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Ligand Bias at Metabotropic Glutamate 1a Receptors: Molecular Determinants That Distinguish β-Arrestin-Mediated from G Protein-Mediated Signaling

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ABSTRACT

The metabotropic glutamate 1a (mGlu1a) receptor is a G protein-coupled receptor linked with phosphoinositide (PI) hydrolysis and with β-arrestin-1-mediated sustained extracellular signal-regulated kinase (ERK) phosphorylation and cytoprotective signaling. Previously, we reported the existence of ligand bias at this receptor, inasmuch as glutamate induced both effects, whereas quisqualate induced only PI hydrolysis. In the current study, we showed that mGlu1 receptor agonists such as glutamate, aspartate, and L-cysteate were unbiased and activated both signaling pathways, whereas quisqualate and (*S*)-3,5-dihydroxyphenylglycine stimulated only PI hydrolysis. Competitive antagonists inhibited only PI hydrolysis and not the β-arrestin-dependent pathway, whereas a noncompetitive mGlu1 receptor antagonist blocked both pathways. Mutational analysis of the ligand binding domain of the mGlu1a receptor revealed that Thr188 residues were essential for PI hydrolysis but not for protective signaling, whereas Arg323 and Lys409 residues were required for β-arrestin-1-mediated sustained ERK phosphorylation and cytoprotective signaling but not for PI hydrolysis. Therefore, the mechanism of ligand bias appears to involve different modes of agonist interactions with the receptor ligand binding domain. Although some mGlu1a receptor agonists are biased toward PI hydrolysis, we identified two endogenous compounds, glutaric acid and succinic acid, as new mGlu1 receptor agonists that are fully biased toward β-arrestin-mediated protective signaling. Pharmacological studies indicated that, in producing the two effects, glutamate interacted in two distinct ways with mGlu1 receptors, inasmuch as competitive mGlu1 receptor antagonists that blocked PI hydrolysis did not inhibit cytoprotective signaling. Quisqualate, which is biased toward PI hydrolysis, failed to inhibit glutamate-induced protection, and glutaric acid, which is biased toward protection, did not interfere with glutamate-induced PI hydrolysis. Taken together, these data indicate that ligand bias at mGlu1 receptors is attributable to different modes of receptor-glutamate interactions, which are differentially coupled to PI hydrolysis and β-arrestin-mediated cytoprotective signaling, and they reveal the existence of new endogenous agonists acting at mGlu1 receptors.

Introduction

Metabotropic glutamate (mGlu) receptors are G protein-coupled receptors (GPCRs) that have been categorized into three groups on the basis of sequence homology and pharmacological characteristics (Pin and Duvoisin, 1995; Conn and Pin, 1997). Group I mGlu receptors (mGlu1 and mGlu5

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ABBREVIATIONS: mGlu, metabotropic glutamate; DHPG, (*S*)-3,5-dihydroxyphenylglycine; HRP, horseradish peroxidase; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; LY367385, (+)-2-methyl-4-carboxyphenyl-glycine; shRNA, short hairpin RNA; ERK, extracellular signal-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, phosphoinositide; PLC, phospholipase C; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; 3-MATIDA, α-amino-5-carboxy-3-methyl-2-thiopheneacetic acid; YM298198, 6-amino-*N*-cyclohexyl-*N*,3-dimethylthiazolo[3,2-*a*]benzimidazole-2-carboxamide hydrochloride; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline/0.1% Triton X-100; JNJ16259685, (3,4-dihydro-2*H*-pyrano[2,3-*b*]quinolin-7-yl)-(*cis*-4-methoxycyclohexyl)-methanone.

receptors) stimulate phospholipase C (PLC) through coupling to $G_{q/11}$ (Aramori and Nakanishi, 1992), which results in the hydrolysis of membrane phosphoinositides (PIs), followed by increased Ca^{2+} release from intracellular stores. Stimulation of group I mGlu receptors also was shown to cause phosphorylation of extracellular signal-regulated kinase (ERK), both in a transient, PLC-dependent manner (Choe and Wang, 2001; Karim et al., 2001) and in a sustained, G protein-independent, β-arrestin-dependent manner (Emery et al., 2010).

In the presence of glutamate, mGlu1 receptors induce signaling that facilitates cellular growth and development. When stimulated with glutamate, mGlu1 receptors were shown to stimulate axon elongation (Kreibich et al., 2004) and outgrowth of dendritic spines in the developing hippocampus (Vanderklish and Edelman, 2002). We reported that mGlu1 receptors produce dual neuroprotective and neurotoxic signaling in cerebellar and cortical neurons (Pshenichkin et al., 2008). Therefore, the mGlu1 receptor exhibits the properties of a dependence receptor (Pshenichkin et al., 2008), inducing apoptosis in the absence of glutamate and promoting neuronal survival in its presence. In CHO cells transfected with mGlu1a receptors, stimulation of the receptors with glutamate protected cells from serum withdrawal-induced apoptosis (Emery et al., 2010). In this model, protective signaling through mGlu1a receptors was accomplished through β-arrestin-1-dependent sustained phosphorylation of ERK (Emery et al., 2010).

Our previous study on signal transduction of mGlu1a receptors indicated that classic, G protein-mediated, signal transduction and transient ERK phosphorylation differed in their pharmacological profiles from sustained ERK phosphorylation and protective signaling; classic mGlu1a receptor-mediated PI hydrolysis and transient ERK phosphorylation were induced by both quisqualate and glutamate, with quisqualate being the most potent agonist (Emery et al., 2010). These data are consistent with several previous reports on the pharmacological features of mGlu1 receptor-mediated PI hydrolysis (Aramori and Nakanishi, 1992) and ERK phosphorylation (Thandi et al., 2002). In contrast, sustained ERK phosphorylation and protection followed a unique pharmacological profile. Only glutamate produced these effects, which demonstrates an apparent ligand bias at mGlu1a receptors. Ligand bias is a new pharmacological concept that has been shown to exist for other GPCRs, with various agonists preferentially activating receptor conformations that are selectively conducive for different signal transduction pathways. The initial example of ligand bias involved M1 muscarinic receptors, which activate both cAMP production and PLC in response to their endogenous agonist acetylcholine; ligands that selectively activated PLC while blocking cAMP formation through these receptors were identified (Fisher et al., 1993). Ligand bias has since been reported for numerous GPCRs (for review, see Rajagopal et al., 2010) and, in addition to selective activation of different G protein pathways, ligands that preferentially activate β-arrestin-mediated signaling, which is independent of G protein signaling, have been identified (Wei et al., 2003).

Our previous results demonstrated that sustained ERK phosphorylation and protective signaling are β-arrestin-1-dependent, which may suggest that agonists that do not cause these effects, such as quisqualate, are biased toward PI hydrolysis through mGlu1 receptors, whereas agonists such as glutamate are less biased, on the basis of our observations that glutamate activated both signal transduction pathways. Although the structural properties responsible for the observed ligand bias are not known, X-ray crystallographic studies of the ligand binding domain of the mGlu1 receptor (Kunishima et al., 2000) suggested that glutamate, the apparently unbiased ligand, interacts with 14 amino acid residues, whereas quisqualate, the apparently biased ligand, interacts with only nine residues (Sato et al., 2003). The interaction of glutamate with additional residues in the binding domain may represent a mechanism through which glutamate, but not quisqualate, can activate the two signal transduction pathways. This raises the possibility that it may be possible to identify compounds that activate mGlu1 receptors but are biased toward sustained ERK phosphorylation and cell survival. The aim of this study was to identify the mechanism of ligand bias through which glutamate but not quisqualate causes protective signaling in cells expressing mGlu1a receptors. We identified glutaric and succinic acids as compounds that are ligands at mGlu1a receptors and are completely biased toward sustained ERK phosphorylation and protective signaling while being inactive in stimulating PI hydrolysis. These ligands may serve as lead compounds for drug discovery.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium and fetal bovine serum for cell cultures were purchased from Invitrogen (Carlsbad, CA). The receptor agonists glutamate, aspartate, L-cysteic acid, quisqualate, and (*S*)-3,5-dihydroxyphenylglycine (DHPG) and antagonists 6-amino-*N*-cyclohexyl-*N*,3-dimethylthiazolo[3,2-*a*]benzimidazole-2-carboxamide hydrochloride (YM298198), (+)-2-methyl-4-carboxyphenyl-glycine (LY367385), and α-amino-5-carboxy-3-methyl-2-thiopheneacetic acid (3-MATIDA) and the PLC inhibitor 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) were obtained from Tocris Bioscience (Ellisville, MO). Glutaric acid, succinic acid, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Cultures. CHO-K1 cells were stably transfected with mGlu1a receptor cDNA in pcDNA-3.1 vector (Invitrogen) by using Lipofectamine 2000 transfection reagent (Invitrogen). Individual cell lines were isolated and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 5% L-proline, and 0.8 mg/ml G-418 for selection (Invitrogen).

Treatment of Cells with β-Arrestin-1 shRNA. SureSilencing shRNA plasmids against β-arrestin-1 (insert sequence, ATGGAGGAAGCTGATGATACT) and random control sequences were subcloned in the pGeneClip Hygromycin vector (SABiosciences, Frederick, MD). CHO cells stably expressing mGlu1a receptor were transfected with plasmids containing shRNA and were selected with 0.8 mg/ml hygromycin B. Knockdown of β-arrestin-1 was confirmed with Western blotting.

Site-Directed Mutagenesis. Point mutations in mGlu1a receptor cDNA were introduced by using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Briefly, 20 ng of plasmid containing mGlu1a receptor cDNA were mixed with 125 ng of two mutagenic primers, dNTPs (50 μM), and 2.5 units of *Pfu* DNA polymerase, in a final volume of 50 μl. Primer sequenceswere as follows: T188A: forward, 5′-CAGATCGCCTATTCTGCCGCTAGCA; reverse, 5′-GTCACTCAGGTCTATGCTAGCGGGA; R323V:forward,5′-GAAGTGATGGATGGGCAGACGTCGACGAAGCATCGAAGGC; reverse, 5′-GCCTTCGATTTCGTCGACGTCTGCCCATCCATCACTTC; K409A: forward, 5′-GAAAACTATGTCCAGGACAGCGCCATGGGATTTGTCATCAATGCC; reverse, 5′-GGCATT-

GATGACAAATCCCATCGGGCTGTCCTGGACATAGTTTTC. Samples were denatured at 95°C for 30 s and then subjected to 20 cycles of denaturation (95°C, 30 s), annealing (55°C, 1 min), and elongation (72°C, 30 min), with a final 10-min extension step. Ten units of DpnI were added to digest the DNA template. After incubation at 37°C for 1 h, samples were used for transformation of *Escherichia coli* XL1-Blue supercompetent cells. Positive clones were identified through restriction analysis, and the authenticity of each mutation was confirmed through DNA sequencing.

Western Blotting. Cells grown and treated in 35-mm dishes were collected in 25 mM Tris-HCl buffer, pH 7.5, containing Halt protease and phosphatase inhibitors with 1 mM EDTA (Thermo Fisher Scientific, Waltham, MA). Proteins were solubilized in Laemmli buffer containing 50 mM dithiothreitol, and equal amounts of sample proteins were resolved on 8% polyacrylamide gels (Invitrogen). Proteins were transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA) and were probed with antibodies against the mGlu1a receptor (BD Biosciences, San Jose, CA), β-tubulin (Sigma-Aldrich), and β-arrestin-1 (Abcam Inc., Cambridge, MA). Proteins were observed through incubation with goat anti-rabbit secondary antibodies coupled to horseradish peroxidase (HRP) (Thermo Fisher Scientific), followed by exposure to chemiluminescent HRP substrate SuperSignal West Femto (Thermo Fisher Scientific).

Measurement of ERK Phosphorylation. Phosphorylated ERK was measured by using a cell-based, enzyme-linked immunosorbent assay, according to a protocol described previously (Versteeg et al., 2000). Cells were grown and treated with agonists in 96-well plates. After incubation with agonist, cells were fixed in 4% formaldehyde/PBS for 20 min at room temperature. After three washes with PBS/0.1% Triton X-100 (PBST) for membrane permeablization, endogenous peroxidase activity was quenched with a 20-min incubation in PBS containing 0.5% H_2O_2 and 0.2% NaN_3. After three more washes with PBST, cells were blocked with 2% bovine serum albumin for 1 h and incubated overnight with primary antibody against phospho-p44/42 (ERK1/2) (Thr202/Tyr204) (Cell Signaling Technology, Danvers, MA). Cells were then washed for 5 min with PBST three times and with PBS twice. A HRP-coupled, goat anti-rabbit secondary antibody (Thermo Fisher Scientific) was incubated for 1 h at room temperature, and cells were again washed five times. Cells were exposed to the colorimetric HRP substrate 1-Step Ultra TMB (Thermo Fisher Scientific). After 10 min of development, the reaction was stopped with 4 M H_2SO_4, and absorbance was measured at 450 nm.

Assessment of Cell Viability. The viability of cells cultured on 96-well plates was measured through incubation for 1 h at 37°C with 0.2 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), which was purchased from Invitrogen. Formation of the formazan product, which is proportional to the number of viable cells, was measured colorimetrically at 570 nm after extraction with 70 μl of dimethylsulfoxide (Mosmann, 1983).

Measurement of PI Hydrolysis. Cells cultured in 96-well plates were incubated overnight with *myo*-[^{3}H]inositol (0.625 μCi per well; PerkinElmer Life and Analytical Sciences, Waltham, MA), to label cell membrane phosphoinositides. After two washes with 0.1 ml of Locke's buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose, 20 mM Hepes, pH 7.4), incubations with receptor ligands were performed for 45 min at 37°C in Locke's buffer containing 20 mM LiCl, to block inositol phosphate degradation. The reaction was terminated through aspiration of the medium, and inositol phosphates were extracted in 60 μl of 10 mM formic acid for 30 min. Samples (40 μl) were transferred to opaque-welled plates and were incubated at room temperature for 1 h with 60 μl of polylysine-coated yttrium scintillation proximity assay beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), with vigorous shaking. After an additional 8 h of incubation with scintillation proximity assay beads, inositol phosphates were detected with scintillation counting.

Glutamate Assay. Glutamate concentrations in collected medium samples were measured by using an Amplex Red glutamic acid assay kit (Invitrogen), according to the manufacturer's instructions. Briefly, 50-μl medium samples were combined with 50 μl of a solution containing Amplex Red (10 μl/ml), horseradish peroxidase (0.25 U/ml), L-glutamate oxidase (0.08 U/ml), L-glutamate-pyruvate transaminase (0.5 U/ml), and L-alanine (200 μM) in 96-well plates, which were incubated in the dark. After 30 min, fluorescence was measured with a fluorescence microplate reader, with excitation at 540 nm and emission detection at 590 nm. Values were corrected for background fluorescence by subtracting values derived from control samples without glutamate. Data were fit to standard curves obtained with assays of serial dilutions of glutamate.

Molecular Modeling. To obtain the model of the binding site, the crystal structure of the dimerized extracellular region of the mGlu1 receptor bound with glutamate (Kunishima et al., 2000) was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb/) and was imported into Maestro 9.2 (Schrödinger, New York, NY) within the Schrödinger suite of programs, in pdb format. After removal of one of the monomers from the crystallized dimer, the receptor was prepared by using the protein preparation wizard (Epik 2.2; Schrödinger) within Maestro. The prepared protein was exported to the PyMOL 1.3 molecular graphics system (Schrödinger) in pdb format. As shown, the model depicts only the side chains of amino acid residues that were previously implicated in glutamate binding (Sato et al., 2003) and are most relevant to the understanding of our mutation strategy. For clarity, all other residues were shown as a cartoon representation of the protein backbone.

Data Analyses. Analysis of dose-response data and calculation of EC_{50} values, when appropriate, was performed by fitting data points to a four-parameter logistic equation through nonlinear regression with SigmaPlot 11.0 software (Systat Software, San Jose, CA).

Results

Although our previous studies clearly showed that glutamate, acting at mGlu1a receptors, activated two distinct signal transduction cascades (Emery et al., 2010), they also suggested that some mGlu1 receptor agonists, such as quisqualate, might enhance only G protein-mediated and not β-arrestin-dependent signaling. Such a phenomenon, which is known as ligand bias or biased agonism, has been described for several G protein-coupled receptors and refers to the ability of some agonists to activate only selected signal transduction mechanisms, whereas other agonists may activate all signaling associated with the receptor (Violin and Lefkowitz, 2007). Using CHO cells expressing mGlu1a receptors, we tested the ability of various known mGlu1 receptor agonists to activate the two signaling cascades. The activation of G protein-mediated signaling was assessed with measurements of PI hydrolysis and transient (5-min) ERK phosphorylation. The activation of β-arrestin-mediated signaling was evaluated with measurements of sustained (24-h) ERK phosphorylation and protection of cells from trophic deprivation. As shown in Fig. 1A, all tested agonists stimulated PI hydrolysis, with quisqualate being the most potent agonist (EC_{50}, ~1 μM), followed by glutamate and DHPG (EC_{50}, ~10 μM) and by aspartate and L-cysteic acid (EC_{50}, ~100 μM). In addition to the stimulation of PI hydrolysis, these agonists increased transient ERK phosphorylation measured after 5 min of incubation with the agonist (Fig. 1B), with a pharmacological profile similar to that observed for PI hydrolysis. As expected, the mGlu1 receptor-selective, noncompetitive antagonist YM298198 (Kohara et al., 2005) completely abolished agonist-induced PI hydrolysis (Fig. 1A) and transient

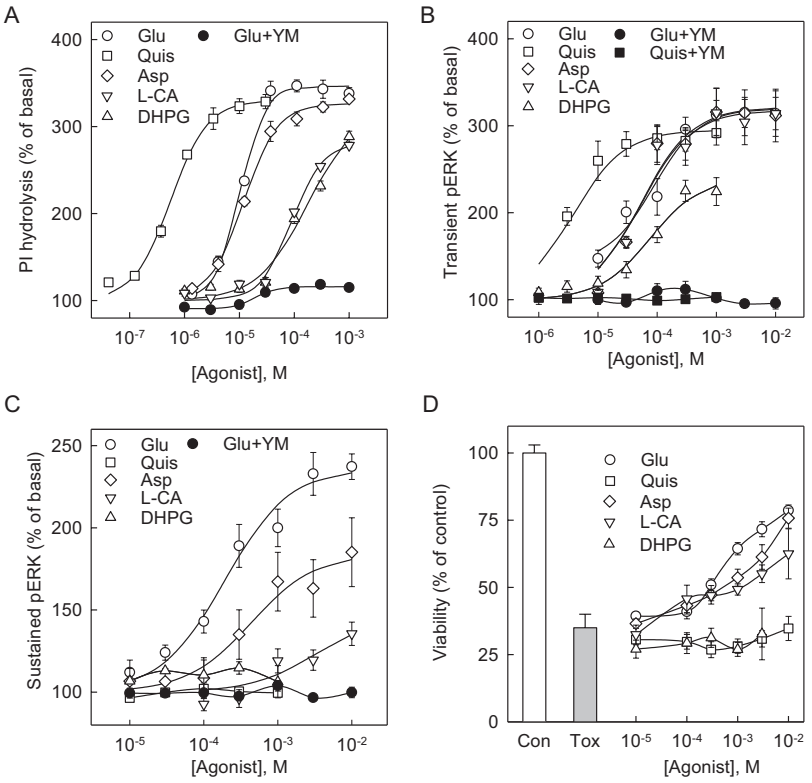

Fig. 1. Agonist profiles of mGlu1a receptor-mediated signaling in transfected CHO cells. A, stimulation of PI hydrolysis, expressed as percentages of basal values. B and C, ERK phosphorylation after 5 min (B) and 24 h (C) of agonist treatment, expressed as percentages of unstimulated control values. D, protection from toxicity (Tox) attributable to serum deprivation for 3 days, compared with the viability of untreated control cells (Con). Cells were treated with the indicated concentrations of glutamate, quisqualate (Quis), aspartate (Asp), L-cysteic acid (L-CA), and DHPG and with the antagonist YM298198 (YM) at 10 μM. All values are means from at least three experiments performed in triplicate, with error bars representing S.E.M.

ERK phosphorylation (Fig. 1B). These results are consistent with our previous report indicating that PI hydrolysis and transient ERK phosphorylation are mediated by the same G protein-dependent mechanism (Emery et al., 2010).

Our previous studies indicated that, in addition to transient ERK phosphorylation, mGlu1a receptors caused long-lasting, β-arrestin-1-dependent, ERK phosphorylation, which was required for mGlu1a receptor-mediated protection from apoptosis (Emery et al., 2010). Sustained ERK phosphorylation, measured after 24 h, had a different pharmacological profile than did transient ERK phosphorylation, being induced only by glutamate, aspartate, and L-cysteic acid and not by quisqualate and DHPG (Fig. 1C). Glutamate-induced sustained ERK phosphorylation was completely blocked by 10 μM YM298198 (Fig. 1C). A similar pharmacological profile for agonist action was observed for the mGlu1 receptor-mediated cytoprotective effect. In those experiments, cells were transferred to serum-free culture medium to induce apoptosis (Zhong et al., 1993), and their viability was assessed with MTT assays. Under those conditions, cells exhibited approximately 30 to 40% viability after 3 days, relative to control cells grown in serum-containing medium (Fig. 1D). The addition of glutamate, aspartate, or L-cysteic acid to the serum-free culture medium produced substantial increases in viability but, in contrast to the pharmacological profile for PI hydrolysis, quisqualate and DHPG were not effective at concentrations up to 10 mM (Fig. 1D). The observed agonist potencies were similar to those observed for mGlu1 receptor-mediated sustained ERK phosphorylation (Fig. 1, C and D). These results emphasize the unique signal transduction and cytoprotective properties of mGlu1a receptors and add to our previous observations that CHO cells transfected with mGlu5 receptors or with the empty vector were not protected by glutamate and glutamate did not induce sustained ERK phosphorylation in those cells (Emery et al., 2010).

Because viability assays involved long incubation times, it was necessary to address the issue of the stability of the drugs used over those extended periods, to confirm that the lack of activity was not attributable to drug depletion. Control experiments were performed to test drug stability under cell culture conditions for the 3-day incubations used in viability assays. Agonists were culture-conditioned with mGlu1a receptor-expressing CHO cells for 3 days, and then the collected medium samples were used to stimulate PI hydrolysis in naive cells. As reported previously, quisqualate and glutamate were equally potent in stimulating PI hydrolysis before and after culture conditioning (Emery et al., 2010). A similar bioassay of DHPG showed no significant breakdown after 3 days, with EC_{50} values being 30 μM for freshly prepared DHPG and 32 μM for preconditioned DHPG. Measurements of glutamate in medium samples indicated that glutamate concentrations after 3 days of incubation with cells did not vary significantly from the concentrations of applied glutamate. Three days after addition of 30 μM, 300 μM, or 3 mM glutamate, measured glutamate concentrations in the medium were 28.7 $\pm$ 3.3 μM, 297 $\pm$ 12 μM, and 3.04 $\pm$ 0.09 mM, respectively. These data indicated that the agonists tested were not subject to degradation or uptake during the extended periods of incubation used and the lack of activity of agonists such as quisqualate and DHPG was not attributable to agonist depletion. Taken together, these results confirm that PI hydrolysis, ERK phosphorylation, and the protective signal transduction pathway are activated by the same receptors, but different mGlu1 receptor agonists appear to activate either one or both signal transduction pathways. Glutamate, aspartate, and L-cysteic acid activate PI hydrolysis, transient and sustained ERK phosphorylation, and protection, whereas quisqualate and DHPG activate only PI hydrolysis and transient ERK phosphorylation.

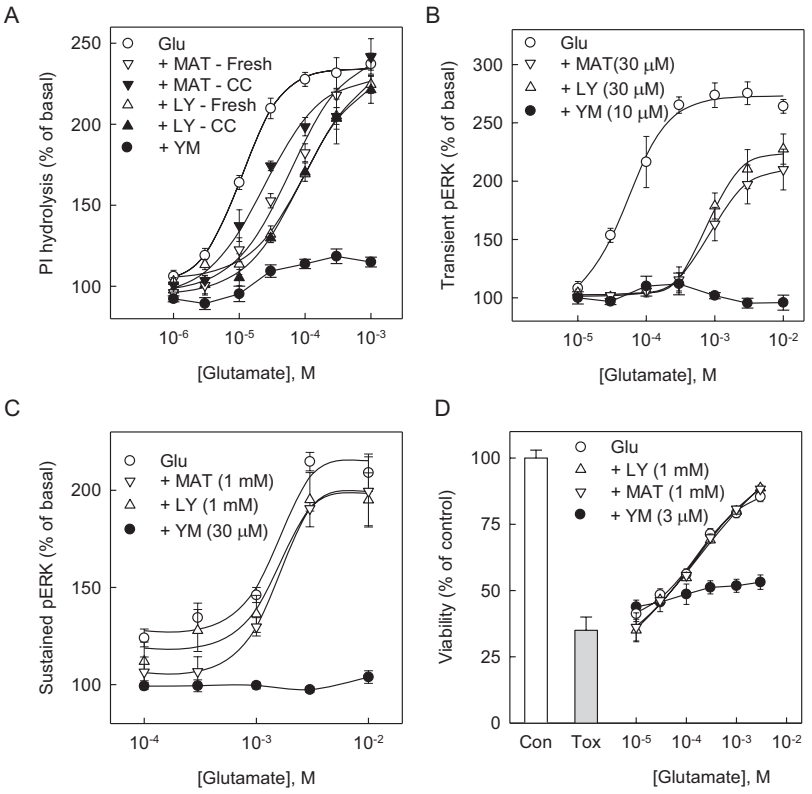

Fig. 2. Antagonism of mGlu1a receptor-mediated signaling in transfected CHO cells. A, inhibition of glutamate-induced PI hydrolysis by the competitive antagonists LY367385 (LY) at 10 μM and 3-MATIDA (MAT) at 30 μM, either freshly prepared (Fresh) or culture-conditioned (CC), and by the noncompetitive antagonist YM298198 (YM) at 10 μM. B and C, inhibition of ERK phosphorylation by antagonists. The competitive antagonists LY367385 and 3-MATIDA inhibited transient (5-min) ERK phosphorylation (B) but not sustained (24-h) ERK phosphorylation (C). The noncompetitive antagonist YM298198 (YM) blocked both transient (B) and sustained (C) ERK phosphorylation. D, protection from toxicity (Tox) attributable to serum deprivation for 3 days. The glutamate-induced protection of CHO cells expressing mGlu1a receptors from toxicity attributable to serum deprivation for 3 days, expressed as percentages of untreated control cell values (Con), was not blocked by the competitive antagonists LY367385 (1 mM) and 3-MATIDA (1 mM) but was abolished by the noncompetitive antagonist YM298198 (3 μM). All values represent means from at least three experiments performed in triplicate, with error bars representing S.E.M.

To investigate further the mGlu1a receptor-mediated pharmacological characteristics and signal transduction, all effects were measured in the presence of mGlu1 receptor-selective antagonists. To this end, LY367385 and 3-MATIDA, selective competitive antagonists of mGlu1 receptors (Clark et al., 1997; Moroni et al., 2002), were used. As shown in Fig. 2A, LY367385 and 3-MATIDA both competitively inhibited glutamate-induced PI hydrolysis, which caused the EC_{50} for glutamate to shift from 11 μM to 108 μM in the presence of LY367385 (10 μM) and to 60 μM in the presence of 3-MATIDA (30 μM). Both competitive antagonists also inhibited transient ERK phosphorylation (Fig. 2B), which shifted the EC_{50} for glutamate from 50 μM to 810 μM in the presence of LY367385 (30 μM) and to 880 μM in the presence of 3-MATIDA (30 μM). As expected, both PI hydrolysis and transient ERK phosphorylation were completely abolished by the noncompetitive antagonist YM298198 (Fig. 2, A and B). In contrast to transient ERK phosphorylation, sustained ERK phosphorylation, which is mediated by the β-arrestin-dependent pathway, was not inhibited by the competitive mGlu1 receptor antagonists. Even high concentrations (1 mM) of either LY367385 or 3-MATIDA failed to inhibit glutamate-induced sustained (24-h) ERK phosphorylation (Fig. 2C). However, glutamate-induced sustained ERK phosphorylation was completely inhibited by 30 μM YM298198, a mGlu1 receptor-selective noncompetitive antagonist (Kohara et al., 2005). As in the case of glutamate-induced sustained ERK phosphorylation, the addition of either LY367385 (1 mM) or 3-MATIDA (1 mM) failed to inhibit glutamate-induced protection (Fig. 2D). Instead, the protective signaling was noncompetitively inhibited by YM298198 (3 μM), which confirms that this effect is mediated by mGlu1 receptors. To confirm that the lack of effect of competitive antagonists was not attributable to their degradation during the long incubation times, both compounds were bioassayed after 3 days of culture conditioning. On the basis of the dose ratios for glutamate dose-response curves shifted by fresh and culture-conditioned antagonists (Fig. 2A), we calculated that, after 3 days of incubation, the initial 1 mM concentrations were reduced to 770 μM for LY367285 and to 270 μM for 3-MATIDA. Therefore, although they were partially degraded, the concentrations of these competitive antagonists were still much greater than those needed for effective inhibition of PI hydrolysis and transient ERK phosphorylation.

Because G protein-mediated mGlu1 receptor signaling was blocked by competitive receptor antagonists but β-arrestin-mediated signaling was not, we hypothesized that the observed ligand bias might reflect different abilities of agonists to interact with the receptor ligand binding domain and different portions of this domain might be involved in stimulating PI hydrolysis and in inducing protective signaling. In such a case, noncompetitive antagonists would still be effective in blocking all signal transduction. The X-ray crystallographic structure of the mGlu1 receptor binding site with bound glutamate was resolved previously (Kunishima et al., 2000). Those studies, as well as mutational analyses of the mGlu1 receptor binding site (Sato et al., 2003), showed that several amino acid residues, including Thr188, Asp208, Tyr236, and Asp318, interacted with the amino group of glutamate (Fig. 3) and those interactions were necessary for receptor activation, as measured with PI hydrolysis and quisqualate binding (Sato et al., 2003). Other residues in the binding site, including Arg323 and Lys409, were postulated to bind the ω-carboxyl group of glutamate (Fig. 3), but mutation of those residues failed to attenuate glutamate-induced PI hydrolysis or quisqualate binding (Sato et al., 2003). To determine whether different glutamate-binding residues might be involved in the activation of different signal trans-

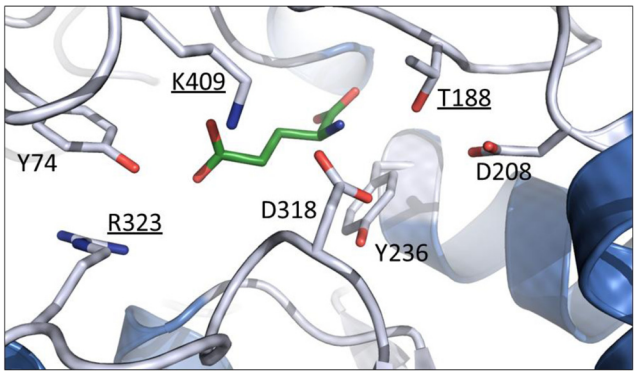

Fig. 3. Putative interactions of glutamate with amino acid residues in the binding pocket of mGlu1a receptors. Glutamate was shown to interact through the α-amino group with Thr188, Tyr236, Asp208, and Asp318 residues. Mutations of these residues (as well as Tyr74) eliminated the ability of mGlu1a receptors to activate PI hydrolysis, whereas mutations of Lys409 and Arg323 had no effect on G protein-mediated mGlu1a receptor signaling (Sato et al., 2003). This model allowed us to formulate the hypothesis that activation of the β-arrestin-mediated pathway may not require agonists with an α-amino group but depends on interactions of the agonist ω-carboxyl group with Lys409 and Arg323 residues. Residues that were mutated in our experiments are underlined. Modeling was performed as described in *Materials and Methods*. The figure does not show all of the amino acid residues that may interact with glutamate; it shows only those involved in the formulation of our hypothesis. For clarity, other residues are shown only in a cartoon of the protein backbone.

duction pathways through the same receptor, we mutated Thr188, Arg323, and Lys409 residues of the mGlu1a receptor ligand binding site, which were selected on the basis of X-ray crystallographic studies of the mGlu1 receptor ligand binding domain (Kunishima et al., 2000).

All mutated constructs of the mGlu1a receptor were stably expressed in CHO cells; for all mutants, protein expression levels were comparable to those for wild-type mGlu1a receptors (Fig. 4A). As expected, the mGlu1a receptor construct with the T188A mutation, which was shown to be critical for the binding of quisqualate and the activation of PI hydrolysis (Sato et al., 2003), was unable to enhance PI hydrolysis, inasmuch as the observed response was not different from that obtained with CHO cells transfected with the control empty vector (Fig. 4C). These data confirmed that the Thr188 residue is required for classic PI-linked signaling through mGlu1a receptors. In contrast, the T188A mutation failed to block the ability of mGlu1 receptors to induce β-arrestin-1-mediated sustained ERK phosphorylation (Fig. 4F) or mGlu1 receptor protective signaling (Fig. 4, D and E). For additional controls to facilitate determination of the role of β-arrestin-1, the expression of β-arrestin-1 was silenced with shRNA in CHO cells stably transfected with the mGlu1a receptor T188A mutant. As shown in Fig. 4B, shRNA targeted to β-arrestin-1 caused an approximately 70% reduction in β-arrestin-1 expression. Cells expressing shRNA against β-arrestin-1 were then transfected with human β-arrestin-1, which is refractory to shRNA because of several mismatched bases. Expression of human β-arrestin-1 caused a substantial increase in protein expression levels for β-arrestin-1 (Fig. 4B). Whereas glutamate stimulated both sustained ERK phosphorylation (Fig. 4F) and protective signaling (Fig. 4E), the silencing of β-arrestin-1 with shRNA resulted in blockade of both responses. These responses were restored when the β-arrestin-1-depleted cells were rescued through transfection with human β-arrestin-1 (Fig. 4, E and F). Taken together, these data indicate that the interaction of glutamate with the Thr188 residue is required for coupling with PI hydrolysis but is not necessary for coupling with sustained ERK phosphorylation and protective signaling.

Because Thr188 was shown to interact with the α-amino group of mGlu1 receptor agonists (Sato et al., 2003), we hypothesized that sustained ERK phosphorylation and protective signaling required ligand interactions with different residues of the binding site. Therefore, mutations were introduced at residues Arg323 and Lys409, which were postulated to interact with the ω-carboxyl group of glutamate but do not functionally interact with quisqualate (Sato et al., 2003). In CHO cells stably transfected with either R323V or K409A mutant mGlu1a receptors, agonist-induced PI hydrolysis was equivalent to that in cells expressing wild-type mGlu1a receptors (Fig. 5A). These data are consistent with a previous report that indicated that these residues are not needed for quisqualate binding or PI hydrolysis (Sato et al., 2003). As expected, glutamate stimulation of both mutated mGlu1a receptors also caused an increase in PLC-dependent transient ERK phosphorylation (Fig. 5B), which was abolished by the PLC inhibitor U73122 (Fig. 5B). In contrast, both R323V and K409A mutant mGlu1a receptors, expressed in CHO cells, failed to stimulate sustained ERK phosphorylation (Fig. 5C) and to protect cells against serum withdrawal-induced apoptosis (Fig. 5D).

On the basis of the data suggesting that interactions with residues Arg323 and Lys409, which are thought to bind the ω-carboxyl group of glutamate, are not necessary for agonist-induced PI hydrolysis but are required for receptor-mediated protective signaling, we reasoned that a ligand that is able to interact with Arg323 and Lys409 but not with Thr188 should induce protection but not PI hydrolysis when applied to wild-type mGlu1a receptors. Glutaric acid, which is such a ligand, is an analog of glutamate that lacks the α-amino group that interacts with Thr188; therefore, it would not be expected to stimulate PI hydrolysis. Succinic acid is an analog of aspartate that also lacks an α-amino group. In CHO cells stably expressing mGlu1a receptors, glutaric acid and succinic acid both failed to enhance PI hydrolysis, even at very high concentrations (Fig. 6A), which suggests that they are not classic mGlu1a receptor agonists. In contrast, glutaric acid stimulated, in a dose-dependent manner, both sustained ERK phosphorylation (Fig. 6B) and the protective signaling of mGlu1 receptors (Fig. 6C). Succinic acid protected cells from apoptosis to a similar extent (Fig. 6D). The effects of glutaric acid and succinic acid were blocked by the noncompetitive mGlu1 receptor antagonist YM298198, which confirmed their site of action at this receptor (Fig. 6, B–D).

The ability of the competitive antagonists LY367385 and 3-MATIDA to inhibit only PI hydrolysis and transient ERK phosphorylation and not sustained ERK phosphorylation or protective signaling suggests that glutamate may have two modes of interaction with the receptor, to stimulate the two different signaling cascades. If the modes of interaction are truly separate, then ligands interacting in one mode should not interfere with the potency of agonists interacting in the second mode. This possibility was investigated by testing for interactions between the biased ligands. First, we tested the ability of quisqualate (biased ligand for PI hydrolysis) to interfere with the potency of glutamate to induce protective

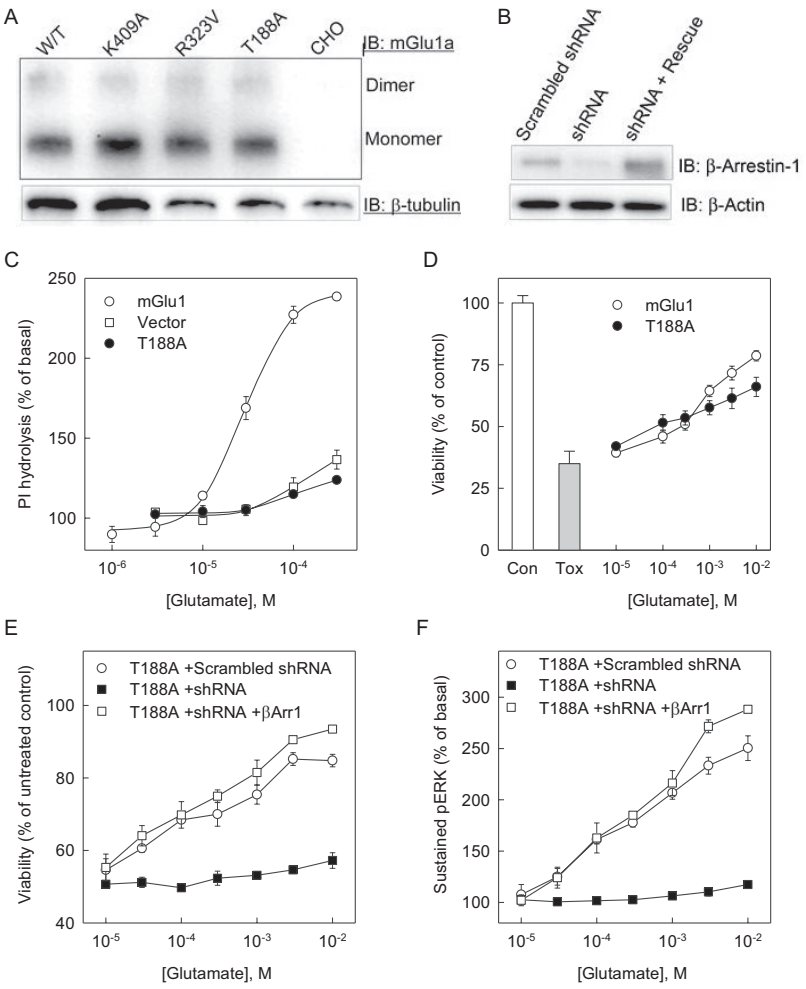

Fig. 4. Pharmacological and signaling properties of T188A mutant mGlu1a receptors expressed in CHO cells. A, comparison of protein expression levels for mutant mGlu1a receptors stably expressed in CHO cells. W/T, wild-type. B, silencing of β-arrestin-1 in CHO cells stably expressing T188A mutant mGlu1a receptors. Expression levels for β-arrestin-1 were restored with human β-arrestin-1 transfection (Rescue). C, abolishment of glutamate-induced PI hydrolysis in CHO cells expressing the T188A mutant, to a level similar to that of CHO cells transfected with the empty vector. D, glutamate-induced protection of CHO cells expressing mGlu1a receptors from toxicity (Tox) attributable to serum deprivation for 3 days. Results, expressed as percentages of untreated control values (Con), indicated that protection was equally effective in cells expressing the T188A mutant and cells expressing wild-type mGlu1a receptors. E, protective signaling through T188A mutant mGlu1a receptors. Protective signaling through T188A mutant mGlu1a receptors was inhibited by shRNA silencing of β-arrestin-1 and was restored with overexpression of human β-arrestin-1 (βArr1). F, sustained ERK phosphorylation through T188A mutant mGlu1a receptors. Sustained ERK phosphorylation measured after 24 h of agonist stimulation was blocked by shRNA silencing of β-arrestin-1 and was restored with overexpression of human β-arrestin-1. All values are means from at least three independent experiments performed in triplicate, with error bars representing S.E.M.

signaling, and we found no effect, even at extremely high (300 μM) concentrations of quisqualate (Fig. 7A). Second, we tested the ability of glutaric acid (biased ligand for sustained ERK phosphorylation and cytoprotection) to interfere with glutamate-induced PI hydrolysis, and we found it equally inactive at concentrations up to 10 mM (Fig. 7B). These data strongly suggest that ligand bias through mGlu1a receptors is attributable to two distinct modes of receptor-ligand interactions and the interaction type of the biased agonists allows for stimulation of different signal transduction cascades.

Discussion

Our previous studies with mGlu1a receptors revealed some unusual properties of these receptors. As shown in primary cultures of cerebellar and cortical neurons, increased mGlu1a receptor expression led to apoptotic cell death (Pshenichkin et al., 2008). This toxic effect was not blocked by mGlu1 receptor antagonists but was blocked by silencing receptor expression. Unexpectedly, mGlu1a receptor toxicity was blocked by the agonist glutamate. These properties allowed us to categorize the mGlu1a receptor as a dependence receptor, defined as a receptor that, when expressed, promotes apoptosis in the absence of its ligand but stimulates survival in its presence (Mehlen and Bredesen, 2004); mGlu1a receptors would make the survival of neuronal cells dependent on the presence of the endogenous agonist glutamate. Although the negative signaling of mGlu1a receptors that leads to apoptosis remains to be elucidated, positive signaling was described in our previous studies (Pshenichkin et al., 2008; Emery et al., 2010). This positive protective signaling is not mediated by the classic G protein- mediated coupling of mGlu1a receptors but instead involves β-arrestin-1-dependent internalization of mGlu1a receptors, followed by stimulation of the mitogen-activated protein kinase/ERK pathway (Emery et al., 2010). However, the protective signaling of mGlu1a receptors showed a different agonist profile than the G protein-mediated stimulation of PLC.

In this study, we investigated the different pharmacological profiles of the two mGlu1a receptor responses and revealed the existence of ligand bias at those receptors. Our studies indicated the existence of three classes of agonists at mGlu1a receptors, i.e., 1) unbiased ligands, such as glutamate, aspartate, and cysteic acid, which activate both G protein-dependent signaling and β-arrestin-dependent protective signaling; 2) ligands biased toward G protein signaling, such as quisqualate and DHPG; and 3) previously unknown ligands biased toward β-arrestin-dependent signaling, such as glutaric acid and succinic acid (Fig. 8). To our knowledge, this is the first report of ligand bias at a metabotropic glutamate receptor, but these findings should come as no surprise, because ligand bias has been reported for numerous other GPCRs, as reviewed by Violin and Lefkowitz (2007).

The initial clue regarding the mechanism responsible for

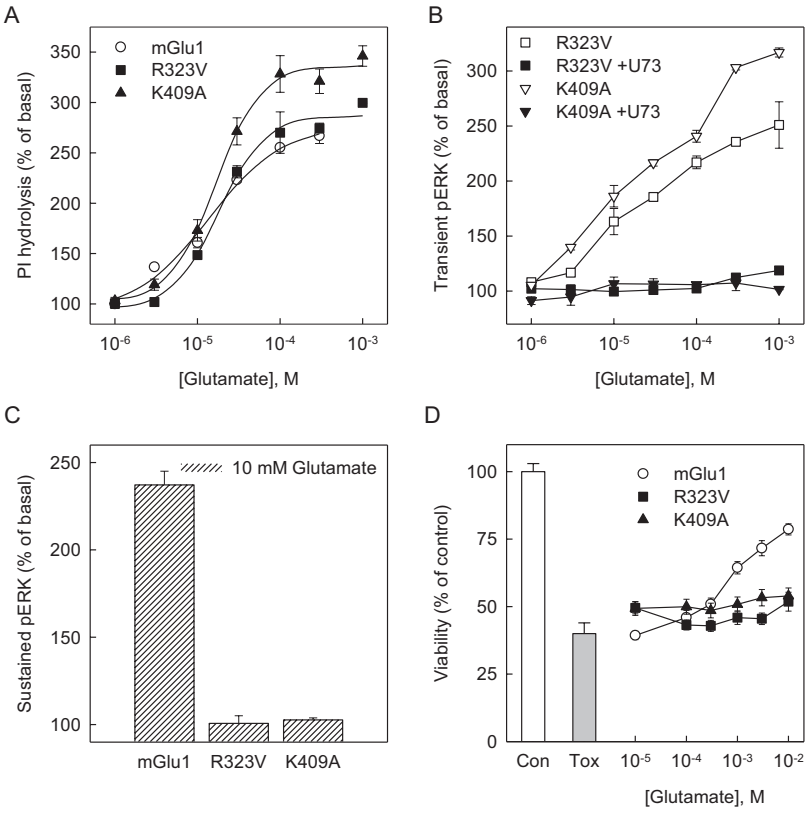

Fig. 5. Pharmacological and signaling properties of R323V and K409A mutant mGlu1a receptors expressed in CHO cells. A, with both R323V and K409A mutants, glutamate-induced PI hydrolysis occurred to an extent similar to that observed for CHO cells expressing wild-type mGlu1a receptors. B, glutamate-induced ERK phosphorylation measured after 5 min in CHO cells expressing R323V or K409A mutants was similar to that with wild-type mGlu1a receptors (Fig. 1B) and was blocked by the phospholipase C inhibitor U73122 (U73). C, R323V and K409A mutants failed to mediate glutamate-induced sustained (24-h) ERK phosphorylation. D, glutamate failed to protect CHO cells expressing R323V and K409A mutant mGlu1a receptors from serum withdrawal-induced toxicity (Tox). Con, control. All values are means from at least three independent experiments performed in triplicate, with error bars representing S.E.M.

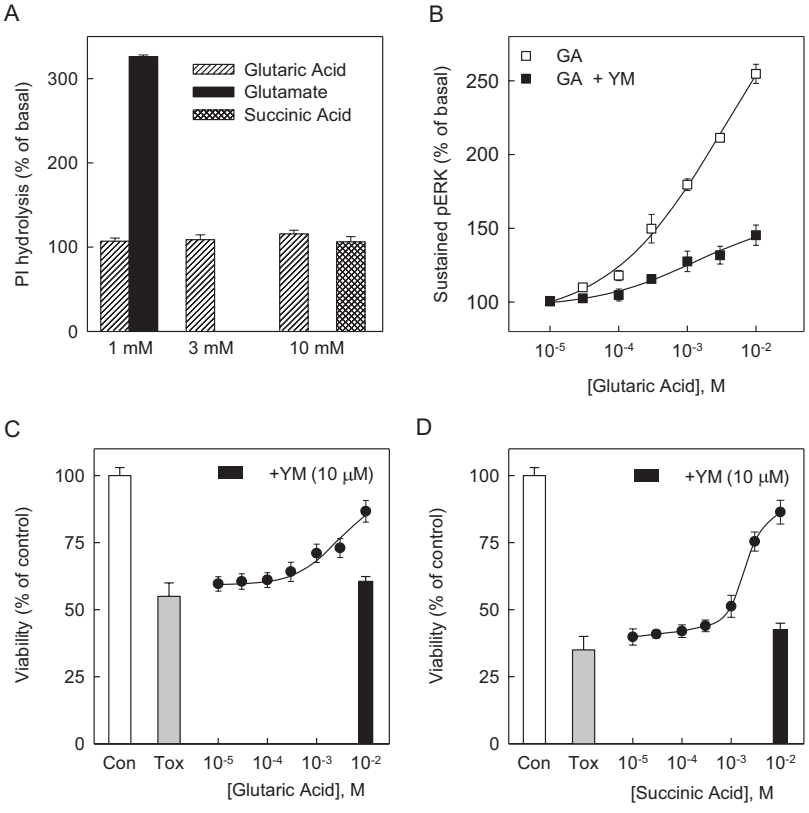

Fig. 6. Pharmacological effects of glutamate and aspartate analogs lacking the α-amino group. A, glutaric acid and succinic acid failed to stimulate PI hydrolysis in mGlu1a receptor-expressing CHO cells. B, glutaric acid (GA) increased sustained (24 h) ERK phosphorylation in CHO cells expressing mGlu1a receptors and was noncompetitively inhibited by YM298198 (YM). C and D, glutaric acid (C) and succinic acid (D) exhibited dose-dependent effects in protecting CHO cells expressing mGlu1a receptors from toxicity (Tox) attributable to serum deprivation for 3 days. Protection was blocked by YM298198 (10 μM). Con, control. Data points are means from three independent experiments, with error bars representing S.E.M.

ligand bias at mGlu1a receptors came from results indicating that, in contrast to PI hydrolysis, protective signaling was not inhibited by competitive mGlu1 receptor antagonists, whereas both responses were blocked by noncompetitive mGlu1 receptor antagonists. This could be explained if glutamate induced protective signaling by interacting with a separate region of the receptor ligand binding domain, rather than that occupied by competitive antagonists. Noncompeti-

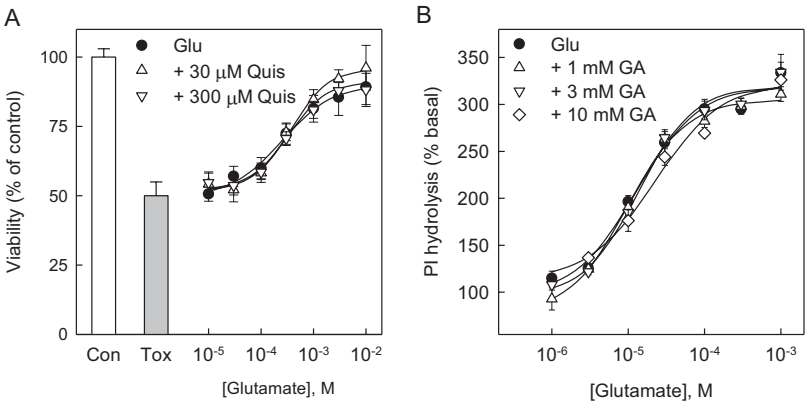

Fig. 7. Lack of interactions between differently biased mGlu1 receptor ligands. A, glutamate-induced protection from serum deprivation-induced toxicity (Tox) in CHO cells expressing mGlu1a receptors was not blocked by high concentrations of quisqualate (Quis). Con, control. B, glutaric acid (GA) failed to interfere with glutamate-induced PI hydrolysis in CHO cells expressing mGlu1a receptors. Data points are means from three independent experiments, with error bars representing S.E.M.

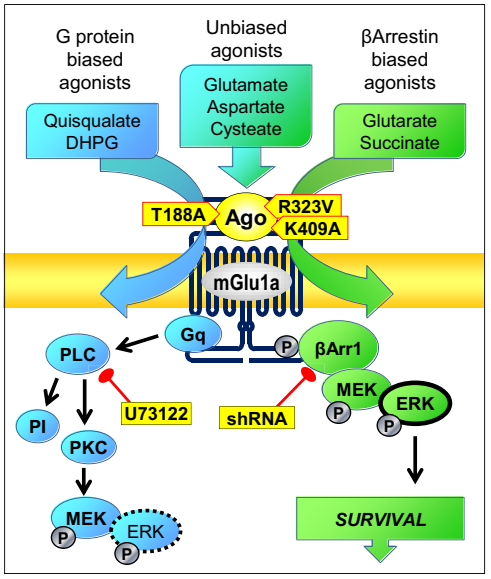

Fig. 8. Biased signaling of mGlu1a receptors. Upon agonist (Ago) stimulation, mGlu1a receptors activate two independent signaling pathways. Agonists such as quisqualate and DHPG activate G protein-mediated signaling leading to the activation of phospholipase C, phosphoinositide hydrolysis, and protein kinase C (PKC) and resulting in transient increases in ERK phosphorylation. This signaling is abolished with mutation of the Thr188 residue in the agonist binding pocket and can be disrupted by the PLC inhibitor U73122. In contrast, glutarate and succinate activate the β-arrestin-1 (βArr1)-dependent pathway, which involves receptor internalization, leads to sustained ERK phosphorylation, and is responsible for the cytoprotective actions of mGlu1a receptors. This signaling is abolished with mutation of Arg323 or Lys409 residues in the agonist binding pocket and can be disrupted through shRNA-mediated inhibition of β-arrestin-1 expression. Glutamate, aspartate, and cysteate are unbiased ligands that activate both pathways. Amino acid mutations that block one of the signaling pathways do not prevent the actions of agonists at the other pathway. MEK, mitogen-activated protein kinase kinase.

tive antagonists could still inhibit both responses, possibly by modifying receptor conformations. The mGlu1 receptor-selective competitive antagonists LY367385 and 3-MATIDA, which both inhibit glutamate-induced PI hydrolysis and transient ERK phosphorylation, had no effect on glutamate-induced sustained ERK phosphorylation or protective signaling at concentrations as high as 1 mM. In contrast, all mGlu1a receptor signaling was inhibited by the mGlu1 receptor-selective, noncompetitive antagonist YM298198, which binds to an allosteric site situated on the seventh transmembrane domain of the receptor (Kohara et al., 2005). Similar results were obtained previously by using other mGlu1 receptor-selective noncompetitive antagonists, including (−)-ethyl-(7*E*)-7-hydroxyimino-1,7a-dihydrocyclopropa[b]chromene-1a-carboxylate and (3,4-dihydro-2*H*-pyrano[2,3-*b*]quinolin-7-yl)-(*cis*-4-methoxycyclohexyl)-methanone (JNJ16259685) (Emery et al., 2010), which indicates that all effects are mediated selectively by mGlu1a receptors but not necessarily through interactions with the same region of the orthosteric binding site that binds competitive antagonists (Clark et al., 1997).

The hypothesis that the ligand binding domain of mGlu1a receptors is responsible for our observed ligand bias is supported by our current mutational studies. The T188A mutation in mGlu1a receptors resulted in a complete block of signaling through G protein-dependent pathways; however, signaling that resulted in sustained ERK phosphorylation and protection from apoptosis through the β-arrestin-1-dependent mechanism remained active. This indicates that the Thr188 residue is required for classic signaling through mGlu1a receptors but interaction with this residue is not necessary for β-arrestin-1-dependent signaling. Conversely, R323V and K409A mutants of mGlu1a receptors did not induce β-arrestin-1-dependent sustained ERK phosphorylation or protective signaling but still enhanced PI hydrolysis in the presence of glutamate, to a similar extent as wild-type mGlu1a receptors. This indicates that glutamate interactions with Arg323 and Lys409 are needed for β-arrestin-1-dependent signaling. These mutational data are consistent with previous mutational analyses of the ligand binding domain of mGlu1a receptors; however, β-arrestin-dependent signaling was not tested in those studies (Sato et al., 2003).

Additional evidence for the existence of different interactions between the particular classes of mGlu1 receptor ligands and the receptor N-terminal binding domain comes from the study of interactions between biased and unbiased mGlu1 receptor ligands. Quisqualate, the most-potent mGlu1 receptor ligand biased toward G protein-mediated signaling, failed to inhibit glutamate-induced protective signaling, even at very high concentrations. Similarly, glutaric acid, an agonist biased toward protective signaling, failed to affect the potency of glutamate in stimulating PI hydrolysis. This lack of interaction may be interpreted to mean that biased ligands bind to two distinct noninteracting sites, but additional studies would be needed to test such a hypothesis. The existence of two distinct binding sites within the ligand binding domain of G protein-coupled receptors in family C was demonstrated previously for the T_1R taste receptor (Zhang et al., 2010). Additional computational modeling studies would be required for detailed evaluation of this hypothesis with respect to mGlu1 receptors.

Although novel, these data suggesting two motifs for glutamate interactions with mGlu1a receptors are in keeping with some studies on mGlu receptors. In a study of mGlu receptor homology, an allosteric ion binding site was located adjacent to the orthosteric glutamate binding site (Ogawa et al., 2010). A ligand has since been discovered that interacts simultaneously with the orthosteric glutamate site and the newly discovered allosteric site in mGlu4 receptors (Acher et al., 2011), which suggests the possibility of multiple agonist binding motifs in other mGlu receptors. In the case of mGlu1 receptors, our mutational data indicated that the two modes of interaction with glutamate occurred in the same general region of the receptor N terminus. If additional studies indicated the presence of two separate glutamate binding sites, then some revisions to the conformational model of the receptor N-terminal domain would be required. The current model assumes that Thr188, Arg323, and Lys409 residues interact with a single molecule of glutamate. Such a conformation would not sterically allow for simultaneous noninteracting binding of two glutamate molecules within the proposed binding pocket. An alternative possibility is the location of the two binding sites on separate subunits of the mGlu1a receptor homodimer; however, we are not aware of any data supporting such a hypothesis.

In the present study, we have identified new ligands for mGlu1a receptors that are biased toward protective signaling. The ligands are glutaric acid and succinic acid, analogs of glutamate and aspartate, respectively, with a deleted α-amino group. Other homologs of these dicarboxylic acids, with shorter or longer carbon chains, were inactive. Although both ligands are endogenous to the brain, they are common metabolites and it is presently unclear whether they could serve a physiological role, acting at native mGlu1 receptors. Consistent with the current findings on the cytoprotective properties of succinate, it was shown previously to ameliorate cognitive defects in a rat model of Alzheimer's disease (Storozheva et al., 2008). These compounds may now be used as lead structures in the design of new biased agonists, and especially antagonists, with the ability to affect mGlu1 receptor-mediated protective signaling without disturbing classic G protein-mediated signal transduction.

In conclusion, we demonstrated that glutamate-induced protective signaling through mGlu1a receptors occurs through a different mode of interaction between the N-terminal domain of the receptor and the cytoprotective ligand, compared with the interactions leading to stimulation of G protein-mediated PI hydrolysis. These observations need to be validated in systems expressing native mGlu1a receptors and in vivo. Studies also are needed to address the mechanism and to establish the conditions under which glutamate, which is classically viewed as an excitotoxin (Siliprandi et al., 1992; Bruno et al., 1994), may produce a protective effect when acting at mGlu1a receptors. Acting as a dependence receptor, the mGlu1 receptor may serve as a sensor of extracellular glutamate, promoting neuronal survival in the presence of glutamate and inducing apoptosis in its absence. Such a mechanism could play an important role in brain physiological processes by allowing glutamate to act as a trophic factor contributing to neuronal development and neuronal selection during synaptogenesis and possibly participating in the restructuring of damaged brain tissue.

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Authorship Contributions

Participated in research design: Emery, DiRaddo, Pshenichkin, Yasuda, Wolfe, and Wroblewski.

Conducted experiments: Emery, Hathaway, Takoudjou, and Grajkowska.

Contributed new reagents or analytic tools: DiRaddo and Miller.

Performed data analysis: Emery and Wroblewski.

Wrote or contributed to the writing of the manuscript: Emery, DiRaddo, Pshenichkin, Wolfe, and Wroblewski.

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