0026-895X/09/7504-991–1003$20.00
MOLECULAR PHARMACOLOGY
Vol. 75, No. 4
52316/3454584
Mol Pharmacol 75:991–1003, 2009
Printed in U.S.A.

Regulation of Group II Metabotropic Glutamate Receptors by G Protein-Coupled Receptor Kinases: mGlu2 Receptors Are Resistant to Homologous Desensitization

L. Iacovelli, G. Molinaro, G. Battaglia, M. Motolese, L. Di Menna, M. Alfiero, J. Blahos, F. Matrisciano, M. Corsi, C. Corti, V. Bruno, A. De Blasi, and F. Nicoletti

Departments of Physiology and Pharmacology (L.I., M.M., M.A., F.M., V.B., F.N.) and Experimental Medicine (A.D.B.), University of Rome Sapienza, Rome, Italy; I.N.M. Neuromed, Pozzilli, Italy (G.M., G.B., L.D.M., V.B., F.N.); Institute of Experimental Medicine, Czech Academy of Science, Prague, Czech Republic (J.B.); and Department of Biology, Psychiatry Centre of Excellence in Drug Discovery, GlaxoSmithKline Medicines Research Centre, Verona, Italy (M.C., C.C.)

Received October 2, 2008; accepted January 22, 2009

ABSTRACT

We examined the regulation of mGlu2 and mGlu3 metabotropic glutamate receptor signaling prompted by the emerging role of these receptor subtypes as therapeutic targets for psychiatric disorders, such as anxiety and schizophrenia. In transfected human embryonic kidney 293 cells, G-protein-coupled receptor kinase (GRK) 2 and GRK3 fully desensitized the agonist-dependent inhibition of cAMP formation mediated by mGlu3 receptors. In contrast, GRK2 or other GRKs did not desensitize the cAMP response to mGlu2 receptor activation. Desensitization of mGlu3 receptors by GRK2 required an intact kinase activity, as shown by the use of the kinase-dead mutant GRK2-K220R or the recombinant GRK2 C-terminal domain. Overexpression of β-arrestin1 also desensitized mGlu3 receptors and did not affect the cAMP signaling mediated by mGlu2 receptors. The difference in the regulation of mGlu2 and mGlu3 receptors was signal-dependent because GRK2 desensitized the activation of the mitogen-activated protein kinase pathway mediated by both mGlu2 and mGlu3 receptors. In vivo studies confirmed the resistance of mGlu2 receptor-mediated cAMP signaling to homologous desensitization. Wild-type, mGlu2(−/−), or mGlu3(−/−) mice were treated intraperitoneally with saline or the mixed mGlu2/3 receptor agonist (−)-2-oxa-4-aminobicyclo[3.1.0]-exhane-4,6-dicarboxylic acid (LY379268; 1 mg/kg) once daily for 7 days. Inhibition of forskolin-stimulated cAMP formation by LY379268 was measured in cortical slices prepared 24 h after the last injection. Agonist pretreatment fully desensitized the cAMP response in wild-type and mGlu2(−/−) mice but had no effect in mGlu3(−/−) mice, in which LY379268 could only activate the mGlu2 receptor. We predict the lack of tolerance when mixed mGlu2/3 receptor agonists or selective mGlu2 enhancers are used continually in patients.

Agonists of group II metabotropic glutamate (mGlu) receptors (mGlu2 and mGlu3 receptors) are under clinical development for the treatment of psychiatric disorders, including generalized anxiety disorder and schizophrenia (Swanson et al., 2005; Corti et al., 2007b). In a recent phase IIa clinical trial, the mixed mGlu2/3 receptor agonist LY404039 (administered orally in the form of a prodrug for 28 days) was as effective as the conventional antipsychotic olanzapine in relieving both positive and negative symptoms of schizophrenia (Patil et al., 2007). This evidence, which has been highlighted in the U.S. media, gave new impetus to the study of mGlu2 and mGlu3 receptors. We decided to examine the mechanisms that regulate desensitization of mGlu2 and mGlu3 receptors, a process that critically affects the outcome of a long-term treatment with receptor agonists and is therefore of great clinical relevance. mGlu2 and mGlu3 receptors share >70% of their amino acid sequence and are both coupled to G_i proteins in heterologous expression systems (Pin and Duvoi-

This work was funded by the Italian Ministero dell'Istruzione, dell'Università e della Ricerca [Grant PRIN 200728AA57].
D.B.A. and N.F. contributed equally to this work.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.108.052316.

ABBREVIATIONS: mGlu, metabotropic glutamate receptor; GPCR, G-protein-coupled receptor; GRK, G protein-coupled receptor kinase; FSK, forskolin; MAPK, mitogen activated protein kinases; (2*R*,4*R*)-APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; LY379268(−)-2-oxa-4-aminobicyclo[3.1.0]exhane-4,6-dicarboxylic acid; GRK2-K220R, kinase-dead G protein-coupled receptor kinase 2 mutant; GRK2-Cter, C-terminal domain of G protein-coupled receptor kinase 2; HEK, human embryonic kidney; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin; PH, pleckstrin homology; wt, wild type; ANOVA, analysis of variance; LY354740, (+)-(1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo(3.1.0)hexane-2,6-dicarboxylic acid; LY404039, 4-amino-2-thiabicyclo(3.1.0)hexane-4,6-dicarboxylic acid.

sin, 1995). Studies on brain tissue and cultured neurons are usually carried out with orthosteric agonists, such as compounds (2*R*,4*R*)-APDC, LY354740, and LY379268, which activate both subtypes with equal affinity (Schoepp et al., 1999). Activation of native mGlu2 and mGlu3 receptors inhibits cAMP formation and stimulates the mitogen-activated protein kinase (MAPK) pathway (De Blasi et al., 2001; Dhami and Ferguson, 2006).

Homologous desensitization of G-protein-coupled receptors (GPCRs) is mediated by a family of enzymes called G-protein-coupled receptor kinases (GRKs). This family includes three subfamilies: GRK1, which corresponds to rhodopsin kinase and GRK7 form the rhodopsin kinase subfamily; GRK2 and GRK3, which are ubiquitous and are activated by G-protein $\beta\gamma$subunits (G$\beta\gamma$), form the βARK subfamily; and GRK4, -5, and -6 form the GRK4 subfamily. Phosphorylation of GPCRs by GRKs causes receptor desensitization and internalization through mechanisms that involve additional proteins, such as β-arrestins (Kohout and Lefkowitz, 2003). Arrestins also behave as scaffolding proteins, linking receptors to downstream signaling pathways, including MAPK activation (Luttrell and Lefkowitz, 2002). The role of different GRKs in the homologous desensitization of mGlu1 receptors has been elucidated (Dhami and Ferguson, 2006). Recombinant mGlu1 receptors expressed in HEK293 cells are desensitized by GRK4 in an agonist-dependent manner, and GRK4 knockdown in cultured cerebellar Purkinje cells (which natively express mGlu1 receptors) impairs receptor desensitization. GRK2 is also involved in desensitization and internalization of mGlu1 receptors, although its action does not require an intact kinase activity (Sallese et al., 2000; Iacovelli et al., 2003; Mundell et al., 2003; Dhami et al., 2004). GRK2 also regulates the expression and function of mGlu5 receptors in heterologous expression systems (Sorensen and Conn, 2003). mGlu4 receptors undergo a complex regulation by GRKs. These receptors are coupled to G_i, and their activation inhibits its adenylyl cyclase activity and stimulates both the MAPK and the phosphatidylinositol-3-kinase pathways in cultured cerebellar granule cells (Iacovelli et al., 2002). In heterologous expression systems, mGlu4 receptor signaling is regulated by GRK2 but not by GRK4: GRK2 desensitizes the MAPK pathway without affecting the inhibition of cAMP formation (Iacovelli et al., 2004). No data are yet available on the mechanisms mediating the homologous desensitization of mGlu2 and mGlu3 receptors and whether or not the two receptors desensitize in response to prolonged agonist stimulation. We have addressed this issues both in recombinant cells and in brain slices prepared from knockout mice lacking either mGlu2 or mGlu3 receptors.

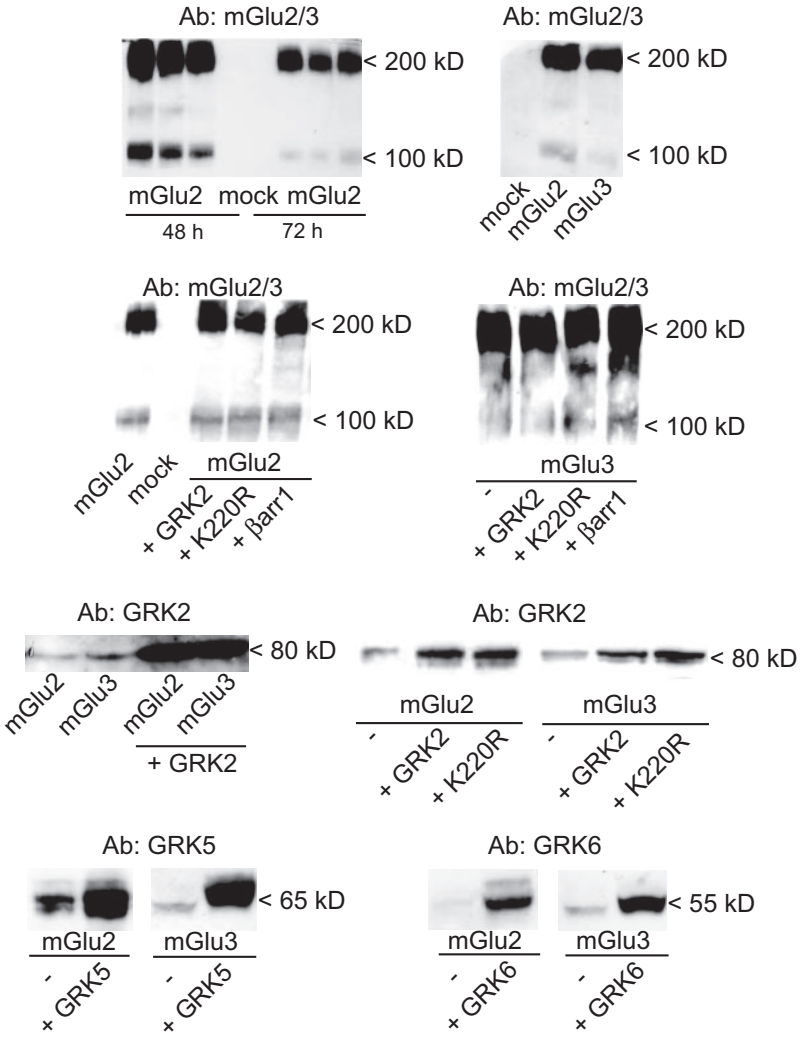

Fig. 1. Immunoblots of mGlu2/3 receptors; GRK2; GRK2 kinase-dead mutant, GRK2-K220R (K220R); GRK5 and GRK6 in HEK293 cells transfected with the respective cDNAs. Mock indicates HEK293 cells transfected with empty vectors. Each lane was loaded with 80 μg of proteins from individual culture dishes. The two bands shown in the immunoblots may correspond to receptor monomers (100 kDa) and dimers (approximately 200 kDa). Note that expression of the monomeric band is lighter for mGlu3 than mGlu2 receptors. The reason for this difference is unknown.

Materials and Methods

Materials. Polyclonal anti-mGlu2/3 receptor was purchased from Millipore Bioscience Research Reagents (Temecula, CA); monoclonal anti-GRK2/3 (clone C5/1) and monoclonal anti GRK4–6 (used to probe GRK5) were purchased from Millipore (Billerica, MA); polyclonal anti-ERK1/2 and polyclonal anti-GRK6 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-phospho-ERK1/2 was from Cell Signaling Technology (Danvers, MA); monoclonal anti-β-arrestin antibody was from BD Transduction Laboratories (Lexington, KY); pertussis toxin (PTX) was purchased from Calbiochem (San Diego, CA). (2*R*,4*R*)-4-Aminopyrrolidine-2,4-dicarboxylate [(2*R*,4*R*)-APDC] was purchased from Tocris Cookson (Bristol, UK); LY379268 was a generous gift from Eli Lilly and Company (Indianapolis, IN). All other drugs were purchased from Sigma-Aldrich (Milan, Italy). The full-length GRK4 cDNA were prepared as described previously (Sallese et al., 2000). The plasmids encoding for the kinase-dead mutant of GRK2 (GRK2-K220R) and the C-terminal domain of GRK2 (Gly^{495}-Leu^{689}) (GRK2-Cter) were kindly provided by C. Scorer (GlaxoSmithKline, Uxbridge, Middlesex, UK); DynK44A cDNA was kindly provided by J. Benovic; mGlu2 receptor cDNA was kindly provided by J. Blahos (Academy of Science, Prague, Czech Republic); and mGlu3 receptor cDNA was kindly provided by F. Ferraguti (Innsbruck Medical University, Innsbruck, Austria).

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were transfected in 10-mm Falcon dishes using 8 μl of LipofectAMINE 2000 in Opti-MEM medium (Invitrogen, Carlsbad, CA) and 10 μg of cDNA for 4 h. The cells used for the determination of cAMP were cotransfected with 2.5 μg/dish of adenylyl cyclase type V cDNA (Aramori et al., 1997). One day later, cells were seeded into six-well plates (for MAPK assay) or in 48-well plates (for cAMP assay) previously coated with poly(L-lysine) (0.01%), and the experiments were performed 48 h after transfection.

Animals. mGlu2 receptor knockout mice were provided by S. Nakanishi (University of Kyoto, Japan). mGlu3 receptor knockout mice were provided by C. Corti (GSK Glaxo, Verona, Italy). All mice were kept under environmentally controlled conditions (ambient temperature, 22°C; humidity, 40%) on a 12-h light/dark cycle with food and water ad libitum. Experiments were performed by following the guidelines for animal care and use of the National Institutes of Health. Wild-type, mGlu2(−/−), and mGlu3(−/−) mice were treated intraperitoneally once daily with LY379268 (1 mg/kg) for 7 days. Control animals were treated with saline. Animals were killed 24 h after the last LY379268 injection.

Immunoblotting. At the end of the final incubation, cells were rapidly rinsed in ice-cold phosphate-buffered saline and solubilized in Triton X-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 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM β-glycerophosphate) as described previously (Iacovelli et al., 2002). Protein cell lysates (80 μg) were separated by SDS-polyacrylamide gel electrophoresis electrophoresis, blotted onto nitrocellulose, and probed using specific antibodies. The antibodies were used at the following dilution: anti-phospho-ERK1/2, 1:500; anti-ERK1/2, 1:2000; anti-mGlu2/3 receptor, 1:1000; anti-GRK2/3 (clone C5/1), 1:7000; anti-GRK4, 1:2000; anti-GRK5 and anti-GRK6 were used 1:1000; anti-β-arrestin antibody was diluted 1:2500. The immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) using horseradish peroxidase-conjugated secondary antibodies.

cAMP Assay. Cell cultures were incubated in Hanks' balanced salt solution buffer, pH 7.4, containing 0.5 mM 3-isobutyl-1-methylxanthine and bovine serum albumin (0.3%). (2*R*,4*R*)-APDC (or vehicle) was added 10 min before forskolin (FSK) stimulation (1 μM). After 20 min, the reaction was stopped by substituting the buffer with ice-cold ethanol. Extraction and measurement of cAMP was carried out as described previously (Iacovelli et al., 1996) by radio-

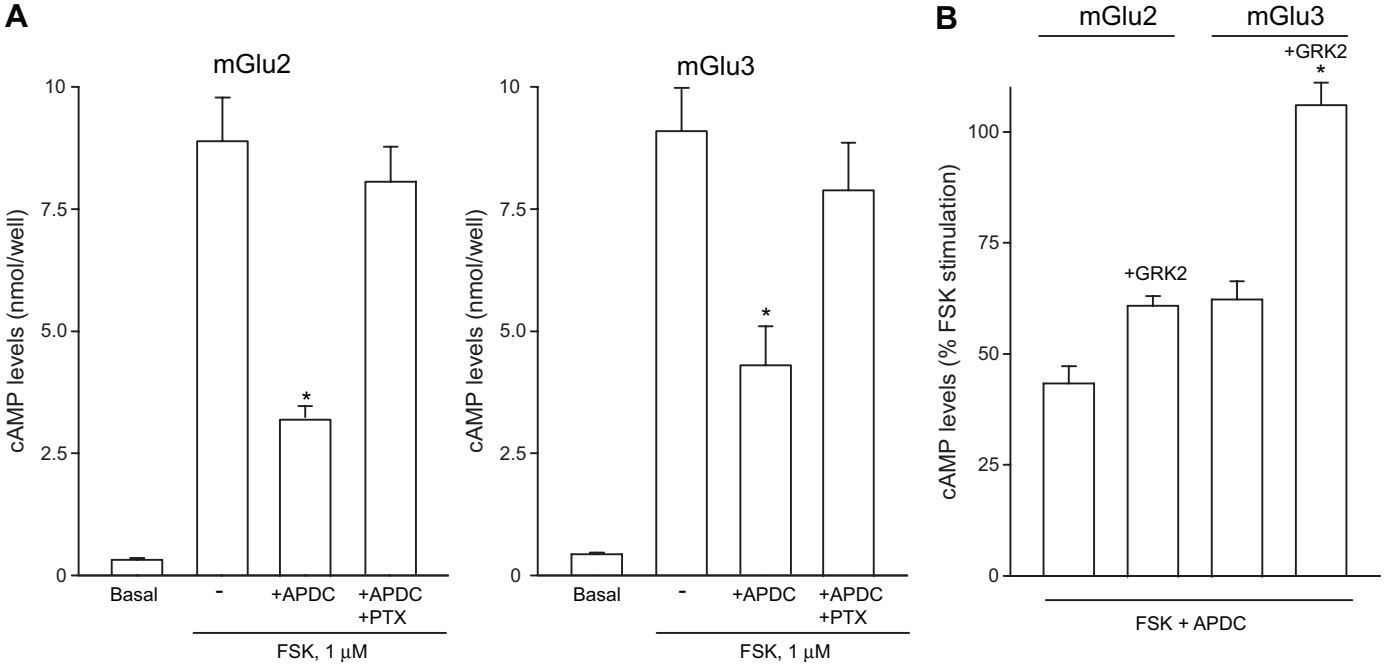

Fig. 2. A, inhibition of FSK-stimulated cAMP formation by (2*R*,4*R*)-APDC is blocked by PTX. HEK293 cells expressing mGlu2 or mGlu3 receptors were pretreated with PTX (1 μg/ml for 16–18 h) and then stimulated with 1 μM FSK ± 100 μM (2*R*,4*R*)-APDC. Values are means ± S.E.M. from three determinations, each in triplicate. *, $p < 0.05$ versus FSK (one-way analysis of variance plus Fisher's least significant difference). B, effects of GRK2 on agonist-dependent adenylyl cyclase inhibition. Inhibition of 1 μM FSK-stimulated cAMP formation by 100 μM (2*R*,4*R*)-APDC was measured in cells expressing mGlu2 or mGlu3 receptors alone or coexpressed with GRK2. Values are means ± S.E.M. from three determinations, each in triplicate. *, $p < 0.05$, Student's *t* test.

immunoassay (PerkinElmer Life and Analytical Sciences, Waltham, MA). Cortical slices were prepared as described by Nicoletti et al. (1986). In brief, animals were killed by decapitation, and cortexes were dissected out and cut into 350 × 350 μm slices with a McIlwain tissue chopper. Slices were incubated in Krebs-Hensleit buffer at 37°C under constant oxygenation to allow metabolic recovery. Forty microliters of gravity-packed slices were then incubated in 500 μl of buffer containing 0.5 mM 3-isobutyl-1-methylxanthine for 15 min. After the addition of FSK and/or mGlu receptor ligands, the incubation was continued for a further 20 min. The reaction was stopped by the addition of an equal volume of ice-cold 0.8 N $HClO_4$. Samples were then frozen or immediately used. Samples were sonicated and centrifuged at low speed (1500*g* for 10 min). One hundred eighty microliters of the supernatant was added to 20 μl of K_2CO_3 (2 M) and after further centrifuged in a microfuge (2 min at maximal speed), 20 μl of the supernatant was used for the cAMP assay. Intracellular cAMP levels were assayed by radioimmunoassay.

Results

GRKs Differentially Regulate mGlu2 and mGlu3 Receptor Signaling in Recombinant Cells. HEK293 cells were transfected with mGlu2 or mGlu3 receptor cDNA. Immunoblot analysis showed maximal receptor expression 48 h after transfection, when levels of mGlu2 and mGlu3 receptors were similar (Fig. 1). Cells were stimulated with compound (2*R*,4*R*)-APDC (100 μM), which behaves as a selective orthosteric agonist of both receptor subtypes (Schoepp et al., 1999). Addition of (2*R*,4*R*)-APDC inhibited FSK-stimulated cAMP formation in both mGlu2- and mGlu3-expressing cells. Inhibition was prevented by an overnight pretreatment with PTX (1 μg/ml), confirming that both receptor subtypes are coupled to Gi/Go proteins in recombinant cells (Fig. 2A). To examine the regulation of mGlu2 or mGlu3 receptor signaling, cells were cotransfected with individual GRK isotypes, starting with GRK2. Figure 1 shows that the levels of expression of GRK2 were similar in mGlu2- and mGlu3-expressing cells. The expression of mGlu2 and mGlu3 receptors was not affected by cotransfection of GRK2 or the kinase-dead mutant GRK2-K220R (Fig. 1). Overexpression of GRK2 produced markedly different effects on mGlu2 and mGlu3 receptor signaling. In cells expressing mGlu2 receptors, the ability of (2*R*,4*R*)-APDC to inhibit FSK-stimulated cAMP formation was only slightly desensitized by GRK2. In contrast, overexpression of GRK2 completely blunted the inhibition of cAMP formation mediated by mGlu3 receptors (Fig. 2B).

GRK4, GRK5, and GRK6 mimicked the action of GRK2 in mGlu2 receptor-expressing cells, producing only a slight reduction in the ability of (2*R*,4*R*)-APDC to inhibit cAMP formation (Fig. 3A). In contrast, only GRK3 shared with GRK2

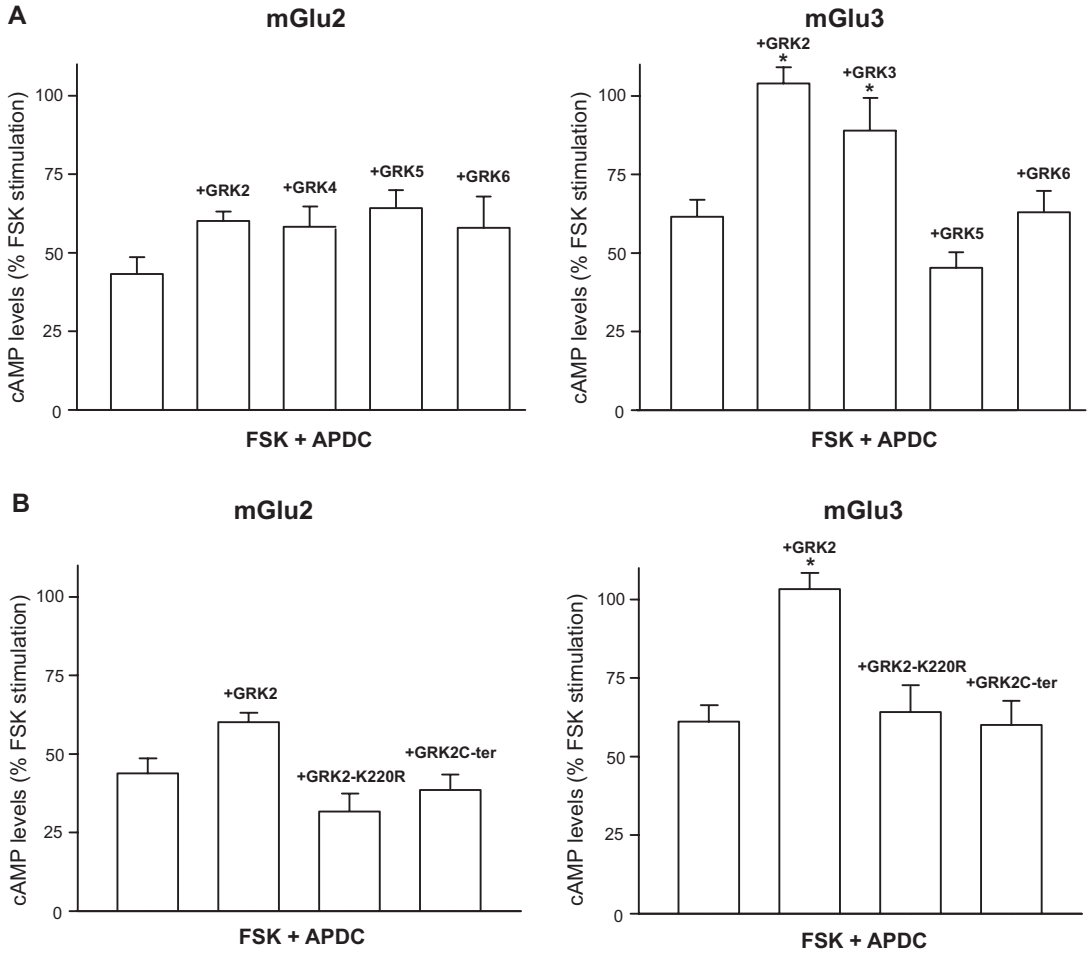

Fig. 3. Differential regulation of mGlu2 and mGlu3 receptor-mediated inhibition of cAMP formation by GRKs. A, inhibition of FSK-stimulated cAMP formation by (2*R*,4*R*)-APDC was measured in HEK293 cells expressing mGlu2 or mGlu3 receptors alone or coexpressed with GRK2, GRK3, GRK4, GRK5, or GRK6 as indicated. B, inhibition of FSK-stimulated cAMP formation by (2*R*,4*R*)-APDC was measured in HEK293 cells expressing mGlu2 or mGlu3 receptors alone or coexpressed with GRK2, GRK2-K220R, or GRK2-Cter. Values are means ± S.E.M. from three determinations, each in triplicate. *, $p < 0.05$ versus receptor alone (one-way ANOVA plus Fisher's *t* test).

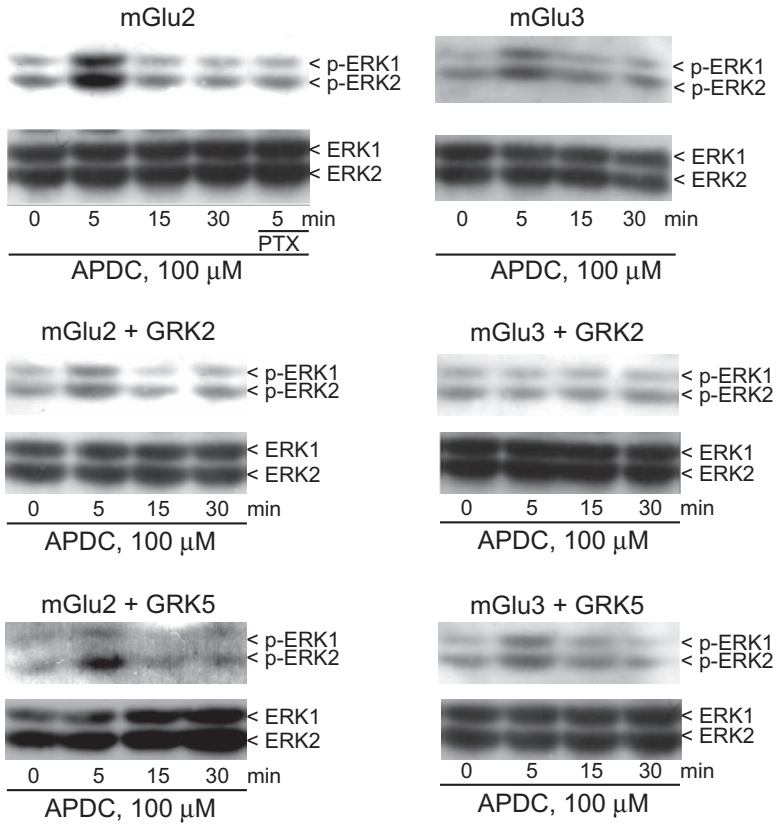

Fig. 4. Regulation of mGlu2/3-receptor-mediated MAPK activation. Representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or mGlu3 receptor alone or coexpressed with GRK2 or GRK5 are shown. Membranes were probed with antibodies specific for phosphorylated ERK1/2 (p-ERK1/2) and then washed and reprobed with antibodies recognizing total ERK1/2 (ERK). Cells were exposed to (2*R*,4*R*)-APDC (100 μM) for the indicated times. These experiments were repeated two additional times with similar results. Densitometric analysis are presented in Table 1. Note that a 16- to 18-h pretreatment with PTX (1 μg/ml) blunted the MAPK response to APDC in mGlu2 receptor-expressing cells (left, first immunoblot). PTX also inhibited MAPK activation by (2*R*,4*R*)-APDC in mGlu3-expressing cells (data not shown).

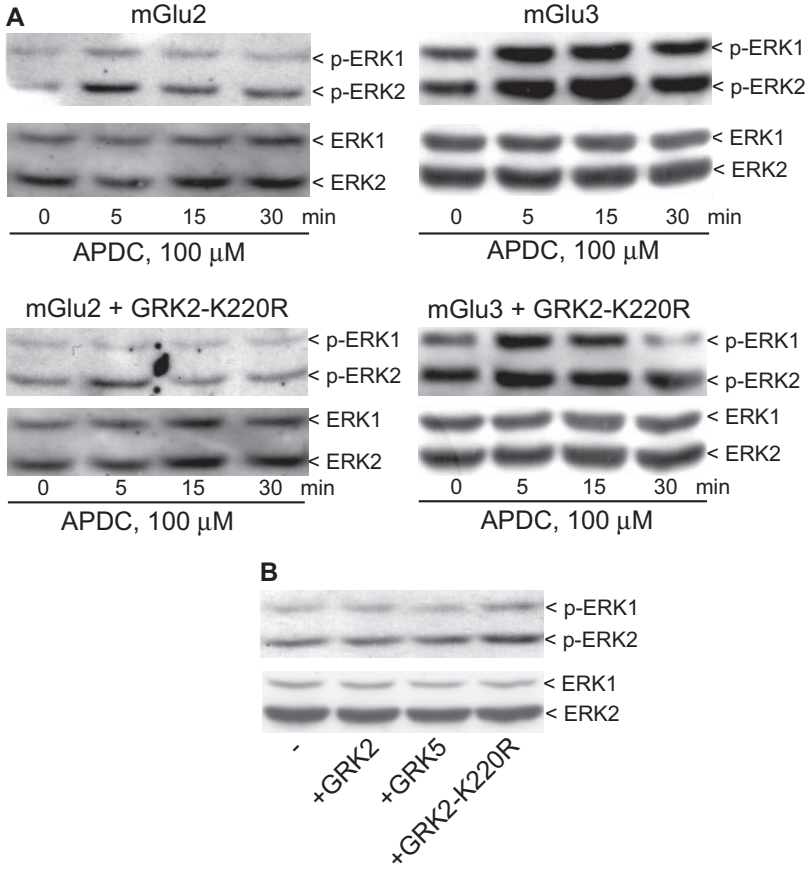

Fig. 5. Effect of GRK2-K220R on mGlu2/3-receptor-mediated MAPK activation. A, representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or mGlu3 receptor alone or coexpressed with GRK2-K220R. Cells were exposed to (2*R*,4*R*)-APDC (100 μM) for indicated times. Densitometric analysis are presented on Table 1. B, basal ERK1/2 was determined in unstimulated HEK293 cells transfected with the empty vector, GRK2, GRK5, and GRK2-K220R. These experiments were repeated two times with similar results.

the ability to fully desensitize the agonist-dependent inhibition of adenylyl cyclase activity in mGlu3-expressing cells, whereas at least GRK5 and GRK6 were inactive (Fig. 3A). Thus, desensitization of mGlu3 receptors in recombinant cells was specifically mediated by GRK2 and GRK3, which are functionally similar, belong to the same GRK subfamily, and share >85% similarity in the amino acid sequence (Benovic et al., 1991).

Desensitization of mGlu2 and mGlu3 Receptors Required an Intact Kinase Activity of GRK2. GRK2 is a multidomain protein that regulates G-protein-mediated signaling by different mechanisms, as shown for many GPCRs, including different mGlu receptor subtypes. GRK2 phosphorylates the agonist-bound GPCR, thus leading to homologous desensitization. In addition, GRK2 can inhibit signal propagation through the $G\beta\gamma$ subunits by means of the PH domain located at its C-terminal domain and can also reduce $G\alpha_q$ signaling by means of the RGS homology domain located at its N-terminal domain. To investigate the mechanisms by which GRK2 desensitizes the mGlu3 receptor-mediated signaling, we used the kinase-dead GRK2 mutant GRK2-K220R (in which the kinase activity is disrupted by site-directed mutagenesis) and the recombinant C-terminal domain (GRK2-Cter), which contains the functional PH domain of GRK2 (see Fig. 1 showing the same levels of expression of GRK2-K220R and GRK2-Cter in different experiments). We found that the mGlu3 receptor signaling, which was fully desensitized by wild-type GRK2, was not affected by cotransfection of the GRK2-K220R and GRK2C-ter mutants. The mGlu2 receptor signaling, which was only slightly desensitized by the wild-type GRK2, was not affected at all by both the GRK2-K220R and the GRK2-Cter (Fig. 3B). This indicates that the kinase activity of GRK2 is absolutely required for the desensitization of mGlu3 receptors and also for the slight desensitization of mGlu2 receptors.

GRK2 Desensitizes Both mGlu2 and mGlu3 Receptor-Mediated MAPK Activation in Recombinant Cells. For the majority of GPCRs that are coupled to the inhibitory heterotrimeric G_i protein, agonist stimulation activates multiple signaling pathways, including cAMP inhibition (mediated by $G\alpha_i$) and ERK1/2 phosphorylation (mediated by $G\beta\gamma$). Accordingly, the addition of (2*R*,4*R*)-APDC (100 μM) to HEK293 cells expressing mGlu2 or mGlu3 receptors induced ERK1/2 phosphorylation (Fig. 4 and Table 1). ERK1/2 phosphorylation was rapid, reversible, and prevented by pretreatment with PTX (Fig. 4 and data not shown), indicating the involvement of G_i/G_o proteins. We examined the regulation of mGlu2 and mGlu3 receptor-dependent ERK1/2 phosphorylation by GRKs. In both mGlu2 and mGlu3 receptor-expressing cells, agonist-stimulated ERK1/2 phosphorylation was fully desensitized by overexpression of GRK2, whereas overexpression of GRK4 and GRK5 was inactive (Fig. 4 and Table 1) (data not shown). Cotransfection with GRK2-K220R did not affect agonist-stimulated ERK1/2 phosphorylation mediated by mGlu2 and mGlu3 receptors (Fig. 5 and Table 1), indicating that the kinase activity of GRK2 is required for receptor desensitization.

Basal ERK1/2 phosphorylation was not affected when cells were transfected with GRKs alone (Fig. 5B) or were cotransfected with the receptors (Figs. 4 and 5). These results show that only agonist-stimulated MAPK activation was desensitized by GRK2 and fulfill the paradigm of receptor homolo-

TABLE 1
Effect of GRKs and GRK mutant on pERK1/2 stimulation: quantification of immunoblots
Single experiments are shown in Figs. 4 and 5. Data are expressed as the ratio of the respective control values (controls = 1). Values are means ± S.E.M. from three to five determinations.

Time	mGlu2R		mGlu2R + GRK2		mGlu2R + GRK5		mGlu2R + K220R		mGlu3R		mGlu3R + GRK2		mGlu3R + GRK5		mGlu3R + K220R	
	pERK1/ERK1	pERK2/ERK2	pERK1/ERK1	pERK2/ERK2	pERK1/ERK1	pERK2/ERK2	pERK1/ERK1	pERK2/ERK2	pERK1/ERK1	pERK2/ERK2	pERK1/ERK1	pERK2/ERK2	pERK1/ERK1	pERK2/ERK2	pERK1/ERK1	pERK2/ERK2
0′	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5′	2.5 ± 0.3	4.1 ± 0.5	1.4 ± 0.2*	1.6 ± 0.1*	2.4 ± 0.0	4.1 ± 0.3	1.6 ± 0.4	4.2 ± 0.0	2.7 ± 0.1	3.1 ± 0.4	1.5 ± 0.1*	1.6 ± 0.1*	2.5 ± 0.1	2.8 ± 0.1	2.5 ± 0.1	3.1 ± 0.2
15′	1.5 ± 0.4	1.6 ± 0.3	1.0 ± 0.0	0.8 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.9 ± 0.3	2.1 ± 0.5	1.1 ± 0.1	0.9 ± 0.1	1.5 ± 0.3	1.6 ± 0.4	1.5 ± 0.4	1.7 ± 0.3
30′	0.9 ± 0.1	1.3 ± 0.2	0.1 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.0 ± 0.0	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.1

* $p < 0.05$ (one-way ANOVA plus Fisher's t test) vs control values.

gous desensitization. Concentration-response curves of agonist-dependent cAMP inhibition and ERK1/2 stimulation in cells expressing mGlu2 receptors were similar (Fig. 6).

β-Arrestin1 Differentially Affects Receptor Signaling in Cells Expressing mGlu2 and mGlu3 Receptors. GPCR phosphorylation by GRK allows the binding of β-arrestin to the receptor, thus leading to complete homologous desensitization. In HEK293 cells expressing mGlu2 receptors, cotransfection of β-arrestin1 slightly reduced the agonist-dependent inhibition of cAMP formation. In contrast, β-arrestin1 abolished this effect in cells expressing the mGlu3 receptor (Fig. 7A). The levels of β-arrestin1 expression were similar in mGlu2- and mGlu3-receptor expressing cells (Fig. 7B). The expression of mGlu2 and mGlu3 receptors was not affected by cotransfection of β-arrestin1 (Fig. 1). The effects of GRK2 and β-arrestin1 on mGlu2 receptor desensitization were additive, although the inhibition of signaling induced by GRK2 plus β-arrestin1 was not complete (Fig. 7C). This is in contrast to what we observed for the mGlu3 receptor, which was fully desensitized by overexpression of either GRK2 or β-arrestin1.

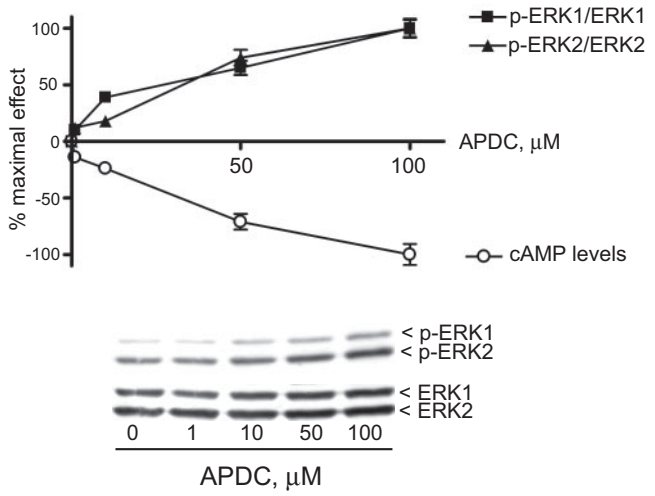

Fig. 6. Concentration-response curves of (2*R*,4*R*)-APDC on cAMP inhibition and MAPK activation. HEK293 cells expressing the mGlu2 receptor were exposed to different concentrations of (2*R*,4*R*)-APDC, and ERK1/2 phosphorylation and FSK-stimulated cAMP inhibition were measured. Data are means ± S.E.M. from three determinations. A representative pERK1/2 immunoblot is also shown.

The analysis of mGlu2 and mGlu3 receptor-dependent ERK1/2 phosphorylation in the presence of β-arrestin1 revealed an unexpected finding. When β-arrestin1 was cotransfected with either the mGlu2 or the mGlu3 receptor, ERK1/2 phosphorylation was increased by 3- to 4-fold, even in the absence of the agonist. This occluded any possible stimulation of MAPK by (2*R*,4*R*)-APDC (Fig. 8). Because β-arrestin has been reported to act as a signaling protein for GPCR-mediated MAPK activation (Iacovelli et al., 2003; Lefkowitz and Whalen, 2004), we hypothesized that β-arrestin could amplify the stimulation of ERK1/2 phosphorylation by constitutively active mGlu2 or mGlu3 receptors. We examined this possibility by transfecting HEK293 cells with the mGlu2 receptor combined or not with β-arrestin1 and measuring ERK1/2 phosphorylation under basal conditions (i.e., without (2*R*,4*R*)-APDC). The levels of ERK1/2 phosphorylation in cells transfected with the mGlu2 receptor were similar to those observed in untransfected or mock-transfected cells. In contrast, the levels of ERK1/2 phosphorylation were increased by overexpression of β-arrestin1 (1.4- to 2.0-fold of mock-transfected cells) and further enhanced by cotransfection of β-arrestin1 and mGlu2 receptors (4- to 6-fold of mock-transfected cells) (Fig. 9A). In cells coexpressing mGlu2 receptors, β-arrestin, and GRK2, basal phosphorylation of ERK1/2 was increased, and the response to (2*R*,4*R*)-APDC was blunted (Fig. 9B).

To test whether mGlu2 and mGlu3 receptor internalization was required for agonist-dependent MAPK activation, we measured (2*R*,4*R*)-APDC-stimulated ERK1/2 phosphorylation in HEK293 cells transfected with the dynamin dominant-negative mutant DynK44A. This mutant is able to prevent the internalization of many GPCRs by competing with endogenous dynamin that cuts the neck of endocytic vesicles. In the presence of DynK44A, the activation of MAPK by (2*R*,4*R*)-APDC was almost completely blunted, suggesting that mGlu2 and mGlu3 receptor internalization is involved in this signaling pathway (Fig. 10).

Different Pattern of Homologous Desensitization in Knockout Mice Lacking mGlu2 or mGlu3 Receptor. We have shown that the cAMP signaling activated by mGlu2 and mGlu3 receptors in HEK293 cells is differentially regulated, with the mGlu3 receptor being desensitized by GRK2 and β-arrestin and the mGlu2 receptor being resistant to homologous desensitization. To extend these observations to an in

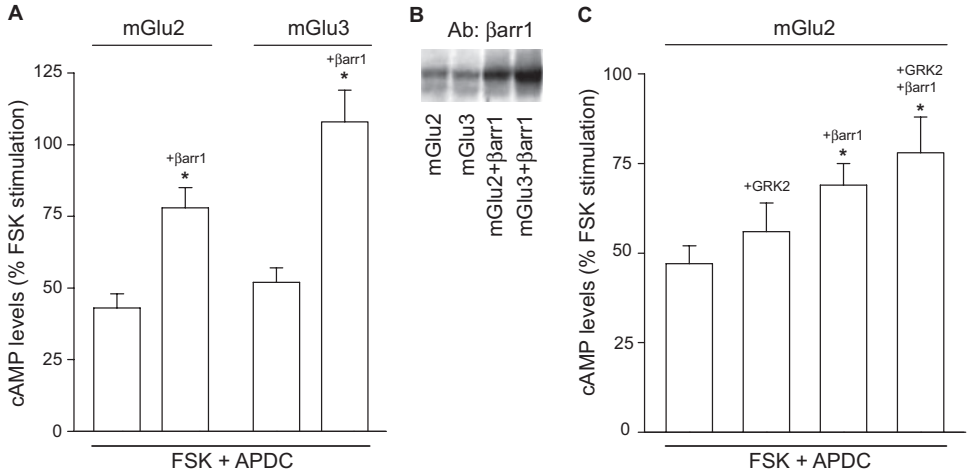

Fig. 7. Regulation of mGlu2/3 receptor-dependent adenylyl cyclase inhibition by β-arrestin1. A, inhibition of FSK-stimulated cAMP formation by (2*R*,4*R*)-APDC was measured in HEK293 cells expressing mGlu2 or mGlu3 receptors alone or coexpressed with β-arrestin1. Values are means ± S.E.M. from three determinations, each in triplicate. *, $p < 0.05$, Student's *t* test. B, the levels of β-arrestin1 coexpressed with the mGlu2 or the mGlu3 receptors are comparable, as assessed by immunoblot. C, inhibition of FSK-stimulated cAMP formation by (2*R*,4*R*)-APDC was measured in HEK293 cells expressing mGlu2 receptors alone or coexpressed with GRK2, β-arrestin1, or GRK2 plus β-arrestin1. Values are means ± S.E.M. from three determinations each in triplicate. *, $p < 0.05$ versus receptor alone (one-way ANOVA plus Fisher's *t* test).

vivo experimental model we used knockout mice lacking either mGlu2 (mGlu2(−/−) mice) or mGlu3 receptors (mGlu3(−/−) mice). Mice were pretreated with saline or with the mixed mGlu2/3 agonist LY379268 for 7 days, and cortical brain slices were prepared 24 h after the last injection. Slices were challenged with FSK alone or FSK plus LY379268 and were used for determinations of cAMP formation. In both wild-type (wt) and mGlu2(−/−) mice, agonist pretreatment

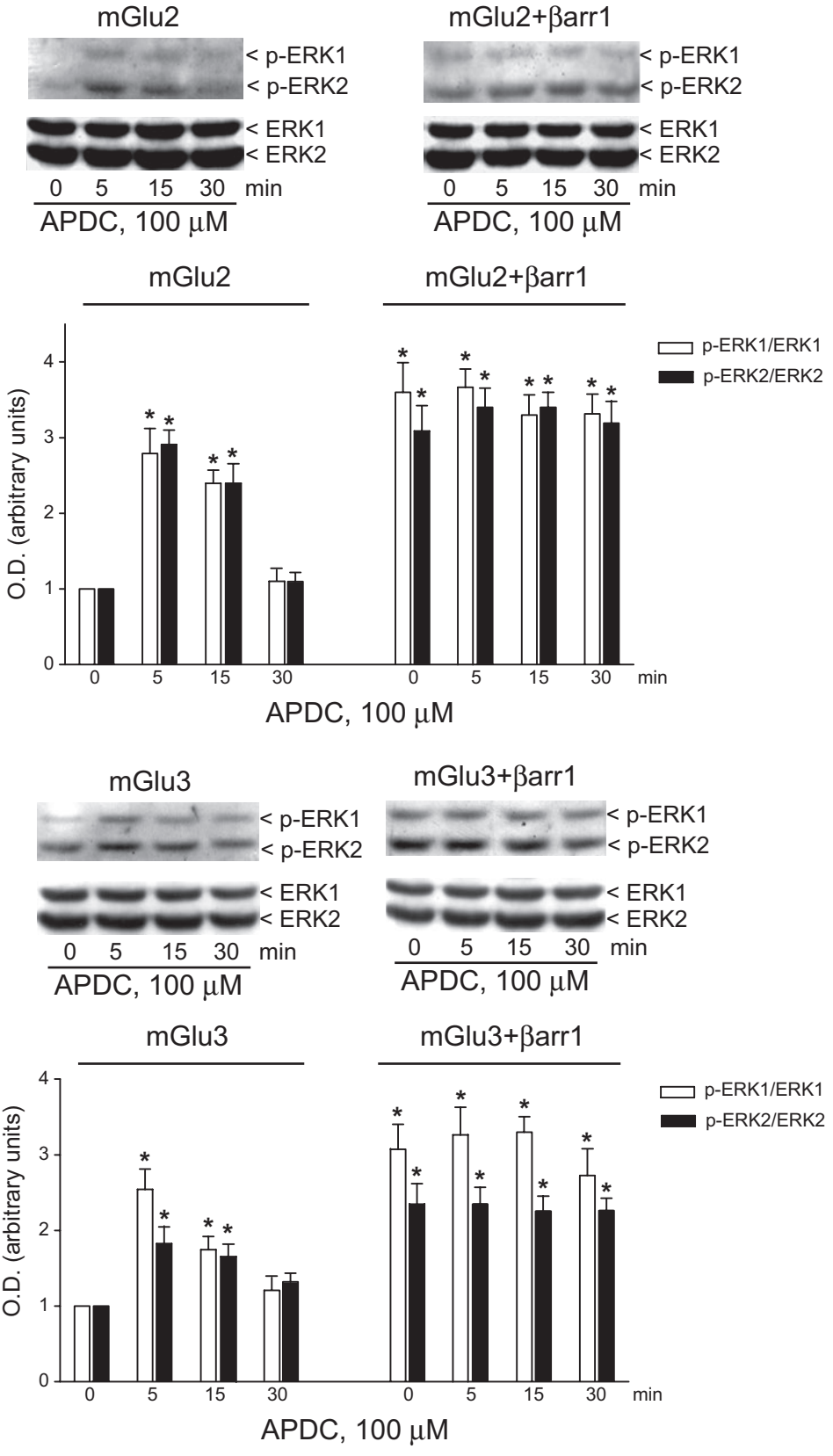

Fig. 8. Regulation of mGlu2/3 receptor-mediated MAPK activation by β-arrestin1. Representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or mGlu3 receptor alone or coexpressed with β-arrestin1 are shown. Cells were exposed to (2*R*,4*R*)-APDC (100 μM) for the indicated times. Densitometric analysis of three to five similar experiments are shown (means ± S.E.M). Data are expressed as the ratio of control values obtained in the absence of both (2*R*,4*R*)-APDC and β-arrestin1 (basal = 1). *, $p < 0.05$ versus control values.

reduced the ability of LY379268 (1 μM) to inhibit FSK-stimulated cAMP formation. In contrast, mGlu3(−/−) mice (in which the agonist could only activate mGlu2 receptors) were completely resistant to homologous desensitization, because the ability of LY379268 to inhibit FSK-stimulated cAMP formation was unaffected by agonist pretreatment (Fig. 11).

Discussion

mGlu2 and mGlu3 share >70% of their amino acid sequence, and their differentiation has been problematic because of the lack of selective ligands and antibodies. This contrasts with the extensive development of mixed orthos-

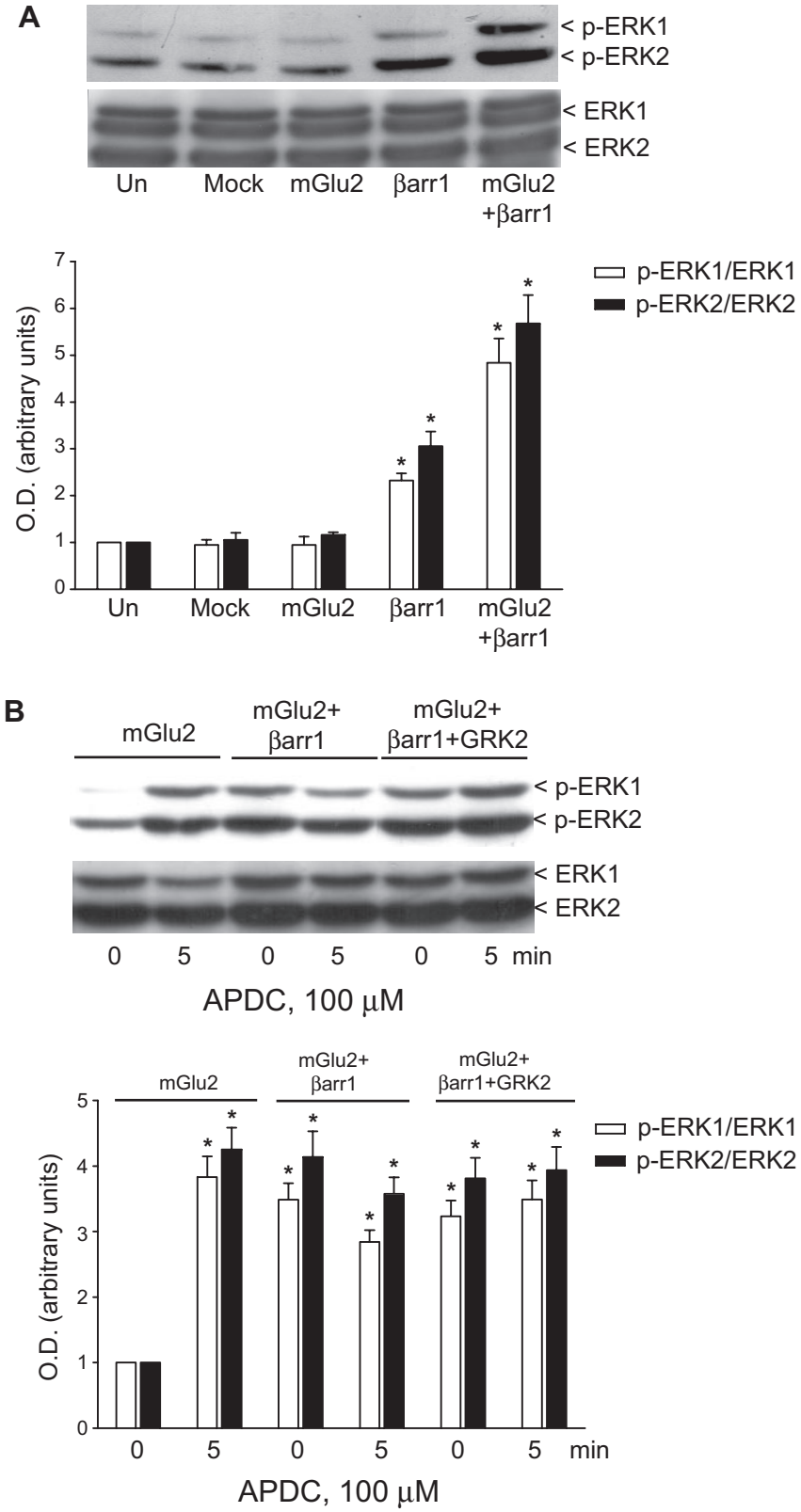

Fig. 9. A, β-arrestin1 increases mGlu2/3 receptor-mediated MAPK activation. ERK1/2 was measured in HEK293 cells were untransfected (Un) or transfected with empty vector (Mock), the mGlu2 receptor (mGlu2), β-arrestin1 (βarr1), or mGlu2 plus β-arrestin1 (mGlu2 + βarr1). All of the conditions presented were in the absence of agonist stimulation. B, effect of β-arrestin1 ± GRK2 transfection on MAPK activation. Cells were exposed to (2*R*,4*R*)-APDC (100 μM) for the indicated times. Densitometric analysis of three similar experiments are shown (means ± S.E.M). Data are expressed as ratio of control values obtained in the absence of both (2*R*,4*R*)-APDC and β-arrestin1 (basal = 1). *, $p < 0.05$ (one-way ANOVA plus Fisher's *t* test) versus control values.

teric mGlu2/3 receptor agonists, some of which are under clinical development (Swanson et al., 2005; Patil et al., 2007). Established clinical targets for mGlu2/3 receptor agonists are schizophrenia and drug-induced psychosis, anxiety, chronic pain, and drug addiction. The relative contribution of mGlu2 and mGlu3 receptors to the experimental treatment of all of

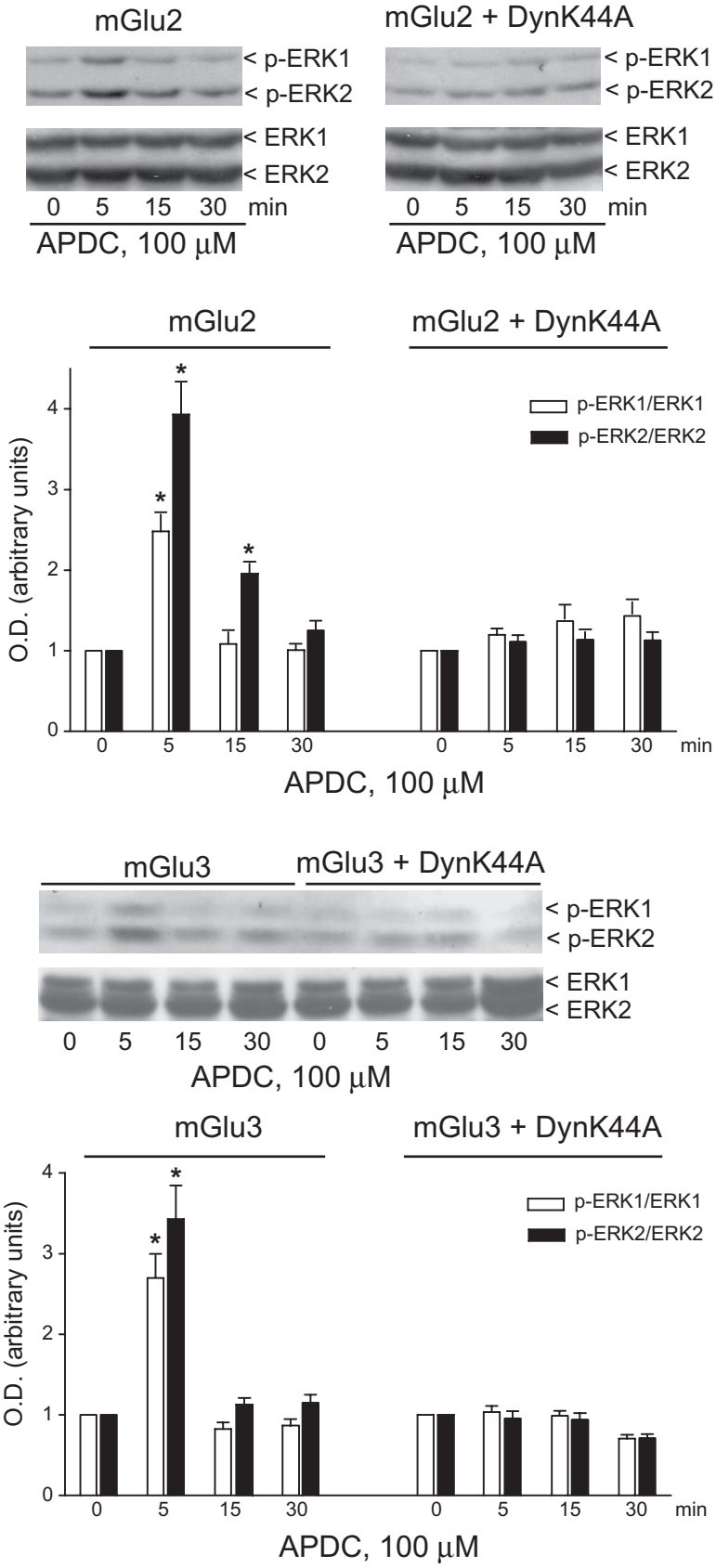

Fig. 10. Effect of dynamin dominant-negative mutant (DynK44A) on receptor-mediated MAPK activation. Representative immunoblots of ERK1/2 from HEK293 cells expressing mGlu2 or mGlu3 receptor alone or coexpressed with DynK44A are shown. Cells were exposed to (2*R*,4*R*)-APDC (100 μM) for the indicated times. Densitometric analysis of three similar experiments are shown (means ± S.E.M). Control values were obtained in the absence of both (2*R*,4*R*)-APDC and DynK44A (basal = 1). *, $p < 0.05$ (one-way ANOVA plus Fisher's *t* test) versus control values.

these disorders can now be dissected using selective mGlu2 receptor enhancers or mice with genetic deletion of either mGlu2 or mGlu3 receptors. For example, mGlu2 receptor enhancers are effective in models predictive of antipsychotic activity (Galici et al., 2005; Johnson et al., 2005), suggesting that it is the mGlu2 receptor that mediates the therapeutic efficacy of mGlu2/3 receptor agonists in schizophrenia. In addition, mGlu2 receptors form functional complexes with 5-HT_{2A} serotonergic receptors (González-Maeso et al., 2008) and mediate the ability of mGlu2/3 receptor agonists to inhibit cellular and behavioral responses to hallucinogenic drugs (Gewirtz and Marek, 2000; Marek et al., 2000, 2001; Gewirtz et al., 2002; Marek et al., 2006; González-Maeso et al., 2008). The individual role of mGlu2 and mGlu3 receptors in the drug treatment of anxiety and panic attacks is more ambiguous because the anxiolytic-like activity of mGlu2/3 receptor agonists is disrupted in both mGlu2 and mGlu3 knockout mice (Linden et al., 2005). Interestingly, the increase in c-Fos expression induced by LY354740 in the central and extended amygdala is mediated by mGlu2 receptors, whereas the suppression of c-Fos expression in the hippocampus is mediated by mGlu3 receptors (Linden et al., 2006). There are only a few studies on the individual role of mGlu2 and mGlu3 receptors in drug addiction and chronic pain. mGlu2 receptor knockout mice show an increase in locomotor sensitization and conditioned place preference in association with repeated cocaine administration, which implicates a critical role for mGlu2 receptors in the reinforcement and addiction of cocaine (Morishima et al., 2005). Acetyl-L-carnitine, a drug currently used for the treatment of neuropathic pain, induces analgesia by selectively up-regulating mGlu2 receptors in the dorsal horns of the spinal cord (Chiechio et al., 2002, 2006). Finally, activation of mGlu3 receptors mediates the protective activity of mGlu2/3 receptor agonists against excitotoxic neuronal death (Corti et al., 2007a), although this particular property has not been translated yet into human studies.

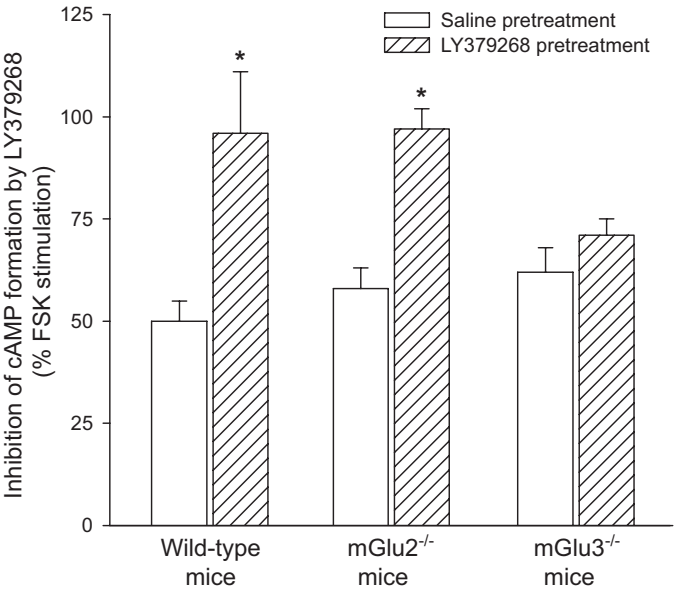

Fig. 11. Knockout mice lacking mGlu3 receptors are resistant to homologous desensitization induced by repeated administrations of the mGlu2/3 agonist, LY379268. Wild-type, mGlu2(−/−), or mGlu3(−/−) mice were pretreated with saline or with LY379268 (1 mg/kg, i.p., for 7 days), and cortical slices were prepared 24 h after the last injection. Slices were challenged with FSK alone or combined with LY379268 (1 μM). Data are expressed as the percentage of FSK-stimulate cAMP formation and were calculated from two independent experiments performed in triplicate. Note that inhibition of FSK-stimulated cAMP formation by LY379268 was no longer visible in cortical slices from wild-type and mGlu2(−/−) mice pretreated with LY379268 but persisted in cortical slices from mGlu3(−/−) mice pretreated with LY379268. This suggests that native mGlu2 receptors are resistant to homologous desensitization. *, $p < 0.05$ (Student's t test) versus the corresponding values obtained in mice pretreated with saline.

Our data show for the first time that mGlu2 and mGlu3 receptors are differently regulated in response to agonist stimulation. Similarly to most GPCRs, heterologously expressed mGlu3 receptors were desensitized by GRK2 and GRK3, two protein kinases that have an established role in mechanisms of homologous desensitization (De Blasi et al., 2001; Kohout and Lefkowitz, 2003; Dhami and Ferguson, 2006). In contrast, mGlu2 receptors showed little or no response to GRK2 or other GRKs when desensitization was assessed by measuring the inhibition of cAMP formation, (i.e., the canonical signaling pathway activated by mGlu2 and mGlu3 receptors). The different behavior of mGlu2 and mGlu3 receptors was supported by experiments in which HEK293 cells were transfected with β-arrestin, which uncouples GRK-phosphorylated GPCRs from the α subunit of G proteins (Luttrell and Lefkowitz, 2002; Kohout and Lefkowitz, 2003; Lefkowitz and Whalen, 2004). Overexpression of β-arrestin desensitized mGlu3 but not mGlu2 receptors. A partial desensitization of mGlu2 receptors could only be observed under extreme conditions (i.e., in cells overexpressing both GRK2 and β-arrestin).

The use of the kinase-dead mutant of GRK2 or the use of the C-terminal domain of GRK2 demonstrated that the kinase activity was necessary for the desensitization of mGlu3 receptors and that other functions of GRK2 (such as the ability to interact with other proteins through the N-terminal RGS domain or the C-terminal PH domain) have no role in this process. Thus, desensitization of the cAMP signaling mediated by the mGlu3 receptors proceeds through receptor phosphorylation by GRK2 or GRK3, which facilitates the association of β-arrestin with ensuing uncoupling of the G_i protein. Obviously, this desensitization process is less efficient with mGlu2 receptors, which are partially refractory to GRK2 or β-arrestin. The evidence that mGlu2 receptor cAMP signaling is resistant to homologous desensitization was strengthened by data obtained in knockout mice. Prolonged to the mGlu2/3 agonist, LY379268 efficiently desensitized the cAMP response in mGlu2 receptor knockout mice, whereas mGlu3 knockout mice, in which the drug could only activate mGlu2 receptors, were fully resistant to homologous desensitization. Thus, the refractoriness to homologous desensitization is a peculiar and intrinsic property of mGlu2 receptors that does not depend on the cellular environment. The complete desensitization of the cAMP response observed in the hippocampus of wt mice was unexpected because wt mice express both mGlu2 and mGlu3 receptors. One possible explanation is that mGlu2 and mGlu3 receptors may form heterodimers, which rely on the presence of mGlu3 receptors for homologous desensitization. It should be highlighted, however, that no evidence for heterodimerization between mGlu2 and mGlu3 receptors has been provided so far.

Interestingly, studies in recombinant cells showed that as opposed to cAMP signaling, MAPK activation by mGlu2 receptors was fully desensitized by GRK2. This signal-depen-

dent regulation was unexpected and has no obvious explanation. Control experiments showed that this was not due to changes in the expression of mGlu2 receptors in cells cotransfected with GRK2. In addition, in cells expressing mGlu2 receptors, (2*R*,4*R*)-APDC inhibited adenylyl cyclase and stimulated ERK1/2 phosphorylation with similar concentration-response curves, and in both cases, responses were mediated by $G_{i/o}$ proteins because they were sensitive to PTX. It is possible that different domains of the mGlu2 receptor trigger the two different signaling pathways and that GRK2 phosphorylation only affects the domains important for ERK stimulation. Alternatively, GRK2 could affect the coupling of the receptor to G_i, but signal propagation through the MAPK pathway is less efficient than the inhibition of adenylyl cyclase and is therefore more sensitive to homologous desensitization. Although the observed signal-dependent regulation of mGlu2 receptors by GRK2 is unusual, an identical pattern of regulation has been described for native G_i-protein-coupled A1 adenosine receptors in FRTL-5 thyroid cell lines (Iacovelli et al., 1999). In these cells, the A1 adenosine receptor-mediated ERK1/2 activation is desensitized by GRK2 (but not by GRK2-K220R), whereas inhibition of adenylyl cyclase response is unaffected.

It should be highlighted that in cells expressing mGlu2 or mGlu3 receptors, cotransfection of β-arrestin1 enhanced MAPK activation even in the absence of agonist. Previous studies on mGlu1 receptors and other GPCRs have shown that β-arrestin can act as a scaffolding protein mediating receptor-dependent MAPK activation (Iacovelli et al., 2003; Lefkowitz and Whalen, 2004). Thus, it is possible that endogenous activation of mGlu2 or mGlu3 receptors (i.e., receptor activation by the glutamate present in the medium or a not-yet-demonstrated constitutive activity of mGlu2 or mGlu3 receptors) becomes sufficient to activate the MAPK pathway when β-arrestin is overexpressed.

Because inhibition of cAMP formation is the canonical signaling pathway activated by mGlu2 and mGlu3 receptors and contributes to the ability of both receptors to inhibit neurotransmitter release (Pin and Duvoisin, 1995), the different sensitivity of the two receptor subtypes to homologous desensitization may have important implications for the therapeutic use of group-II mGlu receptor agonists or enhancers. Given the prominent role of the mGlu2 receptor as a therapeutic target in schizophrenia, we predict a lack of tolerance when mixed mGlu2/3 receptor agonists or selective mGlu2 enhancers are used continually in patients. In contrast, desensitization of mGlu3 receptors may limit the use of receptor agonists/enhancers as protective agents in neurodegenerative disorders. This hypothesis encourages preclinical and clinical studies on the effects of long-term treatment with mGlu2 or mGlu3 receptor ligands.

References

Aramori I, Ferguson SS, Bieniasz PD, Zhang J, Cullen B, and Cullen MG (1997) Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 coreceptor. *EMBO J* **16:**4606–4616.

Benovic JL, Onorato JJ, Arriza JL, Stone WC, Lohse M, Jenkins NA, Gilbert DJ, Copeland NG, Caron MG, and Lefkowitz RJ (1991) Cloning, expression, and chromosomal localization of β-adrenergic receptor kinase 2. A new member of the receptor kinase family. *J Biol Chem* **266:**14939–14946.

Chiechio S, Caricasole A, Barletta E, Storto M, Catania MV, Copani A, Vertechy M, Nicolai R, Calvani M, Melchiorri D, et al. (2002) L-Acetylcarnitine induces analgesia by selectively up-regulating mGlu2 metabotropic glutamate receptors. *Mol Pharmacol* **61:**989–996.

Chiechio S, Copani A, De Petris L, Morales ME, Nicoletti F, and Gereau RW 4th (2006) Transcriptional regulation of metabotropic glutamate receptor 2/3 expression by the NF-kappaB pathway in primary dorsal root ganglia neurons: a possible mechanism for the analgesic effect of L-acetylcarnitine. *Mol Pain* **2:**20.

Corti C, Battaglia G, Molinaro G, Riozzi B, Pittaluga A, Corsi M, Mugnaini M, Nicoletti F, and Bruno V (2007a) The use of knock-out mice unravels distinct roles for mGlu2 and mGlu3 metabotropic glutamate receptors in mechanisms of neurodegeneration/neuroprotection. *J Neurosci* **27:**8297–8308.

Corti C, Crepaldi L, Mion S, Roth AL, Xuereb JH, and Ferraguti F (2007b) Altered dimerization of metabotropic glutamate receptor 3 in schizophrenia. *Biol Psychiatry* **62:**747–755.

De Blasi A, Conn PJ, Pin J, and Nicoletti F (2001) Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol Sci* **22:**114–120.

Dhami GK, Dale LB, Anborgh PH, O'Connor-Halligan KE, Sterne-Marr R, and Ferguson SS (2004) G Protein-coupled receptor kinase 2 regulator of G protein signaling homology domain binds to both metabotropic glutamate receptor 1a and Gαq to attenuate signaling. *J Biol Chem* **279:**16614–16620.

Dhami GK and Ferguson SS (2006) Regulation of metabotropic glutamate receptor signaling, desensitization and endocytosis. *Pharmacol Ther* **111:**260–271.

Galici R, Echemendia NG, Rodriguez AL, and Conn PJ (2005) A selective allosteric potentiator of metabotropic glutamate (mGlu) 2 receptors has effects similar to an orthosteric mGlu2/3 receptor agonist in mouse models predictive of antipsychotic activity. *J Pharmacol Exp Ther* **315:**1181–1187.

Gewirtz JC, Chen AC, Terwilliger R, Duman RC, and Marek GJ (2002) Modulation of DOI-induced increases in cortical BDNF expression by group II mGlu receptors. *Pharmacol Biochem Behav* **73:**317–326.

Gewirtz JC and Marek GJ (2000) Behavioral evidence for interactions between a hallucinogenic drug and group II metabotropic glutamate receptors. *Neuropsychopharmacology* **23:**569–576.

González-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, López-Giménez JF, Zhou M, Okawa Y, Callado LF, Milligan G, et al. (2008) Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* **452:**93–97.

Iacovelli L, Bruno V, Salvatore L, Melchiorri D, Gradini R, Caricasole A, Barletta E, De Blasi A, and Nicoletti F (2002) Native group-III metabotropic glutamate receptors are coupled to the mitogen-activated protein kinase/phosphatidylinositol-3-kinase pathways. *J Neurochem* **82:**216–223.

Iacovelli L, Capobianco L, Iula M, Di Giorgi Gerevini V, Picascia A, Blahos J, Melchiorri D, Nicoletti F, and De Blasi A (2004) Regulation of mGlu4 metabotropic glutamate receptor signaling by type-2 G-protein coupled receptor kinase (GRK2). *Mol Pharmacol* **65:**1103–1110.

Iacovelli L, Franchetti R, Grisolia D, and De Blasi A (1999) Selective regulation of G protein-coupled receptor-mediated signaling by G protein-coupled receptor kinase 2 in FRTL-5 cells: analysis of thyrotropin, α_{1B}-adrenergic, and A_1 adenosine receptor-mediated responses. *Mol Pharmacol* **56:**316–324.

Iacovelli L, Franchetti R, Masini M, and De Blasi A (1996) GRK2 and beta-arrestin 1 as negative regulators of thyrotropin receptor-stimulated response. *Mol Endocrinol* **10:**1138–1146.

Iacovelli L, Salvatore L, Capobianco L, Picascia A, Barletta E, Storto M, Mariggiò S, Sallese M, Porcellini A, Nicoletti F, et al. (2003) 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. *J Biol Chem* **278:**12433–12442.

Johnson MP, Barda D, Britton TC, Emkey R, Hornback WJ, Jagdmann GE, McKinzie DL, Nisenbaum ES, Tizzano JP, and Schoepp DD (2005) Metabotropic glutamate 2 receptor potentiators: receptor modulation, frequency-dependent synaptic activity, and efficacy in preclinical anxiety and psychosis model(s). *Psychopharmacology* (*Berl*) **179:**271–283.

Kohout TA and Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol* **63:**9–18.

Lefkowitz RJ and Whalen EJ (2004) Beta-arrestins: traffic cops of cell signaling. *Curr Opin Cell Biol* **16:**162–168.

Linden AM, Baez M, Bergeron M, and Schoepp DD (2006) Effects of mGlu2 or mGlu3 receptor deletions on mGlu2/3 receptor agonist (LY354740)-induced brain c-Fos expression: specific roles for mGlu2 in the amygdala and subcortical nuclei, and mGlu3 in the hippocampus. *Neuropharmacology* **51:**213–228.

Linden AM, Shannon H, Baez M, Yu JL, Koester A, and Schoepp DD (2005) Anxiolytic-like activity of the mGLU2/3 receptor agonist LY354740 in the elevated plus maze test is disrupted in metabotropic glutamate receptor 2 and 3 knock-out mice. *Psychopharmacology* (*Berl*) **179:**284–291.

Luttrell LM and Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* **115:**455–465.

Marek GJ, Wright RA, Gewirtz JC, and Schoepp DD (2001) A major role for thalamocortical afferents in serotonergic hallucinogen receptor function in the rat neocortex. *Neuroscience* **105:**379–392.

Marek GJ, Wright RA, and Schoepp DD (2006) 5-Hydroxytryptamine2A (5-HT2A) receptor regulation in rat prefrontal cortex: interaction of a phenethylamine hallucinogen and the metabotropic glutamate 2/3 receptor agonist LY354740. *Neurosci Lett* **403:**256–260.

Marek GJ, Wright RA, Schoepp DD, Monn JA, and Aghajanian GK (2000) Physiological antagonism between 5-hydroxytryptamine$_{2A}$ and group II metabotropic glutamate receptors in prefrontal cortex. *J Pharmacol Exp Ther* **292:**76–87.

Morishima Y, Miyakawa T, Furuyashiki T, Tanaka Y, Mizuma H, and Nakanishi S (2005) Enhanced cocaine responsiveness and impaired motor coordination in metabotropic glutamate receptor subtype 2 knockout mice. *Proc Natl Acad Sci U S A* **102:**4170–4175.

Mundell SJ, Pula G, Carswell K, Roberts PJ, and Kelly E (2003) Agonist-induced internalization of metabotropic glutamate receptor 1A: structural determinants for protein kinase C- and G protein-coupled receptor kinase-mediated internalization. *J Neurochem* **84:**294–304.

Nicoletti F, Meek JL, Iadarola MJ, Chuang DM, Roth BL, and Costa E (1986)

Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. *J Neurochem* **46:**40–46.
Patil ST, Zhang L, Martenyi F, Lowe SL, Jackson KA, Andreev BV, Avedisova AS, Bardenstein LM, Gurovich IY, Morozova MA, et al. (2007) Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nat Med* **13:**1102–1117.
Pin JP and Duvoisin R (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* **34:**1–26.
Sallese M, Salvatore L, D'Urbano E, Sala G, Storto M, Launey T, Nicoletti F, Knöpfel T, and De Blasi A (2000) The G-protein-coupled receptor kinase GRK4 mediates homologous desensitization of metabotropic glutamate receptor 1. *FASEB J* **14:**2569–2580.
Schoepp DD, Jane DE, and Monn JA (1999) Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* **38:**1431–1476.
Sorensen SD and Conn PJ (2003) G protein-coupled receptor kinases regulate metabotropic glutamate receptor 5 function and expression. *Neuropharmacology* **44:**699–706.
Swanson CJ, Bures M, Johnson MP, Linden AM, Monn JA, and Schoepp DD (2005) Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. *Nat Rev Drug Discov* **4:**131–144.

Address correspondence to: Dr. Ferdinando Nicoletti, Department of Physiology and Pharmacology, University of Rome Sapienza, Piazzale Aldo Moro, 5, 00185 Rome, Italy. E-mail: ferdinandonicoletti@hotmail.com