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Ligand bias at metabotropic glutamate 1a receptor: Molecular determinants that distinguish β-arrestin from G protein-mediated signaling


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List of Abbreviations: Asp, L-aspartate; DHPG, (S)-3,5-Dihydroxyphenylglycine; DTT, dithiothreitol; GA, L-glutaric acid; Glu, L-glutamate; GPCR, G protein-coupled receptor; IP, inositol phosphate; L-CA, L-cysteic acid; LY367385, (+)-2-Methyl-4-carboxyphenyl glycine; mGlu, metabotropic glutamate; MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]; PI, phosphatidylinositides; PKC, Protein kinase C; PLC, Phospholipase C; Quis, L-quisqualate
ABSTRACT

Metabotropic glutamate receptor 1a (mGlu1a) is a G protein coupled receptor linked with phosphoinositides (PI) hydrolysis and with β-arrestin-1-mediated sustained ERK phosphorylation and cytoprotective signaling. Previously, we reported the existence of ligand bias at this receptor, since glutamate induced both effects, while quisqualate induced only PI hydrolysis. In the current study, we show that such mGlu1 agonists as glutamate, aspartate and L-cysteate were unbiased and activated both signaling pathways, while quisqualate and DHPG stimulated only PI hydrolysis. Moreover, competitive antagonists inhibited only PI hydrolysis, but not the β-arrestin-dependent pathway, while a noncompetitive mGlu1 antagonist blocked both pathways. A mutational analysis of the ligand binding domain of the mGlu1a receptor revealed that Thr\(^{188}\) residues were essential for PI hydrolysis, but not for protective signaling, while Arg\(^{323}\) and Lys\(^{409}\) 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 interaction with the receptor ligand binding domain. While some mGlu1a agonists are biased toward PI hydrolysis, we identified two endogenous compounds, glutaric and succinic acids, as new mGlu1 agonists fully biased toward β-arrestin-mediated protective signaling. Pharmacological studies indicate that in producing the two effects, glutamate interacts in two distinct ways with mGlu1 receptors, since cytoprotective signaling was not inhibited by competitive mGlu1 antagonists which blocked PI hydrolysis. Moreover, quisqualate, a ligand 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 due to different modes of receptor-glutamate interactions which are
differentially coupled to PI hydrolysis and β-arrestin-mediated cytoprotective signaling, and reveal the existence of new endogenous agonists acting at mGlu1 receptors.
INTRODUCTION

Metabotropic glutamate (mGlu) receptors are a group of G protein-coupled receptors (GPCRs) and have been categorized into three groups based on sequence homology and pharmacology (Conn and Pin, 1997; Pin and Duvoisin, 1995). Group I mGlu receptors (mGlu1 and mGlu5) stimulate phospholipase C (PLC) via coupling to Gq/11 (Aramori and Nakanishi, 1992) which results in the hydrolysis of membrane phosphoinositides (PI) followed by increased Ca2+ release from intracellular stores. Additionally, stimulation of group I mGlu receptors has been shown to cause phosphorylation of extracellular signal-regulated kinase (ERK) in both 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 has been shown to stimulate axon elongation (Kreibich et al., 2004) and outgrowth of dendritic spines in the developing hippocampus (Vanderklish and Edelman, 2002). We have recently described that mGlu1 produces dual neuroprotective and neurotoxic signaling in cerebellar and cortical neurons (Pshenichkin et al., 2008). Thus, mGlu1 exhibits the properties of a dependence receptor (Pshenichkin et al., 2008), inducing apoptosis in the absence of glutamate, while promoting neuronal survival in its presence. In CHO cells transfected with mGlu1a receptors, stimulation of the receptor with glutamate protected cells from serum withdrawal-induced apoptosis (Emery et al., 2010). In this model, protective signaling through mGlu1a receptors was accomplished by a β-arrestin-1-dependent sustained phosphorylation of ERK (Emery et al., 2010).

Our previous study on signal transduction of mGlu1a receptors indicates that classical, G protein-mediated signal transduction and transient ERK phosphorylation differ in their
pharmacological profiles from sustained ERK phosphorylation and protective signaling: the classical mGlu1a-mediated PI hydrolysis and transient ERK phosphorylation was induced by both quisqualate and glutamate with quisqualate as the most potent agonist (Emery et al., 2010). These data are consistent with several previous reports on the pharmacology of mGlu1-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, demonstrating an apparent ligand bias at mGlu1a receptors. Ligand bias is a new and emerging concept in pharmacology that has been shown to exist for other GPCRs when various agonists preferentially activate receptor conformations that are selectively conducive for different signal transduction pathways. The initial example of ligand bias was in M1 muscarinic receptors, which activate both cAMP and PLC in response to their endogenous agonist acetylcholine. However, ligands were identified that selectively activate PLC while blocking cAMP formation through these receptors (Fisher et al., 1993). Ligand bias has since been reported in numerous GPCRs (for a review see (Rajagopal et al., 2010)), and in addition to selective activation of different G protein pathways, ligands have been identified which preferentially activate β-arrestin-mediated signaling which is independent of G protein signaling (Wei et al., 2003).

Our previous results demonstrate that sustained ERK phosphorylation and protective signaling are β-arrestin-1-dependent, and this may suggest that agonists that do not cause these effects, such as quisqualate, are biased toward PI hydrolysis through mGlu1, whereas an agonist like glutamate, is less biased owing to our observations that glutamate activated both signal transduction pathways. While the structural properties responsible for the observed ligand bias are unknown, the X-ray crystallographic studies of the ligand binding domain of mGlu1
(Kunishima et al., 2000) have suggested that glutamate, the apparently unbiased ligand, interacts with 14 amino acid residues while quisqualate, the apparently biased ligand, interacts with only 9 residues (Sato et al., 2003). The interaction of glutamate with additional residues in the binding domain may represent a mechanism by which glutamate, but not quisqualate, can activate the two separate signal transduction pathways. This raises the possibility that it may be possible to identify compounds which activate mGlu1 but are biased toward sustained ERK phosphorylation and cell survival. The aim of this study was to identify the mechanism of ligand bias by which glutamate, but not quisqualate causes protective signaling in cells expressing mGlu1a receptors. Herein we identified glutaric and succinic acids as compounds which are ligands at mGlu1a receptors and are completely biased toward sustained ERK phosphorylation and protective signaling while inactive in stimulating PI hydrolysis. These ligands may serve as a lead compounds for drug discovery.

MATERIALS AND METHODS

Materials. DMEM and fetal bovine serum for cell cultures were purchased from Invitrogen (Carlsbad, CA). Receptor agonists glutamate, aspartate, L-cysteic acid, quisqualate, and DHPG, and antagonists YM298198, LY367385, 3-MATIDA, and PLC inhibitor U73122 were obtained from Tocris Cookson (Ellisville, MO). Glutaric and succinic acids and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell cultures. CHO-K1 cells were stably transfected with mGlu1a receptor cDNA in pcDNA-3.1 vector (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 transfection reagent (Invitrogen). Individual cell lines were isolated and cultured in DMEM 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 were contained in the pGeneClip Hygromycin vector (SABiosciences, Frederick, MD). CHO cells stably expressing mGlu1a were transfected with plasmids containing shRNA and selected in 0.8 mg/ml Hygromycin B. Knockdown of β-arrestin-1 was confirmed by Western blotting.

Site directed mutagenesis. Introduction of point mutations in mGlu1a cDNA was made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, 20 ng of plasmid containing mGlu1a cDNA were mixed with 125 ng of two mutagenic primers, dNTPs (50 μM) and 2.5 unit of Pfu DNA polymerase in a final volume of 50 μl. Primer sequences were: T188A, forward 5’CAGATCGCCTATTCTGCCGCTAGCA, reverse 5’GTCACTCAGGTC TATGCTAGCGGA; R323V, forward 5’GAAGTGATGGGCGAGACGTCAATCAGGGC, reverse 5’GCCTTCGATTTCGTCGACGTCTG CCCATCCATCACTTC; K409A, forward 5’GAAAACTATGTCCAGGACAGCGCCATGGGATTTGTCATCAATGCC, reverse 5’GGCATTGATGACAAATCCCATCGGGCTGTCCTGGACATAGTTTTC. Samples were denatured at 95°C for 30 sec and subjected to 20 cycles: denaturation (95°C, 30 sec), annealing (55°C, 1 min), and elongation (72°C, 30 min) with a final 10 min extension. Then 10 units of DpnI were added to digest the DNA template. After incubation at 37°C for 1 hour, samples were used for transformation of E. Coli XL1-Blue Supercompetent cells. Positive clones were identified by restriction analysis and the authenticity of each mutation was confirmed by DNA sequencing.
Western blots. Cells grown and treated in 35 mm dishes were collected in 25 mM Tris-HCl buffer, pH 7.5 containing Halt protease and phosphatase inhibitor cocktails with 1 mM EDTA (Pierce Biotechnology, Rockford, IL). Proteins were solubilized in Laemmli buffer containing 50 mM DDT, and equal amounts of sample protein were resolved on 8% polyacrylamide gels (Invitrogen). Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA) and were probed with antibodies against mGlu1a (BD Biosciences, San Jose, CA), β-tubulin (Sigma), and β-arrestin-1 (Abcam Inc., Cambridge, MA). Proteins were visualized by incubation with goat anti-rabbit secondary antibodies coupled to horseradish peroxidase (Pierce) followed by exposure to chemiluminescent HRP substrate SuperSignal West Femto (Pierce).

Measurement of ERK phosphorylation. Phosphorylated ERK was measured using cell-based ELISA 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 minutes at room temperature. After 3 washes in 0.1% Triton X-100 (PBST) for membrane permeabilization, endogenous peroxidase activity was quenched by 20 minutes incubation in PBS containing 0.5% H₂O₂ and 0.2% NaN₃. After 3 more washes in PBST, cells were blocked with 2% BSA for 1 hour and incubated overnight with primary antibody against Phospho-p44/42 MAPK (ERK1/2) (Thr²⁰²/Tyr²⁰⁴) (Cell Signaling Technology). Cells were then washed for 5 minutes 3 times in PBST and twice in PBS. A HRP-coupled goat anti-rabbit secondary antibody (Pierce) was incubated for 1 hour at room temperature and then cells were again washed 5 times. Cells were exposed to the colorimetric HRP substrate 1-Step Ultra TMB (Pierce). After 10 minutes of developing, the reaction was stopped in 4 M H₂SO₄ and absorbance was read at 450 nm.
Assessment of cell viability. Viability of cells cultured on 96-well plates was measured by incubation for 1 h at 37°C with 0.2 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which was purchased from Invitrogen. The formation of the formazan product, proportional to the number of viable cells, was measured colorimetrically at 570 nm after extraction with 70 µl DMSO (Mosmann, 1983).

Measurements of PI hydrolysis. Cells, cultured in 96-well plates, were incubated overnight with 0.625 μCi/well myo-[3H]inositol (Perkin Elmer, Boston, MA) to label the cell membrane phosphoinositides. After two washes with 0.1 ml of Locke’s buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 1 mM MgCl₂, 1.3 mM CaCl₂, 5.6 mM glucose and 20 mM Hepes, pH 7.4), incubations with receptor ligands were carried out for 45 min at 37°C in Locke’s buffer containing 20 mM LiCl to block inositol phosphate degradation. The reaction was terminated by aspiration of media 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 incubated with 60 µl of polylysine coated yttrium scintillation proximity assay (SPA) beads (GE Healthcare) at room temperature for 1 hour with vigorous shaking. After additional 8 hours of incubation with SPA beads, inositol phosphates were detected by scintillation counting.

Glutamate assay. Glutamate concentrations in collected media were measured using the Amplex Red glutamic acid assay kit (Molecular Probes, Eugene OR) according to the manufacturer’s instructions. Briefly, 50 µl media samples were combined with 50 µl of a solution containing Amplex Red (10µl/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 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 540 nm and emission was detection at 590
For each point, background fluorescence was corrected by subtracting values derived from controls lacking glutamate. Data were fit to standard curves obtained by 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 mGlu1 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 imported into Maestro (version 9.2, Schrödinger, LLC, New York, NY, 2011) within the Schrodinger Suite of programs in pdb format. After removing one of the monomers from the crystalized dimer, the receptor was prepared using Protein Preparation Wizard (Schrödinger Suite 2011 Protein Preparation Wizard; Epik version 2.2, Schrödinger, LLC, New York, NY, 2011D) within Maestro. The prepared protein was exported to the PyMOL Molecular Graphics System (version 1.3, Schrödinger, LLC) in pdb format. As shown, the model depicts only the side chains of amino acid residues which 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 of the protein backbone.

**Calculations and data analysis.** Analysis of dose-response data and calculation of EC$_{50}$ values, when appropriate, was performed by fitting data points to a 4-parameter logistic equation by nonlinear regression using Sigma Plot 11.0 software.

**RESULTS**

While our previous studies have clearly shown that glutamate, acting at mGlu1a receptors, activates two distinct signal transduction cascades (Emery et al., 2010) they have also suggested that some mGlu1 agonists, such as quisqualate, may only enhance the G protein-
mediated, but not the β-arrestin-dependent signaling. Such phenomenon 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 while other agonists may activate all signaling associated with the same receptor (Violin and Lefkowitz, 2007). Using CHO cells expressing mGlu1a receptors, we have tested the ability of various known mGlu1 agonists to activate the two signaling cascades. The activation of the G protein-mediated signaling was assessed by measurements of phosphoinositide (PI) hydrolysis and of the transient (5 min) ERK phosphorylation. The activation of the β-arrestin-mediated signaling was evaluated by measuring the sustained (24 h) ERK phosphorylation and the protection of cells from trophic deprivation. As shown in Fig. 1A, all tested agonists stimulated PI hydrolysis with quisqualate being the most potent agonist (EC50 ~ 1 μM), followed by glutamate and DHPG (EC50 ~ 10 μM), and by aspartate and L-cysteic acid (EC50 ~ 100 μM). In addition to the stimulation of PI hydrolysis, these agonists increased transient ERK phosphorylation measured after 5 minutes of incubation with the agonist (Fig. 1B) with a pharmacological profile similar to that observed for PI hydrolysis. As expected, the mGlu1-selective noncompetitive antagonist YM-298198 (Kohara et al., 2005) completely abolished agonist-induced PI hydrolysis (Fig.1 A) and transient ERK phosphorylation (Fig. 1B). These results are consistent with our previous report indicating that both PI hydrolysis and transient ERK phosphorylation are mediated by the same G protein dependent mechanism (Emery et al., 2010).

Our previous studies have indicated that in addition to transient ERK phosphorylation, mGlu1a receptors also cause a long-lasting, β-arrestin1-dependent, phosphorylation of ERK, which is required for the mGlu1a-mediated protection from apoptosis (Emery et al., 2010). The sustained ERK phosphorylation, measured after 24h, had a different pharmacological profile
from transient ERK phosphorylation being induced only by glutamate, aspartate and L-cysteic acid, but not by quisqualate and DHPG (Fig. 1C). Glutamate-induced sustained ERK phosphorylation was completely blocked by 10 \( \mu \text{M} \)YM-298198 (Fig. 1C). A similar pharmacological profile of agonist action was observed for the mGlu1-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 by MTT assays. Under these conditions, after 3 days, cells exhibited approximately 30-40% of viability relative to controls grown in serum-containing medium (Fig 1D). The addition of glutamate, aspartate or L-cysteic acid to the serum-free culture medium produced a substantial increase in viability, but in contrast to the pharmacology of PI hydrolysis, quisqualate and DHPG were not effective in concentrations up to 10 mM (Fig. 1D). However, the observed agonist potencies were similar to those observed mGlu1-mediated sustained ERK phosphorylation (Fig. 1C and 1D). These results further stress 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, nor did glutamate induce sustained ERK phosphorylation in these cells (Emery et al., 2010).

Because, compared to PI experiments, viability assays involved a long incubation time, it was necessary to address the issue of the stability of drugs used over these extended periods of time to ascertain that the lack of activity is not due to drug depletion. Control experiments were performed to test drug stability under cell culture conditions for the 3-day incubation used in viability assays. Agonists were culture-conditioned with mGlu1a-expressing CHO cells for 3 days, and then the collected media were used to stimulate PI hydrolysis in naïve cells. As reported previously, both quisqualate and glutamate were equally potent at 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\textsubscript{50} values being 30 μM for freshly-prepared DHPG and 32 μM for preconditioned DHPG. Additionally, measurements of glutamate in media indicated that glutamate concentration after 3 days of incubation with cells did not significantly vary from the concentration of applied glutamate. Three days after adding 30 μM, 300 μM, or 3 mM glutamate, measured glutamate concentrations in the media were 28.7 ± 3.3 μM, 297 ± 12 μM, and 3.04 ± 0.09 mM, respectively. These data indicate that the agonists tested are not subject to degradation or uptake during the extended periods of incubation used and that the lack of activity of such agonists as quisqualate and DHPG was not due 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 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; while quisqualate and DHPG activate only PI hydrolysis and the transient phosphorylation of ERK.

To further investigate mGlu1a receptor-mediated pharmacology and signal transduction, all effects were measured in the presence of mGlu1-selective antagonists. To this end, both LY 367385 and 3-MATIDA, selective and competitive antagonists of mGlu1 receptors, (Clark et al., 1997; Moroni et al., 2002) were used. As shown in Fig. 2A, LY 367385 and 3-MATIDA both competitively inhibited glutamate-induced PI hydrolysis, causing the EC\textsubscript{50} for glutamate to shift from 11 μM to 108 μM in presence of LY 367385 (10 μM) and to 60 μM in the presence of 3-MATIDA (30 μM). Both competitive antagonists inhibited also transient ERK phosphorylation (Fig. 2B), shifting the EC\textsubscript{50} for glutamate from 50 μM to 810 μM in presence of LY 367385 (30
μM) and to 880 μM in presence of 3-MATIDA (30 μM). As expected, both PI hydrolysis and transient ERK phosphorylation were completely abolished by the noncompetitive antagonist YM-298198 (Fig. 2A, B). In contrast to transient ERK phosphorylation, the sustained ERK phosphorylation, which is mediated by the β-arrestin-dependent pathway, was not inhibited by the competitive mGlu1 antagonists. Even at high concentrations (1 mM) of either LY 367385 or 3-MATIDA failed to inhibit glutamate-induced sustained (24 hr) ERK phosphorylation (Fig. 2C). However, glutamate-induced sustained ERK phosphorylation was completely inhibited by 30 μM YM-298198, an mGlu1-selective noncompetitive antagonist (Kohara et al., 2005). As in the case of glutamate-induced sustained ERK phosphorylation, the addition of either LY 367385 (1 mM) or 3-MATIDA (1 mM) failed to inhibit glutamate-induced protection (Fig. 2D). Instead, the protective signaling was noncompetitively inhibited by YM-298198 (3 μM), confirming that this effect is mediated by mGlu1 receptors. To ascertain that the lack of effect of competitive antagonists was not due to their degradation during the long incubation times, both compounds were bioassayed after 3 days of culture conditioning. Based on the dose ratios of 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. Hence, while partially degraded, the concentrations of these competitive antagonists were still much greater than those needed for an effective inhibition of PI hydrolysis and transient ERK phosphorylation.

Because G protein-mediated mGlu1 signaling was blocked by competitive receptor antagonist, while β-arrestin-mediated signaling was not, we hypothesized that the observed ligand bias may reflect a differential ability of agonists to interact with the receptor ligand binding domain and that different portions of this domain may be involved in stimulating PI
hydrolysis and in inducing protective signaling. In such case a noncompetitive antagonist would still be effective in blocking all signal transduction. Previously, the X-ray crystallography of the mGlu1 receptor binding site with bound glutamate has been resolved (Kunishima et al., 2000). These studies, as well as mutational analyses of the mGlu1 binding site (Sato et al., 2003), have shown that several amino acid residues, including Thr$^{188}$, Asp$^{208}$, Tyr$^{236}$, and Asp$^{318}$, interacted with the amino group of glutamate (see Fig. 3) and these interactions were necessary for receptor activation as measured by PI hydrolysis and binding of quisqualate (Sato et al., 2003). In contrast, other residues in the binding site, including Arg$^{323}$ and Lys$^{409}$, were postulated to bind the omega-carboxyl group of glutamate (Fig. 3), but mutation of these residues failed to attenuate glutamate-induced PI hydrolysis or the binding of quisqualate (Sato et al., 2003). In order to determine whether different glutamate-binding residues may be involved in activation of different signal transduction pathways through the same receptor, we have mutated Thr$^{188}$, Arg$^{323}$ and Lys$^{409}$ residues of the mGlu1a ligand binding site, selected based on X-ray crystallographic studies of the mGlu1 ligand binding domain (Kunishima et al., 2000).

All mutated constructs of mGlu1a were stably expressed in CHO cells, and in the case of all mutants protein expression levels were comparable to wild-type mGlu1a receptors (Fig. 4A). The mGlu1a construct with the T188A mutation which has been shown to be critical for the binding of quisqualate and activation of PI hydrolysis (Sato et al., 2003) was, as expected, unable to enhance PI hydrolysis, as the observed response was not different from that obtained with CHO cells transfected with the control empty vector (Fig. 4C). These data confirm that the Thr$^{188}$ residue is required for classical PI-linked signaling through mGlu1a receptors. In contrast, the T188A mutation failed to block the ability of mGlu1 to induce the β-arrestin1-mediated sustained ERK phosphorylation (Fig. 4F), or mGlu1 protective signaling (Fig. 4D, 4E).
additional controls to ascertain the role of β-arrestin-1, the expression of β-arrestin-1 was silenced by shRNA in CHO cells stably transfected with mGlu1a 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 due to several mismatched bases. Expression of human β-arrestin-1 caused a substantial increase in protein expression of β-arrestin-1 (Fig. 4B). While glutamate stimulated both sustained ERK phosphorylation (Fig. 4F) and protective signaling (Fig. 4E), the silencing of β-arrestin-1 with shRNA resulted in the blockade of both responses. However, these responses were restored when the β-arrestin-1-depleted cells were rescued by transfection with human β-arrestin-1 (Fig. 4E, 4F). Taken together, these data indicate that the interaction of glutamate with the Thr^{188} residue is required for the coupling with PI hydrolysis, but is not necessary for the coupling with sustained ERK phosphorylation and protective signaling.

Because Thr^{188} has been shown to interact with the alpha amino group of mGlu1 agonists (Sato et al., 2003), we hypothesized that sustained ERK phosphorylation and protective signaling require ligand interactions with different residues of the binding site. Therefore, mutations were performed on residues Arg^{323} and Lys^{409}, which were postulated to interact with the omega 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 of mGlu1a, agonist-induced PI hydrolysis was equivalent to cells expressing wild-type mGlu1a receptors (Fig. 5A). These data are consistent with a previous report indicating 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 mGlu1a mutants, expressed in CHO cells, failed to stimulate the sustained ERK phosphorylation (Fig. 5C), and to protect against serum withdrawal-induced apoptosis (Fig. 5D).

Based on the data suggesting that interaction with residues Arg$^{323}$ and Lys$^{409}$, which are thought to bind the omega carboxyl group of glutamate, are not necessary for agonist-induced PI hydrolysis but, instead, are required for receptor-mediated protective signaling, we reasoned that a ligand able to interact with Arg$^{323}$ and Lys$^{409}$, but not with Thr$^{188}$ should induce protection, but not PI hydrolysis when applied to wild-type mGlu1a receptors. Such a ligand, glutaric acid, is an analog of glutamate that lacks the alpha amino group, which interacts with Thr$^{188}$, therefore it would not be expected to stimulate PI hydrolysis. Similarly, succinic acid is an analog of aspartate which also lacks an alpha amino group. In fact, in CHO cells stably expressing mGlu1a receptors, glutaric and succinic acids both failed to enhance PI hydrolysis, even at very high concentrations (Fig. 6A), suggesting that they are not classical mGlu1a agonists. In contrast, glutaric acid stimulated, in a dose-dependent manner, both the 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 and succinic acid were blocked by the noncompetitive mGlu1 antagonist YM298198, confirming their site of action at this receptor (Fig. 6B, 6C, 6D).

The ability of the competitive antagonists LY 367385 and 3-MATIDA to inhibit only PI hydrolysis and transient ERK phosphorylation, but not the 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 modes of interaction were 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 signaling, and 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 found it equally inactive at concentrations up to 10 mM (Fig. 7B). These data strongly suggest that ligand bias through mGlu1a receptors are due to two distinct modes of receptor-ligand interaction, and the interaction type of the biased agonists allows for the stimulation of different signal transduction cascades.

**DISCUSSION**

Our previous studies with mGlu1a receptors have 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 antagonists but by silencing receptor expression. Unexpectedly, mGlu1a toxicity was also blocked by its agonist glutamate. These properties allow us to categorize mGlu1a as a dependence receptor, defined as a receptor which, when expressed, promotes apoptosis in the absence of its ligand, but stimulates survival in its presence (Mehlen and Bredesen, 2004). Hence, mGlu1a receptors would make the survival of neuronal cells “dependent” on the presence of the endogenous agonist glutamate. While the negative signaling of mGlu1a which leads to apoptosis still remains to be elucidated, the positive
signaling has been described in our previous studies (Emery et al., 2010; Pshenichkin et al., 2008). This positive, protective signaling is not mediated by the classical G protein-mediated coupling of mGlu1a receptors, but, instead, involves a β-arrestin-1-dependent internalization of mGlu1a, followed by the stimulation of the MEK/ERK pathway (Emery et al., 2010). However, the protective signaling of mGlu1a showed a different agonist profile than the G protein-mediated stimulation of PLC.

In this study we have investigated those different pharmacological profiles of the two mGlu1a responses and revealed the existence of ligand bias at these receptors. Our studies indicate the existence of three classes of agonists at mGlu1a receptors: (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 towards G protein signaling, such as quisqualate and DHPG, and (3) previously unknown ligands biased towards β-arrestin-dependent signaling, such as glutaric or 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, as ligand bias has been described at numerous other GPCRs, as reviewed by Violin and Lefkowitz (2007).

The initial clue as to the mechanism responsible for ligand bias at mGlu1a receptors came from results indicating that, in contrast to PI hydrolysis, the protective signaling was not inhibited by competitive mGlu1 antagonists, while both responses were blocked by noncompetitive mGlu1 antagonists. This could be explained if glutamate induced protective signaling by interacting with a separate region of the receptor ligand binding domain than that occupied by competitive antagonists. Instead, noncompetitive antagonists would still inhibit both responses, possibly by modifying receptor conformation. In fact the mGlu1-selective competitive
antagonists LY 367385 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 signaling was inhibited by the mGlu1-selective noncompetitive antagonist YM 298198, which binds to an allosteric site situated on the 7th transmembrane domain of the receptor (Kohara et al., 2005). Previously, similar results were obtained using other mGlu1-selective noncompetitive antagonists, including CPCCOEt and JNJ16259685 (Emery et al., 2010), indicating that all effects are selectively mediated by mGlu1a, but not necessarily due to interactions with the same region of the orthosteric binding site which also 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 further supported by our current mutational studies. The T188A mutation on mGlu1a receptors results in a complete block of signaling through G protein-dependent pathways, however signaling which results in sustained ERK phosphorylation and protection from apoptosis through the β-arrestin-1-dependent mechanism remains active. This indicates that the Thr^{188} residue is required for classical signaling through mGlu1a receptors, but an interaction with this residue is not necessary for β-arrestin-1-dependent signaling. Conversely, R323V and K409A mutants of mGlu1a receptors do not induce β-arrestin-1-dependent, sustained, ERK phosphorylation or protective signaling, but still enhance PI hydrolysis in the presence of glutamate to a similar extent as wild-type mGlu1a receptors. This indicates that glutamate interaction with Arg^{323} and Lys^{409} is needed for β-arrestin-1-dependent signaling. These mutational data are consistent with a previous mutational analysis of the ligand binding domain
of mGlu1a receptors, however β-arrestin-dependent signaling was not tested in these studies (Sato et al., 2003).

Additional evidence for the existence of differential interactions between the particular classes of mGlu1 receptor ligands and the receptor N-terminal binding domain comes from the study of interactions between the biased and unbiased mGlu1 ligands. Quisqualate, the most potent ligand of mGlu1 receptors, biased towards G protein-mediated signaling, failed to inhibit glutamate-induced protective signaling, even at very high concentrations. Similarly, glutaric acid, an agonist biased towards protective signaling, failed to affect the potency of glutamate to stimulate PI hydrolysis. This lack of interactions may be interpreted to mean that biased ligands may bind to two distinct, non-interacting sites, but further studies would be needed to test such a hypothesis. The existence of two distinct binding sites within the ligand binding domain of Family C G protein-coupled receptors has been demonstrated previously in the T1R taste receptor (Zhang et al., 2010). However, additional computational modeling studies would be required for a detailed evaluation of this hypothesis in mGlu1 receptors.

While novel, these data suggesting two motifs of glutamate interaction with mGlu1a receptors are in keeping with some recent 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 which simultaneously interacts with the orthosteric glutamate site and newly discovered allosteric site in mGlu4 receptors (Acher et al., 2011), suggesting the possibility of multiple agonist binding motifs in other mGlu receptors. In the case of mGlu1, our mutational data indicate that the two modes of interaction with glutamate both occur in the same general region of the receptor N-terminus. If further studies were to indicate the presence of two separate glutamate binding sites, this would require
some revisions to the conformational model of the receptor N-terminal domain. The current model assumes that Thr^{188}, as well as Arg^{323} and Lys^{409} residues interact with a single molecule of glutamate. Such a conformation would not sterically allow for a simultaneous non-interacting 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 which are biased towards the protective signaling. They are glutaric and succinic acids, analogues of glutamate and aspartate, respectively, with a deleted alpha-amino group. Other homologues of these dicarboxylic acids, with shorter or longer carbon chains were inactive. While 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 of cytoprotective properties induced by succinate, it has previously been shown to ameliorate cognitive defects in a rat model of Alzheimer’s disease (Storozheva et al., 2008). However, these compounds may now be used as lead structures in the design of new biased agonists, and especially antagonists, with ability to affect mGlu1 receptor-mediated protective signaling, without disturbing the classical G protein-mediated signal transduction.

In conclusion, we have demonstrated that glutamate-induced protective signaling through mGlu1a receptors occurs due to a different mode of interaction between the N-terminal domain of the receptor and the cytoprotective ligand than the interactions leading to stimulation of G protein-mediated PI hydrolysis. These observations need now to be validated in systems expressing native mGlu1a receptors and in vivo. Further studies are also needed to address the mechanism and establish the conditions in which glutamate, classically viewed as an excitotoxin...
(Bruno et al., 1994; Siliprandi et al., 1992), may also produce a protective effect when acting at mGlu1a receptors. Acting as a dependence receptor, mGlu1 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 physiology by allowing glutamate to act as a trophic factor contributing to neuronal development and neuronal selection during synaptogenesis and, possibly, by participating in the restructuring of damaged brain tissue.
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Authorship Contributions

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

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Footnotes

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Figure Legends

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

Figure 2. Antagonism of mGlu1a-mediated signaling in transfected CHO cells. (A) Inhibition of glutamate-induced PI hydrolysis by competitive antagonists 10 μM LY367385 (LY) and 30 μM 3-MATIDA (MAT) using either freshly-prepared (Fresh) or cultured-conditioned (CC) antagonists, and by the noncompetitive 10 μM YM298198 (YM). Competitive antagonists LY367385 (LY) 3-MATIDA (MAT) inhibit the transient (5 min) ERK phosphorylation (B), but not the sustained (24h) ERK phosphorylation (C). The noncompetitive YM298198 (YM) blocks both transient (B) and sustained (C) ERK phosphorylation. (D) Glutamate-induced protection of CHO cells expressing mGlu1a receptors from toxicity (Tox) due to serum deprivation for 3 days, expressed as the percent of untreated controls (Con), was not blocked by either competitive antagonist LY367385 (1 mM) or 3-MATIDA (1 mM), but was abolished by noncompetitive YM298198 (3 μM, YM). All values represent means from at least three experiments performed in triplicate with error bars representing S.E.M.
Figure 3. Putative interactions of glutamate with amino acid residues in the binding pocket of mGlu1a receptors. Glutamate has been shown to interact through the alpha-amino group with the Thr\textsuperscript{188}, Tyr\textsuperscript{236}, Asp\textsuperscript{208} and Asp\textsuperscript{318} residues. Mutations of these residues (as well as Tyr\textsuperscript{74}) eliminated the ability of mGlu1a to activate PI hydrolysis, while mutations of Lys\textsuperscript{409} and Arg\textsuperscript{323} had no effect on G protein-mediated mGlu1a 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 alpha amino group but, instead, depends on the interaction of the agonist omega-carboxyl group with the Lys\textsuperscript{409} and Arg\textsuperscript{323} residues. Residues which have been mutated in our experiments are underlined. Modeling was performed as described in Methods. The figure does not show all of the possible amino acid residues interacting with glutamate, but only those involved in the formulation of our hypothesis. For the purpose of clarity other residues are shown only as a cartoon of the protein backbone.

Figure 4. Pharmacology and signaling properties of T188A mGlu1a mutant receptor expressed in CHO cells. (A) Comparison of protein expression levels of mutant mGlu1a receptors stably expressed in CHO cells. (B) Silencing of β-arrestin-1 in CHO cells stably expressing mGlu1a T188A mutant receptors. Expression levels of β-arrestin-1 were restored by transfection with human β-arrestin-1. (C) Glutamate-induced PI hydrolysis is abolished in CHO cells expressing the T188A mutant to a level similar to CHO cells transfected with the empty vector. (D) Glutamate-induced protection of CHO cells expressing mGlu1a receptors from toxicity (Tox) due to serum deprivation for 3 days, expressed as the percent of untreated controls (Con), was equally effective in cells expressing the T188A mutant as the wild type mGlu1a. (E) Protective signaling through T188A mutant mGlu1a was inhibited by shRNA silencing of β-arrestin-1, and
was restored upon overexpression of human β-arrestin-1. (F) In T188A mutant mGlu1a receptors, sustained ERK phosphorylation measured after 24 hours of agonist stimulation is blocked by shRNA silencing of β-arrestin-1 and is restored by 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.

**Figure 5.** Pharmacology and signaling properties of R323V and K409A mGlu1a mutant receptor expressed in CHO cells. (A) In both R323V and K409A mutants, glutamate-induced PI hydrolysis occurs to a similar extent as in CHO cells expressing the wild-type mGlu1a receptors (B) Glutamate-induced ERK phosphorylation measured after 5 minutes in CHO cells expressing R323V and K409A mutants is similar to that of wild type mGlu1a (see Fig. 1B) and is blocked by the phospholipase C inhibitor U73122 (U73). (C) R323V and K409A mutants fail to mediate glutamate-induced sustained (24h) ERK phosphorylation. (D) Glutamate fails to protect CHO cells expressing R323V and K409A mutant mGlu1a receptors from serum withdrawal-induced toxicity (Tox). All values are means from at least three independent experiments performed in triplicate with error bars representing S.E.M.

**Figure 6.** Pharmacological effects of the glutamate and aspartate analogs lacking the alpha-amino group. (A) Glutaric acid and succinic acid fail to stimulate PI hydrolysis in mGlu1a-expressing CHO cells. (B) Glutaric acid (GA) increases sustained (24 h) ERK phosphorylation in CHO cells expressing mGlu1a receptors and is noncompetitively inhibited by YM298198 (YM). Dose-dependent effect of glutaric acid (C) and succinic acid (D) in protecting CHO cells expressing mGlu1a receptors from toxicity (Tox) due to serum deprivation for 3 days. Protection
was blocked by YM298198 (10 μM). Data points are means from 3 independent experiments with error bars representing S.E.M.

**Figure 7.** Lack of interactions between differently biased mGlu1 ligands. (A) Glutamate-induced protection from serum deprivation-induced toxicity (Tox) in CHO cells expressing mGlu1a receptors is not blocked by high concentrations of quisqualate (Quis). (B) Glutaric acid (GA) fails to interfere with glutamate-induced PI hydrolysis in CHO cells expressing mGlu1a receptors. Data points are means from 3 independent experiments with error bars representing S.E.M.

**Figure 8.** Biased signaling of mGlu1a receptors. Upon agonist stimulation, mGlu1a receptors activate two independent signaling pathways. Agonist such as quisqualate and DHPG activate G protein-mediated signaling leading to the activation of phospholipase C, phosphoinositide hydrolysis and protein kinase C and resulting in a transient increase of ERK phosphorylation. This signaling is abolished by mutation of Thr^{188} 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 dependent pathway which involves receptor internalization, leads to a sustained ERK phosphorylation and is responsible for the cytoprotective actions of mGlu1a. This signaling is abolished by mutations of Arg^{323} or Lys^{409} residues in the agonist binding pocket and can be disrupted by shRNA-mediated inhibition of β-arrestin-1 expression. Glutamate, aspartate and cysteate are unbiased ligands which activate both pathways. Mutations of amino acid residues which block one of the signaling pathways do not prevent the action of agonists at the other pathway.
Figure 1

A

\[
\text{PI hydrolysis (\% of basal)}
\]

B

\[
\text{Transient pERK (\% of basal)}
\]

C

\[
\text{Sustained pERK (\% of basal)}
\]

D

\[
\text{Viability (\% of control)}
\]
Figure 2

A

B

C

D

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Figure 3
Figure 4

A) Western blot analysis showing the expression levels of mGlu1a, Dimer, and Monomer in different cell lines: W/T, K409A, R323V, T188A, and CHO. Immunoblot (IB) and β-tubulin as loading control.


C) Graph showing PI hydrolysis in response to glutamate at different concentrations (10^{-6} - 10^{-2} M) for mGlu1, Vector, and T188A cells.

D) Graph showing viability response to glutamate at different concentrations (10^{-5} - 10^{-2} M) for mGlu1 and T188A cells.

E) Graph showing viability response to glutamate at different concentrations (10^{-5} - 10^{-2} M) for T188A +Scrambled shRNA, T188A +shRNA, T188A +shRNA +βArr1.

F) Graph showing sustained pERK response to glutamate at different concentrations (10^{-5} - 10^{-2} M) for T188A +Scrambled shRNA, T188A +shRNA, T188A +shRNA +βArr1.
Figure 5

A

B

C

D

[Glutamate], M

PI hydrolysis (% of basal)

Transient pERK (% of basal)

Sustained pERK (% of basal)

Viability (% of control)

mGlu1
R323V
K409A

100 150 200 250 300

100 150 200 250 300

100 150 200 250 300

Con Tox 10^{-5} 10^{-4} 10^{-3}

10^{-5} 10^{-4} 10^{-3}

10^{-5} 10^{-4} 10^{-3}
Glutaric Acid, M

Con Tox 10^-5 10^-4 10^-3 10^-2

Viability (% of control)

0 25 50 75 100

+YM (10 μM)

Sustained pERK (% of basal)

100 150 200 250

GA GA + YM

PI hydrolysis (% of basal)

0 100 200 300

1 mM 3 mM 10 mM

GA Glutamate Succinic Acid

Figure 6
Figure 7

A

Viability (% of control)

0 25 50 75 100

Con Tox 10^{-5} 10^{-4} 10^{-3} 10^{-2}

[Glutamate], M

Glu □ + 30 μM Quis ▲ + 300 μM Quis

B

PI hydrolysis (% basal)

100 150 200 250 300 350

10^{-6} 10^{-5} 10^{-4} 10^{-3}

[Glutamate], M

Glu □ + 1 mM GA ▲ + 3 mM GA ▲ + 10 mM GA
Figure 8