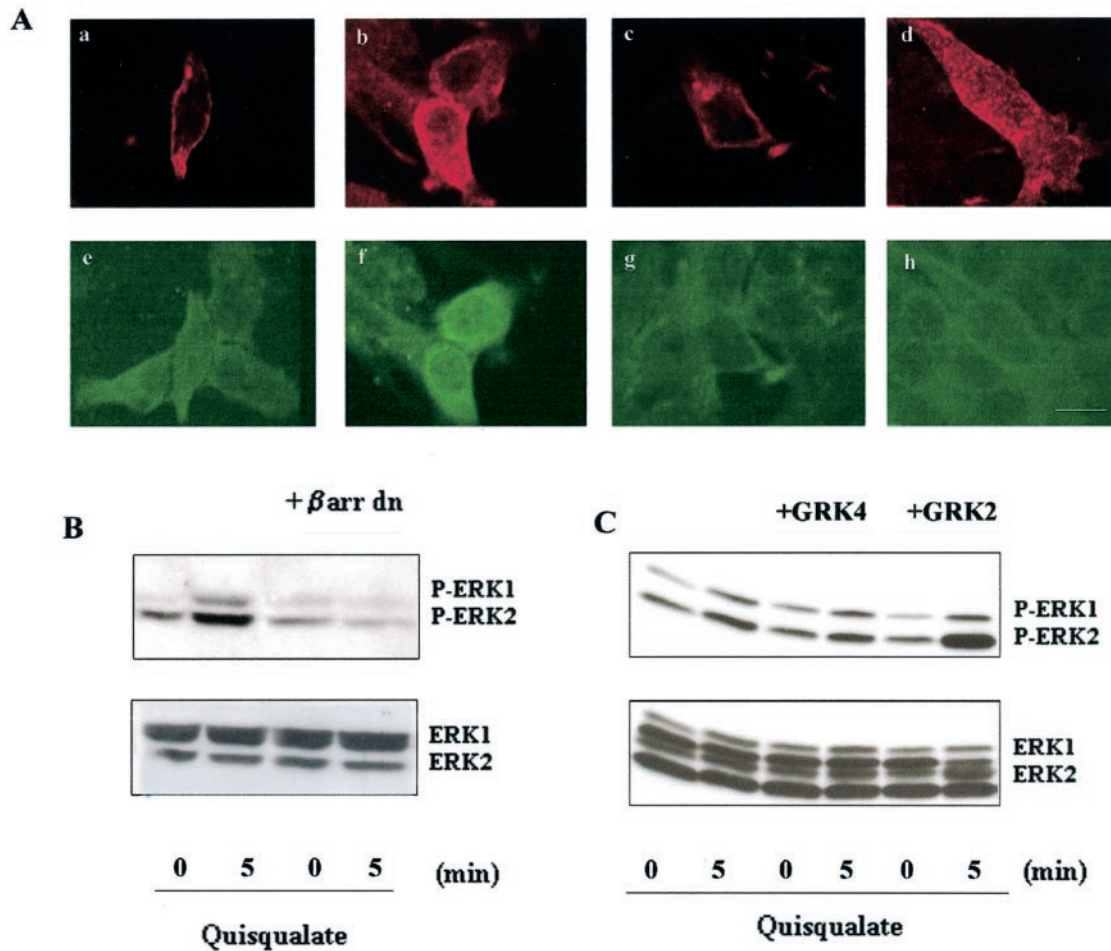
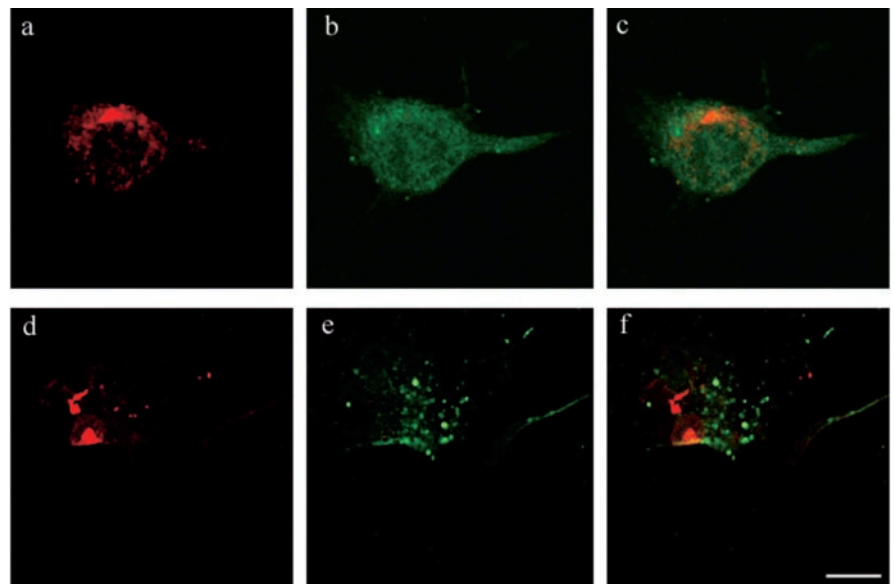


FIG. 6. *A*, overexpression of β arrV53D inhibits ERK1/2 phosphorylation but not receptor internalization. HEK293 cells transfected with the $mGlu_{1a}$ receptor and GRK4 and co-transfected with empty vector (*a*, *b*, *e*, and *f*) or with β arrV53D (10 μ g/dish) (*c*, *d*, *g*, and *h*), were untreated (*a*, *c*, *e*, and *g*) or treated with 100 μ M quisqualate for 5 min (*b*, *d*, *f*, and *h*). The distributions of the $mGlu_{1a}$ receptor (in red; *a*–*d*) and of phospho-ERK1/2 (in green; *e*–*h*) are shown. Scale bar, 5 μ m. *B*, immunoblot of phospho-ERK1/2 and total ERK1/2 in HEK293 cells transfected with the $mGlu_{1a}$ receptor and GRK4 and co-transfected with empty vector or with the β arrV53D (+ β arr dn). *C*, HEK293 cells transfected with the $mGlu_{1a}$ receptor and co-transfected with empty vector or with GRK4 or GRK2 and stimulated with 100 μ M quisqualate for the indicated times, before phospho-ERK1/2 and total ERK1/2 immunoblot. The experiments are representative of three similar ones.

FIG. 7. Agonist-induced redistribution of the $mGlu_1$ receptor and β -arrestin in cerebellar Purkinje cells. Cultured rat cerebellar Purkinje cells treated with vehicle (*a*–*c*) or with 100 μ M quisqualate for 5 min (*d*–*f*) were double-stained with anti- $mGlu_1$ receptor and anti- β -arrestin antibodies for immunofluorescence confocal microscopy analysis. The distribution of the $mGlu_{1a}$ receptor (in red; *a* and *d*) and of β -arrestin (in green; *b* and *e*) are shown in single-channel images. The overlay is shown in *c* and *f*. Scale bar, 7 μ m.



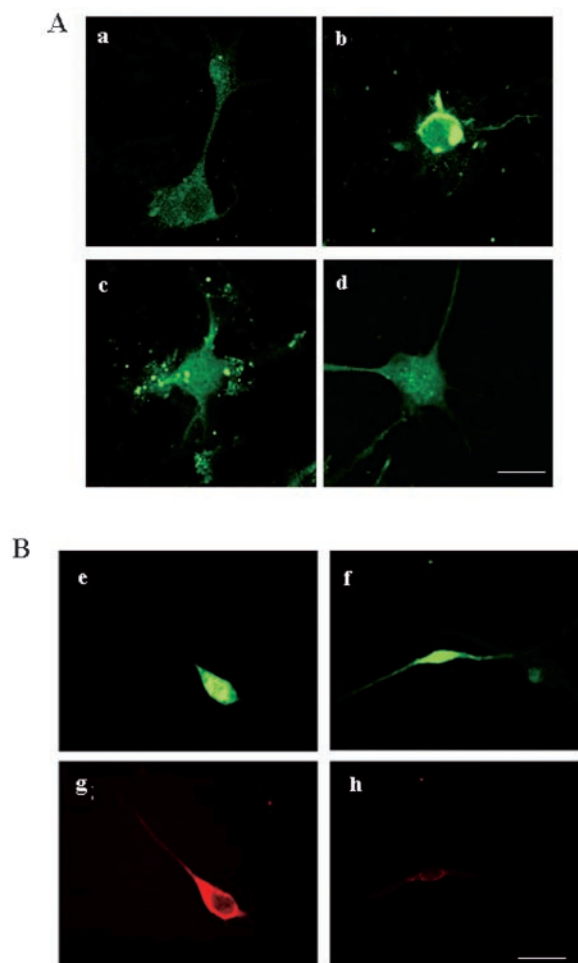


FIG. 8. Agonist-stimulated MAPK activation in cerebellar Purkinje cells. *A*, stimulation of ERK1/2 phosphorylation. Cultured rat cerebellar Purkinje cells were stained with anti-phospho-ERK1/2 (*a* and *b*) or with anti-ERK1/2 (*c* and *d*) antibodies for immunofluorescence confocal microscopy analysis. Serum-starved cells were treated with vehicle (*a* and *c*) or with 100 μ M quisqualate for 5 min (*b* and *d*). Scale bar, 10 μ m. *B*, inhibition of agonist-stimulated ERK1/2 phosphorylation by β arrV53D. Cultured cerebellar Purkinje cells infected with a recombinant adenovirus to express GFP alone (*e* and *g*) or GFP and β arrV53D (*f* and *h*) were treated with 100 μ M quisqualate for 5 min, fixed, and stained with anti-phospho-ERK1/2 antibody for immunofluorescence confocal microscopy analysis. The expression of GFP (in green; *e* and *f*) and of phospho-ERK1/2 (in red; *g* and *h*) is shown. Scale bar, 20 μ m. The experiments are representative of three similar ones.

tor-stimulated β -arrestin-dependent activation of MAPK. GRK2, which is endogenously expressed in HEK293 cells, is the obvious candidate, because the agonist-dependent phosphorylation of the mGlu₁ receptor by GRK2 has been documented (9). We co-transfected mGlu₁ receptor and GRK2 in HEK293 cells, and we found that the quisqualate-promoted activation of MAPK was increased (Fig. 6C). In parallel experiments quisqualate treatment (5 min) increased the level of phospho-ERK1/2 expression by 2.57 \pm 0.08-fold in HEK293 cells transfected with the mGlu₁ receptor alone and by 4.1 \pm 0.18-fold in cells with GRK2 co-transfected. Overexpression of GRK4 did not increase quisqualate-induced ERK1/2 phosphorylation (Fig. 6C).

Agonist-stimulated mGlu₁ Receptor and β -Arrestin Redistribution in Cerebellar Purkinje Cells—We used cerebellar Purkinje cells primary culture to assess agonist-dependent redistribution of the mGlu₁ receptor and β -arrestin in cells that natively express these proteins (Fig. 7). The mGlu₁ receptor, which is expressed at the cell surface in untreated Purkinje

cells, is internalized in intracellular vesicles after 5 min of exposure to quisqualate. β -Arrestin, which is cytosolic under basal conditions, is also rapidly redistributed after agonist exposure. In cells exposed to quisqualate, β -arrestin was found in cytosolic vesicles different from those where the mGlu₁ receptor is internalized. Quantitative analysis of different experiments confirmed that the mGlu₁ receptor and β -arrestin are not co-localized. Under basal conditions 13 \pm 4% of the mGlu₁ receptor and 10 \pm 4% of β -arrestin were reciprocally co-localized ($n = 20$). After quisqualate treatment for 5 min, 22 \pm 5% of the mGlu₁ receptor and 26 \pm 6% of β -arrestin staining were reciprocally co-localized ($n = 20$).

mGlu₁ Receptor-mediated MAPK Activation in Cerebellar Purkinje Cells—The activation MAPK by mGlu₁ receptor was documented in Chinese hamster ovary (21) and HEK293 (present study) cells transfected to overexpress the mGlu₁ receptor. We sought to determine whether this receptor-mediated response also occurs in cells that physiologically express the mGlu₁ receptor. Using confocal microscopy analysis, we evaluated the activation of MAPK by quisqualate in cultured cerebellar Purkinje cells (Fig. 8A). Using the anti-phospho-ERK1/2 antibody, which recognizes the phosphorylated form of ERK, we found that 5-min treatment with quisqualate induced a robust increase of the immunoreactivity (Fig. 8A, *a* and *b*). Phospho-ERK1/2 total immunofluorescence intensity (arbitrary units) was increased from 3586 \pm 416 to 56,796 \pm 2400 following 5 min of exposure to quisqualate ($n = 14$). Phosphorylated ERK1/2 was also redistributed and was localized in the nucleus (Fig. 8A) or in perinuclear regions (data not shown) depending on the cells examined. The labeling of RGS4, used as negative control (data not shown), demonstrated that in the same cells treated with quisqualate the increased immunofluorescence and the redistribution observed with the anti-phospho-ERK1/2 antibody was selective. ERK1/2 were not affected by quisqualate treatment in cultured cerebellar Purkinje cells (Fig. 8A, *c* and *d*). ERK1/2 total immunofluorescence intensity (arbitrary units) was 41,554 \pm 351 and 47,612 \pm 215 in control and treated cells respectively ($n = 16$).

Involvement of GRK4 and β -Arrestin in the Agonist-stimulated mGlu₁ Receptor Internalization and MAPK Activation in Cerebellar Purkinje Cells—Our data in HEK293 cells indicate that GRK4 and β -arrestin are major determinants in the mechanism of agonist-stimulated mGlu₁ receptor internalization and MAPK activation, respectively. We assessed whether these mechanisms are also important in cells that natively express the mGlu₁ receptor and these regulatory proteins.

In cultured cerebellar Purkinje cells, GRK4 was knocked down by the treatment with an antisense oligonucleotide that selectively decreased GRK4 by \sim 70%, whereas the treatment with a scrambled oligonucleotide (used as control) was ineffective (Ref. 8 and data not shown). According to previous findings, the agonist-stimulated mGlu₁ receptor internalization was inhibited by 83 \pm 7% ($n = 12$) in cells treated with the antisense (*versus* scrambled oligonucleotide-treated cells), whereas in the same cells the ERK1/2 phosphorylation induced by quisqualate was similar to that observed in cells treated with the scrambled oligonucleotide (Fig. 9).

To investigate the involvement of β -arrestin, we infected the primary cultured cerebellar Purkinje cells using an adenoviral vector to express the β arrV53D dominant negative (Fig. 8B). We used this approach for the following reasons: (i) the attempt to knock down β -arrestin by the antisense treatment was unsuccessful; (ii) the efficiency of plasmid chemical transfection in primary cultured neurons is too low (<5%) to transfect cerebellar Purkinje cells, which in primary cultures represent \sim 3–4% of the total cell population (8). Using the adenoviral

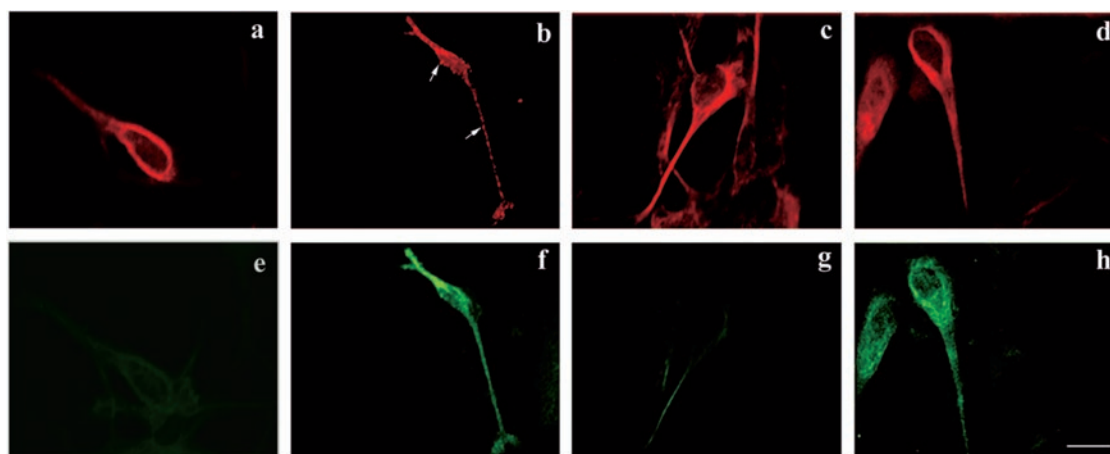


FIG. 9. GRK4 knock-down inhibits receptor internalization but not ERK1/2 phosphorylation in cerebellar Purkinje cells. Cerebellar Purkinje cells treated with the scrambled oligonucleotide (a, b, e, and f) or with the GRK4 antisense oligonucleotide (c, d, g, and h) were untreated (a, c, e, and g) or treated with 100 μ M quisqualate for 5 min (b, d, f, and h). The distribution of the $mGlu_{1a}$ receptor (in red; a–d) and of phospho-ERK1/2 (in green; e–h) are shown. The arrows indicate the punctate receptor after agonist treatment in scrambled oligonucleotide-treated cells. Scale bar, 7 μ m. The experiments are representative of three similar ones.

vector, we obtained a very high infection efficiency (>85% of cerebellar Purkinje cells) as assessed by GFP, which is expressed by infected cells. In cells infected with the adenoviral vector without β arrV53D, exposure to quisqualate induced a robust ERK1/2 phosphorylation (Fig. 8B, e and g). This effect was substantially blunted in cells infected with the adenoviral vector expressing β arrV53D (Fig. 8B, f and h).

DISCUSSION

GRK4 mediates the homologous desensitization and the rapid internalization of the $mGlu_1$ receptor in transfected HEK293 cells and in cultured cerebellar Purkinje cells (8). We have previously shown that, in both cell types, exposure to agonist for 5 min induced the internalization of the $mGlu_1$ receptor and of GRK4 in intracellular vesicles where these proteins were substantially co-localized (8). This delineated a rapid GRK4-dependent mechanism of $mGlu_1$ receptor internalization. The present study documents that, besides inducing internalization and co-localization of the receptor and GRK4, the rapid exposure to agonist promotes the redistribution of β -arrestin, which is likely involved in the $mGlu_1$ receptor-mediated MAPK activation. Upon agonist stimulation the β -arrestin and the $mGlu_1$ were sorted to different intracellular vesicles, and the expression of β arrV53D dominant negative mutant significantly inhibited agonist-promoted MAPK activation. Our results strongly indicate that β -arrestin is directly involved in glutamate-stimulated MAPK activation by acting as a signaling molecule.

GRK4 is a member of the GRK family, which, unlike the other GRKs, is not ubiquitously expressed but instead is found in only few cell types (8, 22, 23). This kinase, initially identified in testis and in sperm cells, is also abundantly expressed in cerebellar Purkinje cells, where it regulates the $mGlu_1$ receptor (8). In transfected HEK293 cells, the $mGlu_1$ receptor signaling can also be regulated by GRK2 (8, 9). Because the expression of the $mGlu_1$ receptor is not limited to neurons, it was suggested that GRK2 can play a role in regulating the $mGlu_1$ receptor in non-neuronal tissues or, alternatively, in neuronal cell types other than the cerebellar Purkinje cells (9). Here we show that GRK2 and GRK4 regulate the $mGlu_1$ receptor signaling by, at least in part, different mechanisms. GRK4-dependent desensitization was fully phosphorylation-mediated, whereas GRK2 is also able to regulate receptor-stimulated IP production by a phosphorylation-independent mechanism, which involves the functional RGS-like domain present within the GRK2 N termi-

nus. The difference between these two kinases likely reflects the different ability of their N-terminal domains to interact with the activated $G\alpha_q$ and supports the idea that only GRK2 and GRK3 (members of the β ARK subfamily) possess a functional RGS-like domain, whereas the members of the GRK4 subfamily (namely GRK4, GRK5, and GRK6) do not interact with the G protein to regulate the signaling (12, 15). While this article was under revision, a paper from Dhami *et al.* (24) was published, reporting that in transfected HEK293 cells the $mGlu_1$ receptor signaling is regulated by GRK2 by a phosphorylation-independent mechanism. These data are entirely consistent with the conclusions reached by our study.

In HEK293 cells transfected with GRK4, exposure to agonist induces the rapid (5 min) internalization of the $mGlu_1$ receptor. This effect resembles the rapid agonist-induced $mGlu_1$ receptor internalization observed in cultured cerebellar Purkinje cells, as the antisense oligonucleotide-induced knock-down of GRK4 (but not of GRK2) prevented receptor internalization (8). We therefore focused on this rapid agonist-stimulated GRK4-dependent $mGlu_1$ receptor desensitization and internalization to assess the role of β -arrestin in receptor trafficking and signaling. In HEK293 cells, upon agonist exposure β -arrestin is rapidly redistributed to plasma membranes and then internalized in intracellular vesicles, which are distinct from those where the receptor is internalized. The agonist-stimulated differential sorting of the receptor and β -arrestin was also observed in cultured cerebellar Purkinje cells. The ability of an agonist to induce a differential sorting of the receptor and β -arrestin was already reported for the 5-HT_{2A} receptor, which is internalized by a β -arrestin-independent mechanism (25). Consistently we found that the β arrV53D dominant negative mutant did not inhibit the agonist-stimulated $mGlu_1$ receptor internalization.

Other groups have reported that, in HEK293 cells, the $mGlu_1$ receptor is internalized in an agonist-stimulated manner even when GRK4 was not co-transfected (10, 11). This mechanism seems to be different from that observed in the presence of GRK4, because the time course is slower (maximal effect at 30–60 min) and the internalization is β -arrestin-dependent. In these studies the β -arrestin dominant negative mutants used to inhibit the $mGlu_1$ receptor internalization were different from the β arrV53D used in the present study, and it should be emphasized that different β -arrestin mutants may interfere differently with the ability of this scaffold protein to interact with other proteins involved in receptor trafficking

and signaling (18). Interestingly a rapid ($t_{1/2} = 3.3$ min) and extensive tonic internalization of the mGlu₁ receptor has been reported (11), which is not blocked by β -arrestin dominant negative mutants. This process is similar to that described in the present study. We suggest that, in the presence of GRK4, the agonist can increase the rate of the "tonic recycling" of the mGlu₁ receptor.

β -Arrestin was first identified as a key regulatory protein important for the homologous desensitization and internalization of many GPCRs. Later it was found that β -arrestin could also act as an adaptor protein, which binds to the GRK-phosphorylated GPCR and, by interacting with clathrin, promotes receptor redistribution and internalization in clathrin-coated vesicles. More recently it has been shown that β -arrestin interacts with different proteins, which are either involved in receptor translocation or in signal transduction events downstream from the receptor (16, 26, 27). The involvement of β -arrestin in the ubiquitination and degradation of the β_2 -adrenergic receptor has also been demonstrated (28). These findings indicate that β -arrestin, besides mediating GPCR internalization, is involved in several GPCR-mediated signaling cascades. We found that β -arrestin is not directly involved in the rapid agonist-induced internalization of the mGlu₁ receptor, because β -arrestin is not physically associated to the mGlu₁ receptor during the receptor internalization and the β arrV53D dominant negative mutant did not prevent receptor internalization. However, the exposure to mGlu₁ receptor agonist induced a rapid redistribution of β -arrestin to plasma membranes, where the receptor and β -arrestin were co-localized. This was followed by internalization of the receptor and β -arrestin in distinct intracellular vesicles. We hypothesized that, under these conditions, β -arrestin may act as a signaling protein for intracellular pathway(s) activated by receptor stimulation. Exposure to the mGlu₁ receptor agonist induces the phosphorylation and activation of ERK1/2 in transfected cells (Ref. 21 and Fig. 5) and in cerebellar Purkinje cells (Fig. 8). We therefore used β arrV53D to assess whether β -arrestin is involved in the mechanism of the mGlu₁ receptor-mediated MAPK activation. A previous study showed that this mutant was able to inhibit agonist-promoted sequestration of the β_2 -AR and 5HT_{1A} receptor and the activation of MAPK mediated by these receptors (18). The authors concluded that the β -arrestin-dependent sequestration of a subset of GPCRs plays a role in the initiation of mitogenic signals. The present study documents that the expression of β arrV53D did not alter the agonist-stimulated mGlu₁ receptor sequestration but did inhibit the agonist-promoted MAPK activation, indicating that the ability of the β -arrestin dominant negative mutant to inhibit ERK1/2 phosphorylation is not the result of the inhibition of receptor sequestration. We suggest that β arrV53D prevents the interaction of the endogenous β -arrestin with protein(s) involved in the formation of the signaling complex that mediates the activation of MAPK. The involvement of β -arrestin in agonist-dependent MAP kinases activation was confirmed in cerebellar Purkinje cells using an adenovirus vector to express β arrV53D.

Our data document that in HEK293 cells and in cerebellar Purkinje cells GRK4 is not necessary for the mGlu₁ receptor-stimulated activation of ERK1/2. Experiments in HEK293 cells rather indicate that GRK2 is involved in this pathway. The mGlu₁ receptor internalization is not sufficient to activate this

pathway, and it is likely that the receptor is not physically associated to the signaling complex, because the mGlu₁ receptor and β -arrestin are internalized in different intracellular compartments.

Based on the available data, we propose the following model for the mGlu₁ receptor signaling and regulation. The activation of the mGlu₁ receptor induces the redistribution of β -arrestin, which works as a signaling protein for receptor-stimulated MAPK activation. In cells that do not express GRK4, GRK2, which is ubiquitous, is involved in agonist-induced rapid activation of ERK1/2, G α_q -mediated signaling desensitization, and delayed receptor internalization. GRK2 likely acts by phosphorylation-independent and/or by phosphorylation-dependent mechanisms. In cells expressing GRK4 (such as cerebellar Purkinje cells), the agonist-dependent receptor activation also induces the rapid internalization of the receptor, which may reinforce the control of the signaling and could activate other as yet unidentified signaling pathway(s) requiring receptor internalization.

REFERENCES

- De Blasi, A., Conn, P. J., Pin, J.-P., and Nicoletti, F. (2001) *Trends Pharmacol. Sci.* **22**, 114–120
- Nakanishi, S. (1994) *Neuron* **29**, 1031–1037
- Conn, P. J., and Pin, J.-P. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37**, 205–237
- Catania, M. V., Aronica, E., Sortino, M. A., Canonico, P. L., and Nicoletti, F. (1991) *J. Neurochem.* **56**, 1329–1335
- Saugstad, J. A., Marino, M. J., Folk, J. A., Hepler, J. R., and Conn, P. J. (1998) *J. Neurosci.* **18**, 905–913
- Berman, D. M., and Gilman, A. G. (1998) *J. Biol. Chem.* **273**, 1269–1272
- Hepler, J. R. (1999) *Trends Pharmacol. Sci.* **20**, 376–382
- Sallese, M., Salvatore, L., D'Urbano, E., Sala, G., Storto, M., Launey, T., Nicoletti, F., Knopfel, T., and De Blasi, A. (2000) *FASEB J.* **14**, 2569–2580
- Dale, B. D., Bhattacharya, M., Anborgh, P. H., Murdoch, B., Bhatia, M., Nakanishi, S., and Ferguson, S. S. G. (2000) *J. Biol. Chem.* **275**, 38213–38220
- Mundell, S. J., Matharu, A., Pula, G., Roberts, P. J., and Kelly, E. (2001) *J. Neurochem.* **78**, 546–551
- Dale, L. B., Bhattacharya, M., Seachrist, J. L., Anborgh, P. H., and Ferguson, S. S. G. (2001) *Mol. Pharmacol.* **60**, 1243–1253
- Sallese, M., Mariggio, S., D'Urbano, E., Iacovelli, L., and De Blasi, A. (2000) *Mol. Pharmacol.* **57**, 826–831
- Furuya, S., Makino, A., and Hirabayashi, Y. (1998) *Brain Res. Protoc.* **3**, 192–198
- He, T.-C., Zhou, S., Da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2509–2514
- Carman, C. V., Parent, J. L., Day, P. W., Pronin, A. N., Stenweis, P. M., Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999) *J. Biol. Chem.* **274**, 34483–34492
- Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) *Nature* **383**, 447–450
- Chen, Z., Dupret, D. J., Le Guillou, C., Rola-Pleszczynski, M., and Stanková, J. (2002) *J. Biol. Chem.* **277**, 7356–7362
- Luttrell, L. M., Ferguson, S. S. G., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F.-T., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) *Science* **283**, 655–661
- DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnnett, N. W. (2000) *J. Cell Biol.* **148**, 1267–1281
- Tohgo, A., Pierce, K. L., Choy, E. W., Lefkowitz, R. J., and Luttrell, L. M. (2002) *J. Biol. Chem.* **277**, 9429–9436
- Ferraguti, F., Baldani-Guerra, B., Corsi, M., Nakanishi, S., and Corti, C. (1999) *Eur. J. Neurosci.* **11**, 2073–2082
- Sallese, M., Mariggio, S., Collodel, G., Moretti, E., Piomboni, P., Baccetti, B., and De Blasi, A. (1997) *J. Biol. Chem.* **272**, 10188–10195
- Ambrose, C., James, M., Barnes, G., Lin, C., Bates, G., Altherr, M., Duyao, M., Groot, N., Church, D., Wasmuth, J. J., Lehrach, H., Housman, D., Buckler, A., Gusella, J. F., and MacDonald, M. E. (1992) *Hum. Mol. Genet.* **1**, 697–703
- Dhami, G. K., Anborgh, P. H., Dale, L. B., Sterne-Marr, R., and Fergusson, S. G. (2002) *J. Biol. Chem.* **277**, 25266–25272
- Bhatnagar, A., Willins, D. J., Gray, J. A., Woods, J., Benovic, J. L., and Roth, B. L. (2001) *J. Biol. Chem.* **276**, 8269–8277
- Miller, W. E., and Lefkowitz, R. J. (2001) *Curr. Biol.* **13**, 139–145
- Ferguson, S. S. (2001) *Pharmacol. Rev.* **53**, 1–24
- Shenoy, S. K., McDonald, P. H., Konhout, T. A., and Lefkowitz, R. J. (2001) *Science* **294**, 1307–1313

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