NEUROPROTECTIVE SIGNALING THROUGH METABOTROPIC GLUTAMATE RECEPTOR 1

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By

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Metabotropic glutamate receptor 1 (mGlu1) is a G protein-coupled receptor which enhances the hydrolysis of membrane phosphoinositides (PI). Additionally, mGlu1 receptors have been shown to stimulate cytoprotective signaling in the presence of the endogenous ligand glutamate. This goal of the experiments in the following thesis was to (1) characterize the signal transduction pathway through which mGlu1 receptors protect cells expressing these receptors from toxicity. Instead of the classical G protein-mediated (PI) signaling, the following experiments indicate that mGlu1a receptors cause a sustained phosphorylation of ERK that is β-arrestin-1 dependent. This G protein-independent signaling pathway was necessary for protective signaling and was stimulated by glutamate, but not quisqualate, demonstrating ligand bias at this receptor. (2) Therefore, pharmacological and mutational studies were carried out to investigate the molecular basis of this ligand bias. Together, these studies indicate the existence of 3 classes of mGlu1a receptor agonists: (a) unbiased agonists, such as glutamate, which stimulate both signal transduction pathways, (b) biased agonists, such as quisqualate, which only stimulate PI hydrolysis, and (c) agonists which are biased toward sustained ERK phosphorylation and protective signaling. In these studies, glutaric and succinic acid were identified as protection-biased mGlu1a receptor agonists. Further pharmacological studies indicate that quisqualate does not inhibit glutamate-induced protection and glutaric acid fails to inhibit glutamate-induced PI
hydrolysis, suggesting the existence of two distinct, non-overlapping agonist binding sites on mGlu1a receptors.
I wish to extend my sincere gratitude to my family and colleagues who have been so helpful and supportive throughout the process of this thesis. In particular I wish to thank my wife, Maria Jena Emery, for all of her love and care, as well as my parents, Clay and Wendy Emery. My advisor, Dr. Jarda Wroblewski, has served as a kindly and sage mentor throughout my entire tenure in his laboratory and was instrumental through the entire process of this thesis. Many of the experiments performed herein were successful due to the assistance of Dr. Sergey Pshenichkin, Ewa Grajkowska, and Rodrigue Takoudjou, S.J. Furthermore, Drs. Barry Wolfe and Bob Yasuda were extremely helpful in regular discussion of experimental methods and results. I remain especially grateful to Dr. Jean Wrathall, who provided grant support to me from the Training Program in Neural Injury and Recovery for the entirety of my thesis research.

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Asp, L-aspartate; CHO, Chinese hamster ovary; CPCCOEt, 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester; DHPG, (S)-3,5-Dihydroxyphenylglycine; DTT, dithiothreitol; ELISA, Enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; GA, L-glutaric acid; Glu, L-glutamate; GPCR, G protein-coupled receptor; IP, inositol phosphate; JNJ16259685, (3,4-dihydro-2H-pyrano[2,3]b quinolin-7-yl) (cis-4-methoxycyclohexyl) methanone; L-CA, L-cysteic acid; LY367385, (+)-2-Methyl-4-carboxyphenyl glycine; MAPK, mitogen-activated protein kinase; MEK, ERK kinase; mGlu, metabotropic glutamate; MTT, [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]; PI, phosphatidylinosites; PKC, Protein kinase C; PLC, Phospholipase C; Quis, L-quisqualate; TPA, 12-O-tetradecanoylphorbol-13-acetate; YM 298198, 6-Amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide hydrochloride.
CHAPTER 1

Thesis Introduction
INTRODUCTION

Properties of metabotropic glutamate receptors

Metabotropic glutamate (mGlu) receptors are a family of G protein-coupled receptors linked to multiple second messenger systems (Conn and Pin, 1997). Eight mGlu receptors have been cloned and have been categorized into three groups based on sequence homology and pharmacology (Conn and Pin, 1997; Pin and Duvoisin, 1995). Group I includes mGlu1 and mGlu5, group II mGlu2 and mGlu3, and group III mGlu4, mGlu6, mGlu7, and mGlu8. These receptors form family C of G protein coupled receptors, which also includes the parathyroid calcium-sensing receptor (Garrett et al., 1995), the GABAβ receptor (Kaupmann et al., 1997), and pheromone receptors (Matsunami and Buck, 1997). Structural features of this family of receptors include a large extracellular domain containing an agonist binding site (Takahashi et al., 1993), seven transmembrane spanning domains, and a variable-length intracellular C-terminal domain (Bhave et al., 2003). The second intracellular loop and portions of the C-terminus are responsible for binding of G proteins and therefore for the coupling of mGlu receptors to the different second messenger systems (Gomeza et al., 1996a; Pin et al., 1994).

Initial evidence for the existence of mGlu1 receptors came from reports which described the ability of glutamate to stimulate the accumulation of inositol phosphates in cultured striatal and cerebellar granule neurons (Nicoletti et al., 1986; Sladeczek et al., 1985). Two laboratories later cloned these receptors (Houamed et al., 1991; Masu et al., 1991) and further studies demonstrated that group I mGlu receptors stimulate phospholipase Cβ (PLC) via coupling to G_{q/11} (Aramori and Nakanishi, 1992; Pickering et al., 1993). Activated PLC cleaves
phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol. Inositol phosphates then cause calcium release from intracellular stores, which happens due to activation of both IP$_3$ receptors and ryanodine receptors, both of which are located on the surface of the endoplasmic reticulum (del Rio et al., 1999). Released calcium and diacylglycerol cause the phosphorylation of protein kinase C (Nishizuka, 1995). Furthermore, agonist stimulation of group I mGlu receptors has more recently been shown to cause a transient protein kinase C-dependent phosphorylation of extracellular signal-regulated kinase (ERK) (Choe and Wang, 2001; Ferraguti et al., 1999; Karim et al., 2001).

**Physiological roles of mGlu1 receptors**

Metabotropic glutamate receptor 1 is highly expressed in the granule and Purkinje cells of the cerebellum, in the substantia nigra pars compacta, in the septal nuclei, in the relay nuclei of the dorsal thalamus, and throughout the dorsal pallidum (Martin et al., 1992). Studies of the localization of mGlu1 receptors have indicated that they are most highly-expressed on postsynaptic membranes at excitatory synapses (Baude et al., 1993). Most physiological studies on mGlu1 receptors have been conducted to investigate its roles in cerebellar long-term depression at the parallel fiber-Purkinje cell synapse (Kano et al., 2008). Knockout mice lacking the gene (GRM1) for mGlu1 receptors are ataxic and have impaired motor learning skills and cerebellar LTD (Aiba et al., 1994). Reintroduction of mGlu1 receptors in these mice corrects ataxia and aberrant cerebellar LTD (Ichise et al., 2000). Additionally, mGlu1 receptors seem to play a role in the synaptogenesis of the cerebellar cortex since GRM1 knockout mice have impaired synapse elimination at parallel fiber-Purkinje cell synapses (Kano et al., 1997). In
addition to its roles in synaptic plasticity, mGlu1 receptors have been shown to mediate slow EPSPs at these synapses (Batchelor et al., 1997).

**Pharmacology of Group I Metabotropic Glutamate Receptors**

Pharmacologically, group I mGlu receptors can be distinguished from other groups of mGlu receptors by the action of their most potent agonist quisqualate, and by the group I-selective agonist 3,5-dihydroxyphenylglycine (DHPG) (Ito et al., 1992; Schoepp et al., 1994). The nonselective agonist of mGlu receptors 1-Aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD) stimulates PI hydrolysis through mGlu1 receptors, however it is less potent than the endogenous agonist glutamate (Thomsen et al., 1993). L-aspartate also acts as an agonist at mGlu1 receptors, but it is also active at NMDA receptors (Chen et al., 2005), which is probably responsible for limiting its use on mGlu1 receptors in the literature. Additionally, sulfur-containing amino acids, such as L-cysteic acid and L-homocysteic acid have been shown to possess the properties of agonists at mGlu1 receptors (Porter and Roberts, 1993; Shi et al., 2003). Despite the relatively high number of available ligands for mGlu1 receptors, an agonist which is selective for mGlu1 receptors and inactive at mGlu5 receptors has not yet been identified.

Competitive antagonism of mGlu1 receptors was initially accomplished using several analogs of phenylglycine, most notably MCPG (Eaton et al., 1993). None of these compounds are selective for mGlu1 receptors (Hayashi et al., 1994), but further modifications led to the development of LY367385, which is moderately potent and for mGlu1 receptors with little action at mGlu5 receptors (Clark et al., 1997). Additionally, 3-MATIDA, a rigidified
phenylglycine analog, selectively competitively inhibits mGlu1 receptors (Moroni et al., 2002). The most potent and selective antagonists at mGlu1 receptors are the noncompetitive antagonists, which bind to an allosteric site situated on the seventh transmembrane domain of the receptor (Litschig et al., 1999). A number of these compounds exist and are typified by the first to be synthesized, CPCCOEt (Hermans et al., 1998). Subsequently, more selective and potent noncompetitive antagonists which are systemically active have been discovered, and these compounds include YM-298198 (Kohara et al., 2005), and JNJ 16259685 (Lavreysen et al., 2004).

**Metabotropic Glutamate Receptors in Neuroprotection**

Several studies indicate that activation of group I mGlu receptors promotes neuronal death. Such results have been demonstrated in an *in vivo* model of rat traumatic brain injury and in an *in vitro* model of traumatic injury of rat cortical neurons (Mukhin et al., 1996). The toxic effects of group I mGlu receptors appear to be mediated through mechanisms including the activation of protein kinase C (Bruno et al., 1995), potentiation of NMDA and AMPA currents (Aniksztejn et al., 1992; Bleakman et al., 1992; Harvey and Collingridge, 1993; Meguro et al., 1992), and the activation of voltage-gated calcium channels (Chavis et al., 1995). In contrast, multiple other studies indicate that in the presence of glutamate, mGlu1 induces signaling that facilitates growth and development as opposed to neurotoxicity. Activation of mGlu1 receptors has been shown to increase tolerance to ischemia in the hippocampus, thus minimizing cellular damage due to ischemia (Werner et al., 2007). When stimulated with glutamate, mGlu1 has been shown to stimulate axon elongation in the developing central nervous system (Kreibich et al., 2004) and
outgrowth of dendritic spines in the developing hippocampus (Vanderklish and Edelman, 2002). It has recently been described that mGlu1a produces dual neuroprotective and neurotoxic signaling in cerebellar and cortical neurons (Pshenichkin et al., 2008). Thus, mGlu1a exhibits the properties of a dependence receptor (Chao, 2003; Mehlen and Bredesen, 2004), inducing apoptosis in the absence of glutamate, while promoting neuronal survival in its presence. However, the molecular mechanisms of this neuroprotective signaling are unknown.

### Regulation of mGlu1 Receptors by β-arrestin

Like other G protein-coupled receptors, desensitization and internalization of mGlu1 receptors occurs both constitutively and in response to agonist stimulation. Initial studies on the desensitization of mGlu1 receptors implicated protein kinase C (PKC), since phorbol esters apparently increased desensitization of mGlu1 receptors and these increases were blocked by inhibitors of PKC (Catania et al., 1991). Further studies on PKC regulation of mGlu1 receptors indicate that PKC phosphorylates the receptor on the second intracellular loop (Alaluf et al., 1995), which is where the G protein binds (Gomeza et al., 1996b), thus reducing G protein-dependent activity through mGlu1 receptors. Along with PKC-mediated desensitization, in the presence of an agonist, mGlu1 receptors undergo rapid homologous desensitization and are endocytosed in a dynamin and β-arrestin-1-dependent manner (Mundell et al., 2001). During this process, mGlu1 may be phosphorylated by several kinases. Homologous desensitization and endocytosis likely require the activity of G-protein coupled receptor kinases (GRKs). Multiple GRK isoforms, including GRK2, GRK4, GRK5, and GRK6 have been shown to have the potential to phosphorylate mGlu1 (Dhami and Ferguson, 2006), however it remains unclear
which of these kinases phosphorylate native mGlu1 receptors. One study indicates that GRK2 regulates the desensitization of mGlu1 via a physical interaction with the second intracellular loop (Dhami et al., 2004). However, phosphorylation of the C-terminus of mGlu1 receptors by GRK4 appears to be necessary for β-arrestin1 binding (Iacovelli et al., 2003). Additionally, the association of mGlu1 receptors with β-arrestin has been also shown to induce receptor-mediated transient phosphorylation of ERK (Iacovelli et al., 2003).

**Cellular Signaling through β-arrestin**

ERK functions in the MAP kinase pathway downstream of MEK1/2 and the phosphorylation of ERK is a critical step in signal transduction from the membrane to the nucleus and usually causes protective or mitogenic cellular responses (Seger and Krebs, 1995). Although a relatively recently described signal transduction pathway, numerous G protein-coupled receptors have been shown to stimulate ERK phosphorylation in a β-arrestin-dependent manner (Kenakin, 2007). Through numerous receptors, ERK can be activated by both β-arrestin dependent and G protein-dependent signaling pathways (Wei et al., 2003). In several systems, including PAC1 and VPAC receptors, these parallel pathways have been described where ERK phosphorylation due to G protein activation is transient, whereas phosphorylation is sustained over time when ERK is bound to β-arrestin (Broca et al., 2009). Transient, G protein-dependent ERK phosphorylation is terminated by the action of β-arrestin, which is critical for the desensitization and internalization of G protein-coupled receptors (Lohse et al., 1990).

Anti-apoptotic signaling through β-arrestin-dependent pathways has been demonstrated for numerous G protein-coupled receptors. There is evidence that β-arrestin-mediated ERK
phosphorylation is independent from G protein signaling when β-arrestin is bound to a receptor. Some receptors where this signaling mechanism has been characterized include the angiotensin II type 1a receptor (Luttrell et al., 2001), the D3 dopamine receptor (Beom et al., 2004), the β2-adrenergic receptor (Shenoy et al., 2006), and the mu opioid receptor (Macey et al., 2006). While β-arrestin-mediated signaling has not previously been demonstrated due to mGlu1 receptor activation, it is known that β-arrestin1 is involved in rapid agonist-induced internalization of mGlu1 (Dale et al., 2001).

**Ligand Bias**

Through the same receptor, β-arrestin-mediated signaling may not follow an identical pharmacological profile as classical G protein-mediated modes of signal transduction. This phenomenon is known as ligand bias, which is a new and emerging concept in pharmacology that has been shown to exist for multiple G protein-coupled receptors when various agonists preferentially activate receptor conformations that are selectively conducive for different signal transduction pathways (Violin and Lefkowitz, 2007). The earliest reported example of ligand bias was in M1 muscarinic receptors, which activate both cAMP and PLC in response to their endogenous agonist acetylcholine. However, several ligands were identified that selectively activate PLC while blocking cAMP formation through these receptors (Fisher et al., 1993). Since this initial report of ligand bias at different modes of G protein-mediated signaling through a single receptor, ligands have been identified preferentially activate β-arrestin-mediated signaling which is independent of G protein signaling (Rajagopal et al., 2010).
MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's media (DMEM) and Neurobasal media, B27 supplement, fetal bovine serum, and all other reagents used for cell cultures were purchased from Invitrogen (Carlsbad, CA). All restriction enzymes used for molecular biology were purchased from New England Biolabs (Ipswich, MA). Receptor agonists glutamate, aspartate, cysteic acid, quisqualate, and DHPG, antagonists LY367385, 3-MATIDA, CPCCOEt, YM298198, JNJ16259685, and inhibitors U73122, dynasore, U0126, and PD98059 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 (American Type Culture Collection, Manassas, VA) were stably transfected with mGlu1a receptor cDNA in pcDNA-3.1 vector (Invitrogen) using Lipofectamine 2000 transfection reagent (Invitrogen). Cell lines were created from mass stable transfections and selected with the antibiotic G-418 (Invitrogen). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 5% L-proline (Invitrogen). Primary cultures of cerebellar granule neurons were prepared as described previously (Wroblewski et al., 1985). Cerebella were dissected from 7-day old Sprague-Dawley rats and granule cells were grown on poly-D-lysine coated 96-well plates (Nunc, Wiesbaden, Germany) in Neurobasal medium containing B27 supplement, 2 mM glutamine, 100 µg/ml gentamicin, and either 5 mM KCl or 25 mM KCl. Cytosine arabinoside (10 µM) was added to granule cells the day after plating to prevent growth of non-neural cells.
Transfection of cells with shRNA. SureSilencing shRNA plasmids against β-arrestin-1 (insert sequence: ATGGAGGAAAGCTGATGATACT) and scrambled control were contained in the pGeneClip Hygromycin vector (SABiosciences, Frederick, MD). CHO cells stably expressing mGlu1a were transfected with plasmids containing shRNA using Effectene transfection reagent (Quiagen, Valencia, CA) and selected in 0.8 mg/ml Hygromycin B. Knockdown of β-arrestin-1 was confirmed by Western blotting. Rescue experiments were performed by transfecting cells expressing shRNA with human β-arrestin-1, which was subcloned in pcDNA-6.2 (Invitrogen).

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 was mixed with 125 ng of two mutagenic primers (sequences in Table 3), dNTPs (50 μM) and 2.5 unit of Pfu DNA polymerase in a final volume of 50 μl. 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), p44/42 MAPK (ERK1/2; Cell Signaling Technology, Danvers, MA), Phospho-p44/42 MAPK (ERK1/2) (Thr\textsuperscript{202}/Tyr\textsuperscript{204}; Cell Signaling Technology), β-actin (Sigma), β-tubulin (Sigma), and β-arrestin-1 (Abcam, Cambridge, MA). Proteins were visualized by incubation with secondary antibodies coupled to horseradish peroxidase (Pierce) followed by exposure to chemiluminescent HRP substrate SuperSignal West Femto (Pierce) and imaged with a CCD camera. Blots were quantified using ImageJ software.

Receptor internalization assays. Receptor endocytosis was assayed as described previously (Jeanneteau et al., 2006). Cells grown in 6 cm dishes were washed in PBS and labeled for 30 minutes with cleavable Sulfo-NHS-SS-Biotin (Pierce), which was in a solution of PBS. For 20 minutes, excess biotin was quenched with 100 mM glycine. Labeled cells were then incubated for 30 minutes in the absence or presence of glutamate at 37°C. Stimulation was halted by three washes with ice cold PBS. Extracellular biotin was then cleaved by 2 washes with 50 mM glutathione, 75 mM NaCl, 5 mM NaOH, 10% FBS in water for 15 minutes. Excess glutathione was then quenched for 30 min in PBS containing 50 mM iodoacetamide and 1% BSA. Cells were then lysed in RIPA buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, and Halt protease inhibitor cocktails) and clarified, and biotinylated proteins were precipitated with NeutrAvidin-agarose (Pierce). Proteins were rinsed 4 times with RIPA buffer, solubilized in Laemmli buffer, and resolved by SDS-PAGE.

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 permeablization, endogenous peroxidase activity was quenched by 20 minutes incubation in PBS containing 0.5% H$_2$O$_2$ and 0.2% NaN$_3$. After three more washes in PBST, cells were blocked with 2% BSA for one hour and incubated overnight with primary antibody against Phospho-p44/42 MAPK (ERK1/2) (Thr$^{202}$/Tyr$^{204}$) (Cell Signaling Technology). Cells were then washed for five minutes three times in PBST and twice in PBS. A HRP-coupled goat anti-rabbit secondary antibody (Pierce) was incubated for one hour at room temperature and then cells were again washed five times. Cells were exposed to the colorimetric HRP substrate 1-Step Ultra TMB (Pierce). After at least 10 minutes of developing, the reaction was stopped in 4 M H$_2$SO$_4$ 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 hour 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 NaHCO$_3$, 1 mM MgCl$_2$, 1.3 mM CaCl$_2$, 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 10 mM formic acid for 30 min. Samples were transferred to opaque-welled plates and incubated with polylysine coated yttrium scintillation proximity assay (SPA) beads (Perkin Elmer) and incubated at room temperature for 1 hour with vigorous shaking. After 8 hours of incubation with SPA beads, inositol phosphates were detected by scintillation counting.

*Calculations and Statistics.* For dose-response data, curves were fitted by nonlinear regression to data points using the four-parameter logistic equation in Sigma Plot. Significance testing was performed using Student’s *t* tests when comparing two groups or by Bonferroni-corrected *t* tests when comparing multiple groups. Statistical significance was deemed by *p* < 0.01.
CHAPTER 2
The Protective Signaling of Metabotropic Glutamate Receptor 1 is Mediated by Sustained β-Arrestin-1-Dependent ERK Phosphorylation

INTRODUCTION

Metabotropic glutamate receptor 1 (mGlu1) is a G protein-coupled receptor that is linked to various second messenger systems through its association with Gq. Numerous studies have indicated a role of mGlu1 receptors in synaptic physiology, notably including systems of long-term depression at the parallel fiber-Purkinje cell synapse in the cerebellum (Kano et al., 2008). Additionally, several other studies indicate that mGlu1 receptors induce signaling that facilitates cellular growth and development. In the presence of glutamate, mGlu1 receptors have been shown to stimulate axon growth in the developing brain (Kreibich et al., 2004) and the outgrowth of dendritic spines in the developing hippocampus (Vanderklish and Edelman, 2002).

Recent evidence has demonstrated that mGlu1 receptors stimulate signaling which protects cultured cerebellar and cortical neurons from apoptosis (Pshenichkin et al., 2008). However, the mechanisms of this neuroprotective signaling remained unknown. In these studies, the protective effect of glutamate followed a different pharmacological profile than for PI hydrolysis, the known signaling mechanism of the receptor. Therefore, the following studies were designed to ascertain whether this signaling followed the known G protein-dependent signaling mechanism or a novel G protein-independent mechanism.

The aim of these studies was to identify the signal transduction pathway through which glutamate causes protective signaling in cells expressing mGlu1a receptors. The following data determined that glutamate, but not quisqualate, stimulates a sustained phosphorylation of ERK through mGlu1a receptors. This phenomenon was unique to mGlu1a receptors and required the expression of β-arrestin-1. Moreover, inhibition of ERK phosphorylation and silencing of β-
arrestin-1 expression abolished the protective effects of glutamate. Therefore, the protective signaling of mGlu1a receptors does not rely on the classical, G protein-mediated, mechanism of signal transduction, but, instead involves a β-arrestin-dependent receptor internalization and ERK phosphorylation.
RESULTS

Previous studies in primary cultures of cortical and cerebellar neurons have shown that glutamate, acting selectively at mGlut1a receptors, rescued these neurons from apoptotic cell death induced by conditions of trophic deprivation (Pshenichkin et al., 2008). This protective effect of glutamate was revealed in the presence of antagonists of ionotropic glutamate receptors which suppressed the excitotoxic actions of glutamate. Moreover, in these cells, the protective effect was elicited by high concentrations of glutamate, about 10 times higher than those needed to activate mGlut1-mediated PI hydrolysis.

In order to study the pharmacological and protective properties of mGlut1a without interference from other glutamate receptors, CHO cells with stably expressed mGlut1a receptors were used to assess the potency of mGlut1 agonists in producing the protective effect. Cells were transferred to serum-free culture medium to induce apoptosis (Zhong et al., 1993) and their viability was monitored by the MTT assay. Under these conditions, after 3 days, cells exhibited approximately 35-50% viability relative to controls grown in serum-containing medium. Cells exposed to 3 mM glutamate were fully protected from serum deprivation-induced apoptosis for up to 6 days, having similar viability to control cells grown in serum-containing growth media while lower concentrations of glutamate (0.3 mM) produced a partial protection from serum deprivation (Figure 1). The protective effect of glutamate required the expression of mGlut1a receptors since untransfected CHO cells were not protected from serum deprivation (Figure 2). Also, among PLC-coupled mGlu receptors only mGlu1, but not mGlu5, was effective in protecting from toxicity induced by serum deprivation (Figure 2).
Figure 1. Time course of protection by glutamate in CHO cells expressing mGlu1a receptors. CHO cells stably expressing mGlu1a receptors were placed in serum-free media to induce toxicity. In the presence of glutamate (Glu), these cells were protected from toxicity. Data points represent means and error bars are S.E.M. from three independent time-course series of MTT assays performed in triplicate.
Figure 2. Efficacy of glutamate to protect CHO cells. CHO cells were stably transfected with mGlu1a receptors or mGlu5a receptors cells and were subjected to serum deprivation for three days in the presence or absence of 1 mM glutamate (Glu). Glutamate protected only CHO cells expressing mGlu1a receptors. Values represent means and error bars represent S.E.M. from three independent series of MTT assays performed in triplicate. **p<0.001 as compared to untreated controls using Student's t-test.
The addition of glutamate to the serum-free culture medium produced a dose-dependent increase in viability with an EC$_{50}$ of 153 ± 31 μM; however, quisqualate, the most potent mGlu1 agonist, was not effective in concentrations up to 3 mM (Figure 3). In order to further ascertain that the protective effect is in fact due to the activation of mGlu1a receptors, and not to a nonspecific effect involving glutamate transporters or another mechanism, glutamate-induced protection was tested in the presence of three distinct noncompetitive mGlu1-selective antagonists. Application of 100 μM CPCCOEt, 10 μM YM-298198, or 30 μM JNJ16259685 completely inhibited the activity of 1 mM glutamate (Figure 4). Effective concentrations of these antagonists were derived from pilot experiments evaluating their effects on mGlu1 receptor-mediated PI hydrolysis (data not shown). A more detailed analysis showed that YM-298198 inhibited glutamate-induced protection in a noncompetitive manner with increasing concentrations of the antagonist decreasing glutamate efficacy but having no effect on its potency as shown by similar glutamate EC$_{50}$ values in the presence of different concentrations of antagonist (Figure 5, Table 1).

The observed pharmacological properties of mGlu1a in inducing the protective effect were inconsistent with its known properties in stimulating G protein-mediated phosphoinositide (PI) hydrolysis (Aramori and Nakanishi, 1992). To study this discrepancy, the potency of the agonists to stimulate PI hydrolysis in CHO cells expressing mGlu1a that were used in these experiments was determined. As shown in Figure 6, both glutamate and quisqualate stimulated PI hydrolysis in a dose-dependent manner with quisqualate being more potent (EC$_{50}$ = 0.63 ± 0.13 μM) than glutamate (EC$_{50}$ = 16 ± 1.2 μM). Hence, the potency of glutamate to induce protection was approximately 10 times lower than its potency to stimulate PI hydrolysis.
**Figure 3. Potency of glutamate to protect CHO cells expressing mGlu1a receptors.** CHO cells stably expressing mGlu1a receptors were subjected to serum withdrawal for three days in the absence or presence of receptor agonists. Glutamate (Glu) was effective, \( \text{EC}_{50} = 154 \pm 31 \, \mu\text{M} \), whereas quisqualate (Quis) failed to protect cells at concentrations as high as 3 mM. Data points are means and error bars are S.E.M. from three independent MTT assays performed in triplicate.
**Figure 4. Inhibition of the protective effect of glutamate by mGlu1-selective noncompetitive antagonists.** CHO cells stably expressing mGlu1a receptors were subjected to serum withdrawal for three days in the absence or presence of 1 mM glutamate (Glu). CP –CPCCOEt (100 μM), YM – YM-298198 (10 μM), and JN – JNJ16259685 (30 μM). Values are means from at least three independent experiments performed in triplicate with error bars representing S.E.M. **p<0.001 as compared to untreated controls using Student’s t-test.
Figure 5. **Noncompetitive inhibition of protection by glutamate.** Dose-response curves of glutamate-mediated protection in the presence of varying concentrations of YM-298198 (YM). Cell viability was measured by MTT assay after 72 hours of incubation with the ligands in serum-free conditions and is expressed as a percent of cells viable in parallel cultures grown in the presence of serum. All data points are means from at least three independent experiments performed in triplicate with error bars representing S.E.M.
**Table 1. Summary of mGlu1a receptor pharmacology.** Comparison of potency and efficacy of glutamate to induce protection and stimulate PI hydrolysis in the absence and presence of a non-competitive antagonist YM-289198 in CHO cells expressing mGlu1a receptors. Potency (EC\(_{50}\)) refers to the concentration of glutamate producing a half-maximal effect. Efficacy (E\(_{\text{max}}\)) represents the maximal effect of glutamate expressed as a percent of control cell viability in the presence of serum for toxicity experiments and as a percent of basal activity for measurements of PI hydrolysis. Data are averages ± SEM calculated from dose-response curves obtained in 3-6 experiments. *p<0.05 compared to values obtained in the absence of antagonist using Bonferroni-adjusted t-test.

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Table 1. Summary of mGlu1a receptor pharmacology. Comparison of potency and efficacy of glutamate to induce protection and stimulate PI hydrolysis in the absence and presence of a non-competitive antagonist YM-289198 in CHO cells expressing mGlu1a receptors. Potency (EC\(_{50}\)) refers to the concentration of glutamate producing a half-maximal effect. Efficacy (E\(_{\text{max}}\)) represents the maximal effect of glutamate expressed as a percent of control cell viability in the presence of serum for toxicity experiments and as a percent of basal activity for measurements of PI hydrolysis. Data are averages ± SEM calculated from dose-response curves obtained in 3-6 experiments. *p<0.05 compared to values obtained in the absence of antagonist using Bonferroni-adjusted t-test.
Figure 6. **Pharmacology of PI hydrolysis through mGlu1a receptors.** In CHO cells stably expressing mGlu1a receptors, PI hydrolysis is activated in a dose-dependent manner by glutamate (Glu; EC$_{50}$ = 11 ± 1.2 μM) and quisqualate (Quis; EC$_{50}$ = 0.63 ± 0.13 μM). Glutamate-induced PI hydrolysis is noncompetitively inhibited by 10 μM YM298198 (YM). Data points represent means from three independent experiments performed in triplicate with error bars corresponding to S.E.M.
One possible explanation for these differences would be that the potencies observed in protection reflect an artefactual decrease of agonist concentrations occurring during the 3 day incubation that would not appear in the 45 minute incubation used when measuring PI hydrolysis. In control experiments testing agonist stability, glutamate and quisqualate were incubated with CHO cells for 3 days and then used to stimulate PI hydrolysis. As shown in Figure 7, both conditioned and freshly prepared agonists stimulated PI hydrolysis with the same potency, demonstrating that these compounds remain intact for the entire 3 day incubation and that the observed discrepancies do not result from a decrease in agonist concentration.

As expected, glutamate-stimulated PI hydrolysis was abolished by the selective noncompetitive mGlu1 antagonist YM-298198 and the antagonism appeared noncompetitive as seen by the decrease of the maximal effect of glutamate with increasing concentrations of the antagonist (Figure 8). However, in contrast with the data obtained for glutamate protective signaling, the calculated potency of glutamate in PI hydrolysis experiments decreased with increasing antagonist concentrations (Table 1). Usually, such right-shifts of agonist potency in the presence of a noncompetitive antagonist indicate the existence of receptor reserve (also known as spare receptors) and are frequently seen in heterologous expression systems. This leads to the conclusion that the apparent difference in potency of glutamate to produce both effects results from the presence of a high receptor reserve for PI hydrolysis but no receptor reserve for protective signaling. The use of the highest concentration of YM-298198 in PI hydrolysis experiments increases the EC_{50} of glutamate to the levels observed in protection experiments. Hence, once this receptor reserve is abolished by the use of a noncompetitive antagonist the potency of glutamate to produce both effects is the same.
Figure 7. **Bioassay demonstrating the stability of glutamate and quisqualate.** Stimulation of PI hydrolysis in CHO cells expressing mGlu1a receptors by fresh agonists and agonists conditioned in the presence of CHO cells for 3 days. EC$_{50}$ for fresh and conditioned agonists was as follows: glutamate (19 ± 3.2 μM and 12 ± 2.6 μM) and quisqualate (0.23 ± 0.11 μM and 0.24 ± 0.13 μM). All data points are means from three independent experiments performed in triplicate with error bars representing S.E.M.
Figure 8. **Receptor reserve for mGlu1a receptor-mediated PI hydrolysis.** Dose-response curves of glutamate-induced PI hydrolysis in the presence of varying concentrations of mGlu1-selective noncompetitive antagonist YM-298198 (YM). With increasing concentrations of YM, the EC$_{50}$ for glutamate increases (see Table 1), indicating the presence of receptor reserve. All data points are means from three independent experiments performed in triplicate with error bars representing S.E.M.
While these pharmacological results confirm that the protective signaling and PI hydrolysis are mediated by the same receptor, they also indicate the existence of ligand bias in producing the two effects. Ligand bias is defined as the ability of some agonists, such as quisqualate, to activate only selected signal transduction mechanisms while other agonists, such as glutamate, may activate all signaling associated with this receptor (Kenakin, 2007).

**Signal transduction of protective signaling through mGlu1.**

To identify the mechanism through which mGlu1a elicits protective signaling, a selective inhibitor of signaling which occurs downstream of G protein activation was used. Since mGlu1 typically couples to phospholipase C (PLC) through Gq/11 (Aramori and Nakanishi, 1992), transfected CHO cells were treated with the PLC inhibitor U73122 (Bleasdale et al., 1990). In conditions of serum deprivation for 3 days U73122 (30 μM) failed to block the protective effect of glutamate (Figure 9A). In control experiments, 30 μM culture-conditioned U73122 effectively blocked glutamate-induced PI hydrolysis (Figure 9B), indicating that this compound is stable in these culture conditions and is effective in blocking PI hydrolysis in this model. These data, therefore, indicate that the protective effect is not mediated by the coupling of mGlu1a receptors to PLC. These data suggest that the protective effect mediated by mGlu1a receptor is not related to its ability to stimulate PI hydrolysis but, rather, is mediated by a different signal transduction mechanism.
Figure 9. **Glutamate-induced protection is PLC-independent but MEK and dynamin-dependent.** (A) CHO cells expressing mGlu1a were subjected to serum deprivation for 72 hours in the presence of glutamate (Glu) and inhibitors U73122 (30 μM), PD98059 (10 μM), U0126 (10 μM), or dynasore (100 μM). Phospholipase C inhibitor U73122 fails to block protection by glutamate, whereas protection is blocked by MEK1 inhibitor PD98059, MEK 1/2 inhibitor U0126, and dynamin inhibitor, dynasore. **p < 0.001 as compared with serum-deprived controls using Bonferroni-adjusted t test. (B) Inhibition of glutamate-induced PI hydrolysis in mGlu1a-expressing CHO cells by U73122 (30 μM) that is freshly prepared (U73) or preconditioned (U73-C) by exposure to CHO cells for 3 days, indicating U73122 is stable for the entire 3 day incubation period. Values are means from at least three independent experiments performed in triplicate with error bars representing S.E.M.
Since mGlu1 has also been reported to activate the MAP kinase pathway (Sheffler and Conn, 2008), the involvement of this pathway in the protective effect of glutamate was investigated. Indeed, treatment of mGlu1a-expressing CHO cells with MEK1 inhibitor PD98059 (Rosen et al., 1994) or with the MEK1/2 inhibitor U0126 (Favata et al., 1998) abolished the efficacy of glutamate to cause protective signaling (Figure 9A).

Furthermore dynasore, an inhibitor of dynamin, a protein in the endocytotic pathway for mGlu1 (Mundell et al., 2001), also abolished glutamate-induced protection. In control experiments, monitoring receptor trafficking, dynasore (100 μM) blocked glutamate-induced internalization of mGlu1 receptors (Figure 10A, 10B). These data suggest that the protective effect of glutamate is not mediated by the classical G protein-mediated mechanism, but instead, a G protein-independent activation of MAP kinase pathway, most probably involving receptor internalization and the phosphorylation of ERK. Additionally, these studies were replicated in cultured cerebellar granule cells, which natively express mGlu1 receptors (Pshenichkin et al., 2008). Cerebellar granule cells require 25 mM extracellular potassium (K25) to survive in culture. Reducing the potassium concentration to 5 mM (K5) caused granule cells to exhibit an approximately 50% reduction in viability compared to K25 controls (Figure 11). Glutamate protected these cells from low potassium-induced apoptosis, and protection was inhibited by both MEK inhibitors, 30 μM U0126 and 30 μM PD98059, as well as dynasore (100 μM). As with transfected CHO cells, the PLC inhibitor U73122 (30 μM) failed to attenuate the protective effect of glutamate (Figure 11).
**Figure 10.** **Internalization of mGlu1a receptors is dynamin-dependent.** (A) Inhibition of glutamate-induced internalization of mGlu1a receptors by the dynamin inhibitor dynasore (100 μM). Cells were labeled with cleavable biotin and stimulated with 1 mM glutamate for 1 hour. Extracellular biotin was removed and internalized mGlu1a receptors were pulled down in NeutrAvidin. P, pellet (internalized during incubation); S, supernatant (remaining receptors). (B) Densitometric quantification of Western blot from Fig. 10A.
Figure 11. Glutamate-induced protection is PLC-independent but MEK and Dynamin-dependent in neurons. Cerebellar granule cells were subjected to low potassium (K5) for 12 hours to induce apoptosis in the presence of glutamate (Glu) and inhibitors U73122 (30 μM), PD98059 (30 μM), U0126 (30 μM), or dynasore (100 μM). Phospholipase C inhibitor U73122 fails to block protection by glutamate, whereas protection is blocked by MEK1 inhibitor PD98059, MEK 1/2 inhibitor U0126, and dynamin inhibitor, dynasore.
Time-course and pharmacology of mGlu1-mediated ERK phosphorylation.

Previous reports have demonstrated that the stimulation of group I mGlu receptors causes transient ERK phosphorylation that occurs due to G protein activation and subsides within 30 minutes of agonist application (Karim et al., 2001). As shown by Western blot (Fig. 12A, 12B), in CHO cells expressing mGlu1, glutamate and quisqualate increased ERK phosphorylation at 5 minutes after agonist application. In addition, when agonists were added for 24 hours, glutamate, but not quisqualate, produced a sustained ERK phosphorylation. As shown in Fig. 12A, both isoforms of ERK were equally phosphorylated.

These effects were further quantified using an ELISA-based assay to compare the levels of ERK phosphorylation in CHO cells expressing either mGlu1a or mGlu5a receptors. In cells expressing mGlu5a both glutamate and quisqualate elicited a transient ERK phosphorylation which was elevated at 5 minutes after the addition of agonists, and subsided after 30 minutes (Figure 13B). However, in cells expressing mGlu1a, while both agonists induced a transient increase in ERK phosphorylation, after the addition of glutamate ERK phosphorylation was elevated up to 24 hours (Figure 13A).

A closer look at the pharmacology of ERK phosphorylation revealed that the transient, 5 minute, phosphorylation was induced by both glutamate and quisqualate (Figure 14A), with a pharmacological profile and agonist potencies similar to those observed for PI hydrolysis (Figure 6). Also like PI hydrolysis, agonist-induced ERK phosphorylation was abolished by the mGlu1-selective noncompetitive antagonist YM-298198 (Figure 14A). In contrast, the sustained 24 hour ERK phosphorylation was only induced by glutamate, but not by quisqualate, and was blocked by YM-298198 (Figure 14B).
Figure 12. ERK phosphorylation due to mGlu1 agonists. (A) Representative Western blot of phosphorylated ERK (pERK) and total ERK (ERK) at 5 minutes and 24 hours after the application of glutamate or quisqualate to CHO cells expressing mGlu1a receptors. (B) Densitometric quantification of Western blot from A, expressed as the ratio of measured intensities for the sum of pERK bands divided by total ERK. 12-O-tetradecanoylphorbol-13-acetate (TPA), which activates PKC, was used as positive control.
Figure 13. **ELISA-based quantification of phosphorylated ERK.** CHO cells expressing mGlu1a receptors (A) or mGlu5a receptors (B) were treated with agonists glutamate (Glu) or quisqualate (Quis) for varying times. Values are means from at least three experiments performed in triplicate with error bars representing S.E.M. and are expressed as the percent of ERK phosphorylation measured in cells not treated with agonists. *, $p < 0.01$ and **, $p < 0.001$ as compared with untreated controls using Bonferroni-adjusted t test.
Figure 14. **Agonist profiles of ERK phosphorylation through mGlu1a receptors.** CHO cells expressing mGlu1a receptors were stimulated with agonists glutamate (Glu) or quisqualate (Quis) for 5 minutes (A) or 24 hours (B). Glutamate caused an increase in phosphorylated ERK at both times, while quisqualate was only active at the 5 minute time point. All effects were inhibited by 10 μM YM298198 (YM). All data points are means from at least three experiments performed in triplicate with error bars representing S.E.M. and are expressed as the percent of ERK phosphorylation measured in cells not treated with agonists.
The observed agonist potencies were lower than those for PI hydrolysis and transient ERK phosphorylation, but approximated agonist potencies observed for the protective signaling through mGlu1a (Figure 3).

Together, these data indicate that transient (5 minute) ERK phosphorylation is activated by both mGlu1a and mGlu5a receptors and has a pharmacological profile similar to the activation of PI hydrolysis, hence is mediated by a G protein-mediated mechanism. In contrast, the sustained ERK phosphorylation, lasting up to 24 hours, is only activated by mGlu1a, and only glutamate is able to produce the protective mGlu1a signaling. These results further support the existence of a ligand bias which extends to ERK phosphorylation and reveals the existence of two distinct signal transduction mechanisms. The classical mechanism, activated by both glutamate and quisqualate, mGlu1 agonists with an apparent high potency, involves a G protein-mediated PI hydrolysis and transient ERK phosphorylation. The second mechanism, which correlates well with protective mGlu1a signaling, is activated with an apparent lower potency by glutamate and is mediated by a G protein-independent mechanism which leads to prolonged activation of MAPK pathway and sustained ERK phosphorylation.
The role of β-arrestin-1 in sustained ERK phosphorylation and the protective signaling of mGlu1a.

Based on similarities with other GPCRs (Brauner-Osborne et al., 2007) we hypothesized that the mGlu1a-induced phosphorylation of ERK may involve a β-arrestin-1-dependent internalization of mGlu1a receptors and formation of a signaling complex mediating this protective signaling. To investigate this hypothesis the expression of β-arrestin-1 in mGlu1a-expressing CHO cells was silenced using a plasmid encoding shRNA targeted against rat β-arrestin-1. Transfection with this plasmid encoding shRNA caused a substantial decrease in protein expression levels of β-arrestin-1 as monitored by Western blots, while a scrambled shRNA used in control experiments had no effect (Figure 15A, 15B). In rescue experiments, cells expressing targeted shRNA were transfected with cDNA for human β-arrestin-1, which is refractory to the silencing effects of rat shRNA due to having mismatches of several bases. Expression of human β-arrestin-1 caused an approximate 3-fold increase of β-arrestin-1 protein levels relative to untransfected controls (Figure 15A, 15B). Additional control experiments showed that shRNA silencing of β-arrestin-1 failed to decrease agonist-induced PI hydrolysis in CHO cells expressing mGlu1a receptors (Figure 16), suggesting that this shRNA did not significantly affect classical mGlu1 receptor function. In contrast, β-arrestin-1 shRNA effectively abolished the protective effect of glutamate against serum deprivation (Figure 16). Protection by glutamate was restored after transfection of human β-arrestin-1 (Figure 17).
Figure 15. Silencing of β-arrestin-1 with shRNA. (A) Protein levels of β-arrestin-1 in mGlu1a-expressing CHO cells. Samples in lane 3 were transfected with plasmids encoding shRNA targeted against rat β-arrestin-1, which reduces expression to 24% of untransfected controls (lane 1). Scrambled control shRNA does not affect protein levels of β-arrestin-1 (lane 2). Knockdown due to shRNA is reversed by transfection of human β-arrestin-1 (lane 4). Knockdown due to shRNA is reversed by transfection of human β-arrestin-1 (lane 4). Transfection with human β-arrestin-1 causes expression levels of 314% relative to untransfected controls. (B) Densitometric quantification of the Western blot expressed as a ratio of band densities measured for β-arrestin-1 and β-actin.
Figure 16. Knockdown of β-arrestin-1 of does not impair classical mGlu1a receptor signaling. PI hydrolysis was measured in CHO cells expressing mGlu1a and scrambled shRNA; or mGlu1a and shRNA targeted against β-arrestin-1, which were stimulated with agonists glutamate (Glu) or quisqualate (Quis). All data points are means from two experiments performed in quadruplicate with error bars representing S.E.M.
Figure 17. **Protective signaling through mGlu1a is abolished by shRNA silencing of β-arrestin-1.** In shRNA-expressing cells, transfection of human β-arrestin-1 restores protective signaling by glutamate. All CHO cells expressed mGlu1a receptors and were treated with serum deprivation for three days to induce toxicity, which was measured by MTT assays. Data points reflect experiments performed at least four times in triplicate with data points representing means and error bars as S.E.M.
Internalization assays showed that a 30 minute pulse of 1 mM glutamate caused 50% of mGlu1a receptors to become internalized in contrast to control cells, where approximately 18% of mGlu1a receptors were internalized during the 30 minute control incubation (Figure 17A, 17B). Transfection with shRNA targeted toward β-arrestin-1 caused internalization in the presence of 1 mM glutamate to be equivalent with the constitutive internalization seen in cells not exposed to glutamate (Figure 18A, 18B).

Further experiments showed that β-arrestin-1 silencing differentially affected the transient and sustained ERK phosphorylation mediated by mGlu1a receptors. The transient ERK phosphorylation induced by glutamate and quisqualate was reduced by the PLC inhibitor U73122 but not by β-arrestin-1 silencing (Figure 19A). In contrast, the sustained ERK phosphorylation, measured 24 hours after the application of agonist, was activated only by glutamate and was abolished by β-arrestin-1 silencing, but not by the PLC inhibitor U73122 (Figure 19B).

These results show that the classical mGlu1a receptor signaling through PI hydrolysis, which also includes transient ERK phosphorylation, does not depend on the presence of β-arrestin-1. In fact, Figure 16 suggests that PI hydrolysis after silencing of β-arrestin-1 was slightly elevated, which corresponds well to the reported ability of β-arrestin to inhibit G protein-mediated signal transduction (Diviani et al., 1996). In contrast, these results indicate that the presence of β-arrestin-1 is required for both the sustained ERK phosphorylation and the protective mGlu1a signaling.
**Figure 18. Endocytosis of mGlu1a receptors is β-arrestin-1-dependent.** (A) Representative Western blot showing that glutamate (Glu)-induced internalization of mGlu1a is attenuated by shRNA silencing of β-arrestin-1. CHO cells expressing mGlu1a receptors were labeled with cleavable biotin and stimulated with 1 mM glutamate for 1 hour. Extracellular biotin was removed and internalized mGlu1a receptors were pulled down in NeutrAvidin. P, pellet (internalized during incubation); S, supernatant (remaining receptors). (B) Densitometric quantification of three independent internalization assays.
A. Sustained ERK phosphorylation through mGlu1a receptors is β-arrestin-1-dependent. Effect of shRNA silencing of β-arrestin1 on ERK phosphorylation induced by agonists glutamate (Glu) and quisqualate (Quis) after 5 minutes (A) and 24 hours (B). Transient ERK phosphorylation is sensitive to the inhibition of PLC by U73122 (30 μM) (A). Sustained ERK phosphorylation is blocked by shRNA silencing of β-arrestin-1 but is not affected by the inhibition of PLC (B). ERK phosphorylation was measured using ELISA, and the data are expressed as a percent of ERK phosphorylated in the absence of agonists. All values are means from at least three experiments performed in triplicate with error bars representing S.E.M. **p < 0.001) as compared with untreated controls using Bonferroni-adjusted t test.

B.
Figure 20. Schematic representation of signal transduction through mGlu1 receptors.

During short-term treatment with glutamate, mGlu1 receptors stimulate PI hydrolysis via coupling to Gq. After long-term treatment, the receptor is hypothesized to be phosphorylated by a GRK and is internalized in by dynamin and β-arrestin-1 (βarr-1). Once internalized, mGlu1 receptors cause a long-term phosphorylation (P) of MEK and ERK, which leads to cellular survival.
DISCUSSION

Previously it has been shown that glutamate, acting selectively at mGlu1 receptors, protects neurons from apoptotic death induced by trophic deprivation (Pshenichkin et al., 2008). This study demonstrates the existence of a novel signal transduction mechanism that allows mGlu1 receptors to mediate this protective signaling. This mechanism is distinct from the well described classical G protein-mediated signal transduction of group I mGlu receptors (Aramori and Nakanishi, 1992; Pickering et al., 1993) and is not shared by mGlu5 receptors, likely due to differences in the two receptors respective C-termini. Shown here is that in CHO cells expressing mGlu1a receptors, glutamate caused protection from toxicity due to serum deprivation, and this protection was blocked not only by noncompetitive antagonists of mGlu1, but also by the inhibition of receptor internalization by silencing β-arrestin-1 expression and inhibition of dynamin. Furthermore, protective signaling was blocked by inhibitors of MEK1/2. The pathway through which mGlu1 acts appears to be unrelated to classical G protein signaling, as protection was not attenuated by inhibition of phospholipase C. Instead, these data reveal the existence of a previously unreported signaling mechanism associated with mGlu1a receptors, namely a G protein-independent protective signaling pathway that requires β-arrestin-1 and activation of MEK, followed by ERK phosphorylation.

Anti-apoptotic signaling through β-arrestin has been demonstrated for numerous GPCRs (DeWire et al., 2007). As signaling molecules, β-arrestins have been shown to mediate the stimulation of phosphorylation of several protein kinases, including ERK (Daaka et al., 1998). Additionally, β-arrestin-mediated signaling has been shown to be independent of G protein signaling for multiple receptors including the angiotensin II type 1a receptor (Luttrell et al.,
2001), the D3 dopamine receptor (Beom et al., 2004), the β2-adrenergic receptor (Shenoy et al., 2006), and the mu opioid receptor (Macey et al., 2006). Consistent with previous reports that β-arrestin-1 is required for agonist-induced internalization of mGlu1 (Dale et al., 2001), these findings suggest that the protective signaling of mGlu1a may be initiated by agonist-dependent receptor internalization, followed by a non-classical, G protein-independent mechanism of signal transduction. The current results also indicate that, similarly to several other GPCRs (Kenakin, 2007), mGlu1a receptors show a ligand bias towards the different signaling mechanisms, manifested by the ability of glutamate to activate both signaling pathways, while quisqualate activates only G protein-mediated PI hydrolysis.

The protective mGlu1a signaling appears to be mediated by a different mechanism of ERK phosphorylation than that described previously. Several reports show that agonists acting at mGlu1 receptors induce a transient increase of ERK phosphorylation which peaks at 5 minutes and subsides within 30 minutes (Mundell et al., 2001). Consistent with these reports, in CHO cells expressing mGlu1, at 5 minutes, agonists induced a transient ERK phosphorylation which had a pharmacological profile similar to that observed for PI hydrolysis, was blocked by the inhibition of PLC activity, and was not dependent on β-arrestin expression. At higher glutamate concentrations, β-arrestin silencing slightly reduced ERK phosphorylation also at 5 minutes, which is consistent with a previous report showing that a portion of mGlu1-mediated transient MAP kinase phosphorylation is β-arrestin-dependent (Iacovelli et al., 2003). Together, these data suggest that transient ERK phosphorylation is mediated by the classical mechanism of G protein-mediated PI hydrolysis.
In addition, these data show that glutamate, acting at mGlu1 receptors, activates also a long lasting phase of ERK phosphorylation, which was maintained in the presence of glutamate for 24 hours. This is the first report of a sustained phosphorylation of ERK due to stimulation of a metabotropic glutamate receptor. Similar to the protective effect of glutamate, the sustained phase of ERK phosphorylation was insensitive to PLC inhibition and was completely abolished by shRNA silencing of β-arrestin-1 expression. In contrast to transient ERK phosphorylation, the sustained phase was induced by much higher glutamate concentrations and was not elicited by quisqualate. As demonstrated in these studies, this difference in glutamate potency reflected the presence in transfected CHO cells of receptor reserve for PI hydrolysis. The pharmacological profile of the sustained ERK phosphorylation resembled that of the protective mGlu1a signaling, but not that of PI hydrolysis and transient ERK phosphorylation. These data strongly suggest that the protective mGlu1a signaling is mediated by glutamate-induced sustained ERK phosphorylation. While the specific downstream targets of phosphorylated ERK have not been identified, phosphorylated ERK, acting at the cell nucleus, was shown to cause protective cellular responses (Seger and Krebs, 1995).
CHAPTER 3

Metabotropic Glutamate Receptor 1 Possesses Two Glutamate Binding Sites Coupled to Different Signal Transduction Systems
INTRODUCTION

The previous chapter demonstrated that in CHO cells transfected with mGlu1a receptors, stimulation of mGlu1a receptors with glutamate protected cells from serum withdrawal-induced apoptosis. These results were published in Emery et al., 2010. In this manuscript we showed that, protective signaling through mGlu1a receptors was accomplished by a β-arrestin-1-dependent sustained phosphorylation of ERK. Moreover, this 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. In these studies, the classical mGlu1a-mediated PI hydrolysis and transient ERK phosphorylation was induced by both quisqualate and glutamate with quisqualate as the most potent agonist.

Our 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 (Rajagopal et al., 2010). The earliest reported example of ligand bias was in M1 muscarinic receptors, which activate both cAMP and PLC in response to their endogenous agonist acetylcholine. However, several ligands were identified that selectively activate PLC while blocking cAMP formation through these receptors (Fisher et al., 1993). Ligand bias, also referred
to as “biased agonism”, has since been reported in numerous GPCRs, and has been recently reviewed by Rajagopal et al. (2010). In addition to selective activation of different G protein-dependent pathways, ligands have been identified which preferentially activate β-arrestin-mediated signaling which is independent of G protein signaling for several receptor systems (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 receptors, whereas an agonist such as glutamate is less biased owing to the 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 led to the suggestions that glutamate, the apparently unbiased ligand, interacts with 14 amino acid residues while quisqualate, the apparently biased ligand, interacts with only nine of these 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 an interesting possibility that it may be possible to identify compounds which activate mGlu1 receptors but are biased toward sustained ERK phosphorylation and cellular survival. The aim of the following studies is to identify the mechanism of ligand bias by which glutamate, but not quisqualate causes protective signaling in cells expressing mGlu1a receptors. In these studies, glutaric and succinic acids are identified as compounds which act as ligands at mGlu1a receptors that are completely biased toward sustained ERK phosphorylation and protective signaling and
are inactive in stimulating PI hydrolysis. These dicarboxylic acids may serve as lead compounds for drug discovery.
RESULTS

Previous studies in primary cultures of cortical neurons and cerebellar granule neurons have demonstrated that glutamate, acting selectively at mGlu1 receptors, enhanced PI hydrolysis, but also rescued these neurons from apoptotic cell death induced by conditions of trophic deprivation (Pshenichkin et al., 2008). This protective effect of glutamate was revealed in the presence of antagonists of ionotropic glutamate receptors which suppressed the excitotoxic actions of glutamate. Studies in CHO cells stably transfected with mGlu1a receptors have shown that protective signaling of mGlu1a is not mediated by its coupling with G proteins, but instead, it is due to β-arrestin-1-mediated sustained ERK phosphorylation (Emery et al., 2010). This sustained ERK phosphorylation is distinct from the transient, G protein–mediated and PLC-dependent phosphorylation of ERK also seen after activation of mGlu1a receptors. In both cerebellar granule cells, which express native mGlu1a receptors, and in CHO cells transfected with mGlu1a receptors, protective signaling through mGlu1a receptors was induced only by glutamate, the native ligand for the receptor, but not by quisqualate (Emery et al., 2010; Pshenichkin et al., 2008), the most potent agonist in inducing PI hydrolysis through mGlu1a receptors (Aramori and Nakanishi, 1992; Emery et al., 2010). These data suggest the existence of ligand bias at mGlu1a receptors, where some agonists, such as quisqualate, can only stimulate the G protein-mediated signaling, while others, such as glutamate, can induce both PI hydrolysis and sustained ERK phosphorylation.
In order to further study the pharmacological properties of mGlu1a receptors without interference from ionotropic or other metabotropic glutamate receptors, CHO cells stably expressing mGlu1a receptors were used. As shown in Figure 21, 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 caused an increase in transient ERK phosphorylation measured after five minutes of incubation with the agonist (Figure 22) which, as shown previously, is PLC-dependent (Emery et al., 2010). The current data also indicate that the transient ERK phosphorylation was induced by all mGlu1 agonists tested (Figure 22), with a pharmacological profile and agonist potencies similar to those observed for PI hydrolysis (Figure 21). As expected, the mGlu1-selective noncompetitive antagonist YM-298198 (Kohara et al., 2005) completely abolished both glutamate and quisqualate-induced ERK phosphorylation (Figure 22).

The previous studies 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 24 hours, had a different pharmacological profile from transient ERK phosphorylation (Figure 23), and was only induced by glutamate, aspartate and L-cysteic acid, but not by quisqualate and DHPG (Figure 23). Glutamate-induced sustained ERK phosphorylation was completely blocked by 10 μM YM-298198 (Figure 23).
Figure 21. **Agonist profile for mGlu1a-mediated PI hydrolysis.** Dose response curves of agonist induced PI hydrolysis in CHO cells stably expressing mGlu1a receptors. Quisqualate (Quis) was the most potent agonist followed by glutamate (Glu), DHPG, aspartate (Asp), and L-cysteic acid (L-CA). Glutamate-induced PI hydrolysis was blocked by 10 μM YM-298198 (YM). All data points are means from three independent experiments performed in triplicate with error bars corresponding to S.E.M.
**Figure 22. Agonist profile for mGlu1a-mediated transient ERK phosphorylation.** Dose response curves of agonist induced ERK phosphorylation measured after 5 minutes of incubation with ligands in CHO cells stably expressing mGlu1a receptors. Quisqualate (Quis) was the most potent agonist followed by glutamate (Glu), aspartate (Asp), L-cysteic acid (L-CA), and DHPG. Quisqualate and glutamate-induced ERK phosphorylation was blocked by 10 µM YM-298198 (YM). All data points are means from three independent experiments performed in quadruplicate with error bars corresponding to S.E.M.
**Figure 2.** Agonist profile for mGlu1a-mediated sustained ERK phosphorylation. Dose response curves of agonist induced ERK phosphorylation measured after 24 hours of incubation with ligands in CHO cells stably expressing mGlu1a receptors. Glutamate (Glu) was the most agonist, followed by aspartate (Asp), and L-cysteic acid (L-CA). Neither quisqualate nor DHPG stimulated sustained ERK phosphorylation. Glutamate-induced ERK phosphorylation was blocked by 10 μM YM-298198 (YM). All data points are means from three independent experiments performed in quadruplicate with error bars corresponding to S.E.M.
In addition, the potency and efficacy of the various mGlu1 agonists to elicit the protective effect were tested. To this end, 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. The addition of glutamate, aspartate or L-cysteic acid to the serum-free culture medium produced a substantial increase in viability, however in contrast to the pharmacology of PI hydrolysis, quisqualate and DHPG were not effective in concentrations up to 10 mM (Figure 24). The observed agonist potencies were similar to those observed for the sustained phosphorylation of ERK through mGlu1a (Figures 23 and 24). The lack of effect by quisqualate and DHPG is not likely due to agonist degradation or uptake since 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 naive cells. As shown previously, both quisqualate and glutamate were equally potent at stimulating PI hydrolysis before and after culture conditioning (Figure 7). Additionally, a similar bioassay of DHPG showed no significant breakdown: EC$_{50} = 30.6 \pm 4.7$ μM for freshly-diluted DHPG, EC$_{50} = 32.4 \pm 5.3$ μM for preconditioned DHPG. These data indicate that the agonists tested are not subject to degradation or uptake during the extended periods of incubation used.
Figure 24. **Agonist profile for mGlu1a-mediated protective signaling.** Dose response curves of agonist induced protection from serum deprivation in CHO cells stably expressing mGlu1a receptors as measured by MTT assays. Glutamate (Glu) was the most agonist, followed by aspartate (Asp), and L-cysteic acid (L-CA). Neither quisqualate (Quis) nor DHPG protected cells. All data points are means from three independent experiments performed in triplicate with error bars corresponding to S.E.M.
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; while quisqualate and DHPG only activate PI hydrolysis and a transient phosphorylation of ERK. Such results can be explained by a phenomenon known as ligand bias, which is the ability of some agonists, such as quisqualate, to activate only selected signal transduction mechanisms while other agonists, such as glutamate, may activate all signaling associated with the same receptor (Violin and Lefkowitz, 2007).

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 seen in Figures 25 and 26, LY 367385 and 3-MATIDA both competitively inhibited glutamate-induced PI hydrolysis, causing the EC$_{50}$ for glutamate to shift from $17 \pm 2.2 \, \mu M$ to $101 \pm 15 \, \mu M$ in presence of LY 367385 (10 μM) and from $9.2 \pm 0.3 \, \mu M$ to $76 \pm 18 \, \mu M$ in the presence of 3-MATIDA (30 μM). Additionally, both competitive antagonists were bioassayed after 3 days of culture conditioning and both were stable (Figures 25, 26). Competitive inhibition of glutamate-induced transient ERK phosphorylation was also observed (Figure 27), causing the EC$_{50}$ for glutamate to shift from $50 \, \mu M$ to $810 \, \mu M$ in presence of LY 367385 (30 μM) and to $880 \, \mu M$ in presence of 3-MATIDA (30 μM).
Figure 25. Competitive antagonism of glutamate-induced PI hydrolysis by LY367685. Dose response curves of glutamate-induced PI hydrolysis with competitive inhibition by freshly-prepared (Fresh) and cultured-conditioned (CC) 10 μM LY367385 (LY). For glutamate, EC<sub>50</sub> = 17 ± 2.2 μM and 101 ± 15 μM in the presence of either freshly-prepared or culture-conditioned LY367385, demonstrating stability of this antagonist. Control refers to cells treated only with indicated concentrations of glutamate and the solvent used to dilute LY367385. All data points are means from three independent experiments performed in triplicate with error bars corresponding to S.E.M.
Figure 26. **Competitive antagonism of glutamate-induced PI hydrolysis by 3-MATIDA.**

Dose response curves of glutamate-induced PI hydrolysis with competitive inhibition by freshly-prepared (Fresh) and cultured-conditioned (CC) 30 μM 3-MATIDA. For glutamate, EC$_{50}$ = 9.2 ± 0.3 μM, and 76 ± 18 μM in the presence of either freshly-prepared or culture-conditioned 3-MATIDA, demonstrating stability of this antagonist. Control refers to cells treated only with indicated concentrations of glutamate and the solvent used to dilute 3-MATIDA. All data points are means from three independent experiments performed in triplicate with error bars corresponding to S.E.M.
**Figure 27. Competitive antagonism of glutamate-induced transient ERK phosphorylation.**

Dose response curves of glutamate-induced transient ERK phosphorylation with competitive inhibition by 30 μM LY367385 or 30 μM 3-MATIDA. For glutamate, EC\textsubscript{50} = 50 μM, shifting to 810 μM in presence of LY 367385 (30 μM) and to 880 μM in presence of 3-MATIDA (30 μM). All data points are means from three independent experiments performed in triplicate with error bars corresponding to S.E.M.
In contrast, even high concentrations (1 mM) of either LY 367385 or 3-MATIDA failed to inhibit glutamate-induced sustained (24 hour) ERK phosphorylation (Figure 28), where the EC$_{50}$ for glutamate did not vary significantly from 301 μM in the presence or absence of either competitive antagonist. 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) had no effect on glutamate-induced protection (Figure 29). Instead, the protective signaling was noncompetitively inhibited by YM-298198 (3 μM), confirming that this effect is mediated by mGlu1 receptors.

Because sustained ERK phosphorylation and protective signaling were inhibited by noncompetitive antagonists, but not by the competitive antagonists LY 367285 and 3-MATIDA, we hypothesized that glutamate may bind to different sites to stimulate PI hydrolysis and to induce protective signaling. Previously, the X-ray crystallography of the mGlu1 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), indicate that several amino acid residues, including Thr$_{188}$, Asp$_{208}$, Tyr$_{236}$, and Asp$_{318}$, interact with the amino group of the glutamate and these interactions are necessary for receptor activation as measured by PI hydrolysis and the binding of quisqualate (Sato et al., 2003). In contrast, there are additional residues in the binding site, including Arg$_{323}$ and Lys$_{409}$, which are postulated to bind the omega-carboxyl group of glutamate, but mutation of these residues does not attenuate glutamate-induced PI hydrolysis or the binding of quisqualate (Sato et al., 2003).
Figure 28. Lack of competitive antagonism on glutamate-induced sustained ERK phosphorylation. Dose response curves of glutamate-induced sustained (24 hour) ERK phosphorylation in the presence of competitive antagonists by LY367385 (1 mM) or 3-MATIDA (1 mM). For glutamate, EC$_{50}$ = 300 μM, neither competitive antagonist changed the potency of glutamate. Glutamate-induced sustained ERK phosphorylation was effectively blocked by the noncompetitive antagonist YM-298198 (30 μM). All data points are means from three independent experiments performed in triplicate with error bars corresponding to S.E.M.
Figure 29. **Lack of competitive antagonism on glutamate-induced protective signaling.** Dose response curves of glutamate-induced protective signaling in the presence of competitive antagonists by LY367385 (1 mM) or 3-MATIDA (1 mM). Neither competitive antagonist changed the potency of glutamate, however glutamate-induced protection was noncompetitively inhibited by the noncompetitive antagonist YM-298198 (3 μM). All data points are means from four independent experiments performed in triplicate with error bars corresponding to S.E.M.
In order to ascertain the mechanism by which different ligands can activate different signal transduction pathways through the same receptor, we have mutated Thr\textsuperscript{188}, Arg\textsuperscript{323} and Lys\textsuperscript{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 (Figure 30). 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 (Figure 31). These data confirm that the Thr\textsuperscript{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 and protective signaling (Figures 33, 34). For 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 seen in Figure 32, 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 (Figure 32). While glutamate stimulated both sustained ERK phosphorylation (Figure 33) and protective signaling (Figure 34), the silencing of β-arrestin-1 with shRNA resulted in the blockade of both responses.
Figure 30. **Protein expression levels of mutant mGlu1a receptors.** Representative Western blot depicting protein expression levels of mutated mGlu1a receptors stably transfected in CHO cells. All receptors were expressed at equivalent levels to wild-type (W/T) control mGlu1a receptors.
Figure 31. Lack of classical mGlu1a receptor signaling in T188A mutant receptors. Glutamate-induced PI hydrolysis occurs to a similar extent in CHO cells transfected with vector (pcDNA 3.1) and CHO cells stably expressing the T188A mutant mGlu1a receptor. Data points are means from three independent experiments performed in quadruplicate with error bars representing S.E.M.
Figure 32. Silencing of β-arrestin-1 in CHO cells expressing T188A mutant mGlu1a receptors. Representative Western blot depicting efficient silencing of β-arrestin-1 by shRNA. Protein expression levels were restored by transfection with human β-arrestin-1.
**Figure 3.** β-arrestin-1 dependent sustained ERK phosphorylation in T188A mutant mGlu1a receptors. In T188A mutant mGlu1a receptors stably expressed in CHO cells, glutamate-induced 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 data points are means from three experiments performed in quadruplicate with error bars representing S.E.M.
**Figure 34. Glutamate-induced protective signaling in T188A mutant mGlu1a receptors.** Glutamate-induced protective signaling through T188A mutant mGlu1a receptors expressed in CHO cells is inhibited by shRNA silencing of β-arrestin-1. Glutamate-induced protection is restored upon 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.
However, these responses were restored when the β-arrestin-1-depleted cells were rescued by transfection with human β-arrestin-1 (Figures 33, 34). Taken together, these data indicate that the interaction of glutamate with the Thr\textsuperscript{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\textsuperscript{188} has been shown to interact with the alpha amino group of mGlu1 agonists (Sato et al., 2003), the next hypothesis was that sustained ERK phosphorylation and protective signaling require ligand interactions with different residues of the binding site. Therefore, mutations were performed on residues Arg\textsuperscript{323} and Lys\textsuperscript{409}, which are 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 (Figure 35). 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 (Figure 36), which was abolished by the PLC inhibitor U73122 (Figure 36). In contrast, both R323V and K409A mGlu1a mutants, expressed in CHO cells, failed to stimulate the sustained ERK phosphorylation (Figure 37), and failed to protect against serum withdrawal-induced apoptosis (Figure 38).
Figure 35. Classical signaling in R323V and K409A mutant mGlu1a receptors. In both R323V and K409A mutant mGlu1a receptors expressed in CHO cells, glutamate-induced PI hydrolysis occurs to a similar extent as in CHO cells expressing the wild-type mGlu1a receptors (EC$_{50}$ = 18 μM, 16 μM, and 13 μM, respectively). All data points are means from three independent experiments performed in triplicate with error bars representing S.E.M.
Figure 36. **ERK phosphorylation is G protein-dependent in R323V and K409A mutant mGlu1a receptors.** Glutamate-induced ERK phosphorylation measured after 5 minutes of agonist stimulation was completely inhibited by PLC inhibitor U73122 (U73), suggesting ERK phosphorylation through R323V and K409A mutant mGlu1a receptors is completely G protein-dependent. All data points are means from two experiments performed with six replications and error bars represent S.E.M.
**Figure 37.** R323V and K409A mutant mGlu1a receptors do not stimulate long-term ERK phosphorylation. Glutamate-induced ERK phosphorylation measured after 24 hours of agonist stimulation was not significantly elevated over basal in R323V or K409A mutant mGlu1a receptors expressed in CHO cells. All data points are means from two experiments performed with six replications and error bars represent S.E.M.
**Figure 38.** **R323V and K409A mutant mGlu1a receptors do not protect CHO cells.**

Glutamate-induced protection from serum deprivation-induced toxicity was measured after three days agonist stimulation by MTT assays. Glutamate failed to increase the viability of CHO cells stably expressing R323V or K409A mutant mGlu1a receptors. All data points are means from three experiments performed with six replications and error bars represent S.E.M.
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 (Figure 39), suggesting that they are not classical mGlu1a agonists. In contrast, glutaric acid stimulated, in a dose-dependent manner, both the sustained ERK phosphorylation (Figure 40) and the protective signaling of mGlu1 receptors (Figure 41). Moreover, glutaric acid protected cerebellar granule cells, which express native mGlu1a receptors, from low potassium-induced apoptosis in a dose-dependent manner (Figure 42). Succinic acid protected cells from apoptosis to a similar extent (Figure 43). The effects of glutaric and succinic acid were blocked by the noncompetitive mGlu1 antagonist YM-298198, confirming their site of action at this receptor (Figures 41-43).
Figure 39. Effects of glutaric and succinic acids on PI hydrolysis. Neither glutaric nor succinic acid caused an elevation in PI hydrolysis in CHO cells stably expressing mGlu1a receptors. Glutamate (1 mM) served as a positive control in these experiments. Values are means from three experiments performed in quadruplicate and error bars represent S.E.M.
Figure 40. Glutaric acid is an mGlu1a receptor agonist that causes ERK phosphorylation. ERK phosphorylation in CHO cells expressing mGlu1a receptors was measured after 5 minutes and 24 hours of incubation with glutaric acid increased in a dose-dependent manner. All effects of glutaric acid were noncompetitively inhibited by YM-298198 (YM). All data points are means from three experiments performed in triplicate with error bars representing S.E.M.
Figure 41. Glutaric acid protects CHO cells through activity at mGlu1a receptors. Dose-dependent effect of glutaric acid on protecting CHO cells expressing mGlu1a receptors from serum deprivation-induced toxicity. Glutaric acid was noncompetitively inhibited by YM-298198 (YM). All data points are means from four experiments performed in triplicate with error bars representing S.E.M.
Figure 42. **Glutaric acid protects cerebellar granule neurons through mGlu1a receptors.**

Dose-dependent effect of glutaric acid (GA) on viability of cerebellar granule neurons subjected to 5 mM potassium (K5) overnight to induce apoptosis. The effect of glutaric acid was noncompetitively inhibited by 10 µM YM-298198 (YM). Viability was measured by MTT assay. All data points were normalized to controls grown in 25 mM potassium (K25) and are means from four experiments performed in triplicate with error bars representing S.E.M.
**Figure 43.** Succinic acid protects CHO cells through activity at mGlu1a receptors. Dose-dependent effect of succinic acid on protecting CHO cells expressing mGlu1a receptors from serum deprivation-induced toxicity. Succinic acid was noncompetitively inhibited by YM-298198 (YM). All data points are means from four experiments performed in triplicate with error bars representing S.E.M.
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 bind to two distinct sites to stimulate the two different signaling cascades. If these sites were truly separate, then ligands binding to one site (site A) should not interfere with the potency of agonists binding to the second site (site B). This possibility was investigated by testing for interactions between the biased ligands.

First, the ability of quisqualate (biased ligand for site A) to interfere with the potency of glutamate to induce protective signaling was tested, and no effect was found, even at extreme (300 μM) concentrations of quisqualate (Figure 44). Second, we tested the ability of glutaric acid (biased ligand at site B) to interfere with glutamate-induced PI hydrolysis, and found it equally inactive at concentrations up to 10 mM (Figure 45). These data strongly suggest that mGlu1a receptors possess two distinct, non-interacting glutamate binding sites, and the binding of agonists to each site allows for the stimulation of a different signal transduction cascade.
Figure 44. Quisqualate fails to inhibit glutamate-induced protection at mGlu1a receptors.

Glutamate-induced protection from serum deprivation-induced toxicity in CHO cells expressing mGlu1a receptors in the presence of varying concentrations of quisqualate. In the presence of quisqualate, the EC$_{50}$ for glutamate to protect did not vary significantly from 320 μM. All data points are means from five experiments performed in triplicate with error bars representing S.E.M.
Figure 45. **Glutaric acid fails to inhibit glutamate-induced PI hydrolysis at mGlu1a receptors.** Glutamate-induced PI hydrolysis in CHO cells expressing mGlu1a receptors in the presence of varying concentrations of glutaric acid. In the presence of glutaric acid, the EC$_{50}$ for glutamate to protect did not vary significantly from 13 μM. All data points are means from three experiments performed in triplicate with error bars representing S.E.M.
DISCUSSION

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, those different pharmacological profiles of the two mGlu1a responses were investigated and revealed the existence of a ligand bias at these receptors. These 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 (Table 2). 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 in (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 would be explained if glutamate induced protective signaling by binding to a site distinct from 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 orthosteric binding site which also binds competitive antagonists (Clark et al., 1997).
<table>
<thead>
<tr>
<th>Agonist</th>
<th>Type of agonism</th>
<th>Wild type mGlu1a</th>
<th>T188A mutant</th>
<th>R323V &amp; K409A mutants</th>
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<tr>
<td>Glutamate, Aspartate, Cysteate</td>
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<tr>
<td>Quisqualate, DHPG</td>
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*Table 2. Different responses induced by three classes of agonists acting at wild type and mutated mGlu1a receptors. PI: PI hydrolysis, PR: protection from apoptosis.*
The hypothesis for the existence of a second glutamate binding site on mGlu1a receptors is further supported by the 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).

Strong evidence for the existence of two distinct glutamate binding sites 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 change the potency of glutamate to stimulate PI hydrolysis. This lack of interactions indicates that biased ligands likely bind to two distinct, non-interacting sites. While novel, these data suggesting the presence of two separate glutamate binding sites on 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). Interestingly, 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 case of mGlu1, our mutational data indicate that the two binding sites, while separate, are still located in the same general region of the receptor N-terminal domain. If confirmed, 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, new ligands for mGlu1a receptors which are biased towards the protective signaling were identified. 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 (oxalic acid and malonic acid) or longer (adipic acid, and pimelic acid) carbon chains were inactive (data not shown). Consistent with the current findings of cytoprotective properties, succinate has previously been shown to ameliorate cognitive defects in a rat model of Alzheimer’s disease (Storozheva et al., 2008). While both glutarate and succinate are endogenous to the brain, they are common metabolites, and it is unclear whether they could serve a physiological role, acting at native mGlu1 receptors. 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.
**Major findings of this thesis**

The existence of a new signal transduction mechanism of mGlu1a receptors is demonstrated in Chapter 2. In contrast to the classical G protein-mediated PI hydrolysis, this mechanism appears to involve agonist-mediated, β-arrestin-1-dependent receptor internalization, followed by the activation of MEK cascade and a long-lasting, sustained phase of ERK phosphorylation. This mechanism appears to be responsible for the protective action of glutamate mediated by mGlu1a receptors. These experiments were described in the first published report of this signal transduction pathway though a metabotropic glutamate receptor (Emery et al., 2010). While novel, these findings should come as no surprise, since β-arrestin-1-dependent sustained ERK phosphorylation has previously been demonstrated through numerous other G protein-coupled receptors (Broca et al., 2009; Quan et al., 2008; Zheng et al., 2008).

Previously, it was proposed that mGlu1a functions as a dependence receptor causing apoptosis in the absence of glutamate, possibly due to the cleavage of its C-terminal domain (Pshenichkin et al., 2008). Now we propose that the protective, positive signaling of mGlu1, which occurs in the presence of glutamate, is mediated by this new, G protein-independent, mechanism of signal transduction. This hypothesis needs now to be more extensively 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., 1995; Siliprandi et al., 1992), may also produce a protective effect when acting at mGlu1 receptors. Acting as a dependence receptor, mGlu1a 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.

In Chapter 3, the molecular mechanism for ligand bias at mGlu1 receptors was
investigated. Multiple experiments indicated that some agonists, such as glutamate, aspartate,
and L-cysteic acid, stimulate both PI hydrolysis and protective signaling through mGlu1a
receptors while other agonists, such as quisqualate and DHPG, only stimulate PI hydrolysis.
Mutations of selected residues of the ligand binding domain of the receptor revealed that Thr^{188}
is necessary for PI hydrolysis while Arg^{323} and Lys^{409} are necessary for protective signaling but
not PI hydrolysis. These data suggest that the interaction of the α-amino group of a ligand and
the receptor is required for PI hydrolysis while interaction between the omega-carboxyl group of
the ligand and the receptor is necessary for protective signaling. This hypothesis was supported
by the fact that glutaric and succinic acids, analogs of glutamate and aspartate lacking the α-
amino group, stimulated sustained ERK phosphorylation and protective signaling selectively
through mGlu1a receptors. This is the first report of these two compounds as agonists at any
mGlu receptor. Finally, glutamate-induced protection was not inhibited by quisqualate, even at
very high concentrations. Likewise, glutaric acid did not inhibit glutamate-induced PI hydrolysis.
Taken together, these data suggest the existence of two distinct non-overlapping glutamate
binding sites on mGlu1a receptors, with agonist action at one site (Site A) being responsible for
G protein-dependent signaling and agonist action at the second site (Site B) being responsible for
sustained ERK phosphorylation and protective signaling.
Future directions

Many elements of the protective signaling cascade initiated by mGlu1a receptors remain unknown. Future studies should investigate the hypothesis that the C-terminus of mGlu1a receptors is phosphorylated with GRK4 prior to the binding of β-arrestin-1. The protective substrates of phosphorylated ERK remain unknown. Since phosphorylated ERK that is linked to β-arrestin generally acts upon cytosolic substrates, the phosphoproteins should be investigated using antibodies. Moreover, the changes in gene regulation that result from this signaling cascade could be investigated through the use of microarrays and further investigated using pharmacological approaches. Such studies could be useful in identifying other potential methods to protect cells from toxicity. Additionally, these studies should be more comprehensively confirmed in primary cultures of neurons and ultimately in vivo. Such studies could be very useful in identifying a role for glutamate to act as a trophic factor in the developing brain, and possibly as a neuroprotective mechanism. Additionally, emerging evidence suggest that activation of mGlu1 receptors plays a role in the proliferation of melanoma cells (Marin and Chen, 2004). Further studies should test the hypothesis that β-arrestin-dependent signaling through mGlu1 receptors is responsible for this increase in proliferation. If confirmed, these results would yield multiple additional molecular targets for the treatment of melanoma.

The studies in Chapter 3 represent interesting possibilities for further studies. Taken together, these results suggest the existence of two glutamate binding sites at mGlu1a receptors. A more thorough mutational analysis of the ligand binding domain of the receptor paired with computer modeling may lead greater insight into the second binding site. Pharmacological studies indicate that glutamate, aspartate, and L-cysteic acid act at Site B, and glutaric and
succinic acids were identified as biased agonists for this site on mGlu1a receptor. Neither of these newly-identified compounds is very potent, but they may serve as lead compounds for further rational drug design, as ligands which do not stimulate PI hydrolysis but do stimulate protective signaling could be quite useful for possible use in neurodegeneration. Moreover, since mGlu1 receptor activation increases the proliferation of melanoma cells (Marin and Chen, 2004), identification of a compound that selectively antagonizes Site B could be extremely useful, since it could potentially be used to inhibit the proliferation of melanoma cells while not affecting G protein-dependent mGlu1 receptor signaling, which occurs due to agonist activity at Site A.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>T188A Forward</td>
<td>5’CAGATCGCCTATTCTGCGCAGCTAGCA</td>
</tr>
<tr>
<td>T188A Reverse</td>
<td>5’GTCACTCAGGTCTATGCTAGCGGA</td>
</tr>
<tr>
<td>R323V Forward</td>
<td>5’GAAGTGATGGAGGGCGACAGACGACGAGTGACGAGTCATCGAAGGC</td>
</tr>
<tr>
<td>R323V Reverse</td>
<td>5’GCCTTCGATTTCGTGACGTCGACGTGAGTCCCATCCATCACCTTC</td>
</tr>
<tr>
<td>K409A Forward</td>
<td>5’GAAAACTATGTCCAGGACAGGCGCCATGGGGATTTGTGATCAATGCC</td>
</tr>
<tr>
<td>K409A Reverse</td>
<td>5’GGCATTGATGACAAATCCCATCGGGCTGTCTCGGACATAGTTTC</td>
</tr>
</tbody>
</table>

*Table 3. Sequences of PCR primers for creating mutant mGlu1 receptors*
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