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Pharmacological characterization of mGlu1 receptors in cerebellar granule cells reveals biased agonism

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ABSTRACT

The majority of existing research on the function of metabotropic glutamate (mGlu) receptor 1 focuses on G protein-mediated outcomes. However, similar to other G protein-coupled receptors (GPCR), it is becoming apparent that $mGlu_1$ receptor signaling is multi-dimensional and does not always involve G protein activation. Previously, in transfected CHO cells, we showed that $mGlu_1$ receptors activate a G protein-independent, β-arrestin-dependent signal transduction mechanism and that some $mGlu_1$ receptor ligands were incapable of stimulating this response. Here we set out to investigate the physiological relevance of these findings in a native system using primary cultures of cerebellar granule cells. We tested the ability of a panel of compounds to stimulate two $mGlu_1$ receptor-mediated outcomes: (1) protection from decreased cell viability after withdrawal of trophic support and (2) G protein-mediated phosphoinositide (PI) hydrolysis. We report that the commonly used $mGlu_1$ receptor ligands quisqualate, DHPG, and ACPD are completely biased towards PI hydrolysis and do not induce $mGlu_1$ receptor-stimulated neuroprotection. On the other hand, endogenous compounds including glutamate, aspartate, cysteic acid, cysteine sulfinic acid, and homocysteic acid stimulate both responses. These results show that some commonly used $mGlu_1$ receptor ligands are biased agonists, stimulating only a fraction of $mGlu_1$ receptor-mediated responses in neurons. This emphasizes the importance of utilizing multiple agonists and assays when studying GPCR function.

1. Introduction

G protein-coupled receptors (GPCRs) make up the largest class of membrane receptors and are the target of the majority of current pharmaceuticals (Reiter et al., 2012). Classically, stimulation of these receptors causes activation of their associated G proteins and production of second messengers. Various GPCRs also partake in G protein-independent signal transduction, the most studied of which is β-arrestin-dependent signaling. These G protein-independent cascades activate downstream enzymes in temporally and spatially different patterns compared to G protein-dependent activation (DeWire et al., 2007). Thus, it is not surprising that G protein-independent signals result in cellular and physiological outcomes that are distinct from those mediated by G protein stimulation (Luttrell and Gesty-Palmer, 2010).

As we understand more about the complexities of GPCR signaling it is apparent that receptor activation is not as simple as on and off (Rajagopal et al., 2010). Indeed, many GPCR ligands exhibit *biased agonism*: unequal ability (i.e. efficacies) to stimulate different responses mediated by a single receptor (Rajagopal et al., 2011). Because of the multidimensionality of GPCR signaling and agonism, it is important to assay numerous agonists and responses when characterizing GPCR signaling. Such rigorous investigations not only provide a more complete picture of receptor function, but also provide the framework for development of selective

Abbreviations: ACPD, 1-Aminocyclopentane-1,3-dicarboxylic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DHPG, 3,5-dihydroxyphenylglycine; CPCCOEt, 7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; CA, Cysteic acid; CSA, Cysteine sulfinic acid; GPCR, G protein-coupled receptor; HCA, Homocysteic acid; mGlu, Metabotropic glutamate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; PI, Phosphoinositide.

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therapeutics that potentially reduce side effects by specifically activating one receptor outcome (Luttrell and Gesty-Palmer, 2010; Violin et al., 2014).

Metabotropic glutamate (mGlu) receptors are GPCRs primarily expressed in the nervous system. mGlu receptors are divided into three groups based on sequence homology and G protein-coupling (Conn and Pin, 1997; Nakanishi, 1992). Group I mGlu receptors, which include $mGlu_1$ receptors and $mGlu_5$ receptors, are coupled to Gαq proteins. Thus, activation of group I mGlu receptors stimulates phospholipase C, resulting in hydrolysis of PIP_2 and formation of the second messengers IP_3 and DAG (Ferraguti et al., 2008). Additionally, glutamatergic activation of $mGlu_1$ receptors stimulates a G protein-independent, β-arrestin-dependent signal transduction mechanism that protects $mGlu_1$ receptor-transfected CHO cells from toxicity after serum withdrawal (Emery et al., 2010). Similar increases in cell viability upon glutamate treatment occur in multiple cell types and are dependent on $mGlu_1$ receptor activation (Gelb et al., 2014; Pshenichkin et al., 2008). Activation of the protective, β-arrestin-dependent pathway does not occur with exogenous, group I mGlu receptor-preferring agonists (e.g. quisqualate), but only with endogenous agonists such as glutamate and aspartate (Emery et al., 2012). These findings suggest that activation of $mGlu_1$ receptors may protect neurons from apoptosis. As targeting of $mGlu_1$ receptors could be a potential neuroprotective strategy (Caraci et al., 2012), it is vital to fully characterize $mGlu_1$ receptor signaling in native systems.

The purpose of this study was to investigate $mGlu_1$ receptor signaling in a physiologically relevant model. Primary cultures of cerebellar granule cells were utilized as $mGlu_1$ receptors are highly expressed in these neurons (Santi et al., 1994). To maximize culture viability, granule cells must be maintained in chronic depolarizing conditions (>20 mM potassium) since granule cells undergo apoptosis in concentrations of potassium that are physiological (5 mM). Granule cell death in this model occurs quickly and is reproducible, which is why the potassium removal paradigm is commonly used to study neurotoxicity and neuroprotection (see Contestabile, 2002 for thorough review). Using primary cultures of cerebellar granule cells we characterized $mGlu_1$ receptors using a panel of eight $mGlu_1$ receptor ligands (glutamate, aspartate, cysteic acid, homocysteic acid, cysteine sulfinic acid, quisqualate, DHPG, and ACPD). The response to each ligand was determined by two $mGlu_1$ receptor-dependent outcomes: protection from toxicity after potassium removal and phosphoinositide (PI) hydrolysis. We report here that native $mGlu_1$ receptors exhibit biased agonism in primary neuronal cultures. These findings confirm the neuroprotective potential of $mGlu_1$ receptors and emphasize the need for further drug design endeavors to selectively activate this protective mechanism while potentially reducing side effects.

2. Methods

2.1. Materials

Neurobasal (NB) media, 2 M KCl, B27 supplement, L-glutamine, and gentamicin were purchased from Life Technologies (Carlsbad, CA, USA). Receptor agonists (glutamate, aspartate, quisqualate, 3,5-dihydroxyphenylglycine (DHPG), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), N-methyl-D-aspartate (NMDA)), receptor antagonists (YM298198-HCl, CPCCOEt, JNJ16259685, MPEP, MK801, CFM2, MCCG, MAP4) were purchased from Tocris Bioscience (Bristol, United Kingdom). All receptor agonists were prepared in equimolar sodium hydroxide (VWR, Radnor, PA, USA) and adjusted to pH 7.3–7.5. All antagonists were diluted in DMSO (Fisher Scientific, Pittsburgh, PA, USA), except for MPEP, which was prepared in water. Glutamate pyruvate transaminase (GPT) was obtained from Roche (Indianapolis, IN, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell cultures

Primary cultures of cerebellar granule neurons were prepared as described previously (Wroblewski et al., 1985) using cerebella dissected from P7 Sprague-Dawley rat pups. Cerebellar granule cells were plated in 100 μl at a density of 8×10^5 cells/cm^2 on Nunclon Delta Surface 96-well plates (VWR) coated with poly-L-lysine. Cytosine arabinoside (10 μM) was added the day after plating to prevent growth of non-neural cells. Cultures were maintained at 37 °C in 5% CO_2 in culture media (Neurobasal media containing 100 μg/ml gentamicin, 2 mM glutamine, 2% B27 supplement, and 25 mM KCl) for 5–6 days in vitro (DIV). For experimentation, granule cells were maintained overnight at 37 °C in 5% CO_2 in Neurobasal containing 100 μg/ml gentamicin and either 25 mM KCl (K25 conditions) or 5 mM KCl (K5 conditions). Granule cells cultured under these conditions express high levels of $mGlu_1$ receptors (Pshenichkin et al., 2008).

2.3. Cell viability assays

Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay where formation of the colored product is proportional to the number of viable cells (Mosmann, 1983). Cerebellar granule cells were maintained for 5 days in vitro in culture media, and then media was removed and replaced with 200 μl K5 media containing drugs in 1% DMSO. Neurons were incubated with drugs overnight. The next day, 50 μl of 1.5 mg/ml MTT in K5 media was added to each well and incubated at 37 °C for 60 min. MTT was then removed and the colored formazan product was dissolved in 65 μl/well DMSO. Absorbance of each well was measured at 570 nm by spectrophotometer (EnVision, Perkin-Elmer, Waltham, MA, USA). Cell viability is expressed as either percent of K25 (positive control), or as percent of glutamate efficacy.

2.4. Measurement of PI hydrolysis

PI hydrolysis was measured using scintillation proximity assay (SPA) beads as previously described (Emery et al., 2010). After 6–7 days in vitro, culture media was replaced with K5 media containing 0.625 μCi/well myo-[^{3}H]inositol (Perkin Elmer) and incubated overnight. Media was then removed and cells were treated with drugs in 0.5% DMSO for 1 h at 37 °C in 100 μl/well 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) with 20 mM LiCl to block the degradation of inositol phosphates. Drug treatments were then removed and inositol phosphates were extracted in 60 μl/well ice cold 10 mM formic acid for 30 min. 40 μl/well were then transferred to a scintillation plate containing 1 mg/well YSi poly-lysine SPA beads (Perkin-Elmer) and incubated at room temperature for 1 h with vigorous shaking. After 10 h of incubation with SPA beads, inositol phosphates were detected by scintillation counting.

2.5. Stability of agonists in media

Agonists were bioassayed after 24-h exposure to cerebellar granule cells to establish prolonged stability. Agonists were "cultured conditioned" by incubating agonists in K5 media with cerebellar granule cells for 24 h. Culture conditioned agonists in K5 media were collected, 20 mM LiCl was added, and agonist were applied to separate cultures of cerebellar granule cells that were previously labeled with 0.625 μCi/well myo-[^{3}H]inositol. Resulting PI hydrolysis in response to culture conditioned agonists was compared in parallel to PI hydrolysis caused by agonists freshly prepared in K5 media with 20 mM LiCl.

2.6. Curve fittings and statistical analysis

Dose–response curves were modeled via four-parameter nonlinear regression using GraphPad Prism 6 software (La Jolla, CA, USA). For each agonist the bottom parameter was constrained to the corresponding basal value (K5 for viability, Locke for PI hydrolysis). Differences between dose–response curves were determined by comparing E_{max} and EC_{50} values; for experiments with data that could not be fit to the non-linear regression model significant effects were determined by comparing one maximal concentration of ligand to basal levels. Each experiment was performed at least two times using independent preparations of granule cells, with measurements taken in duplicate within each experiment. Statistical significance was defined as p-value < 0.05 by Student's t-test or Sidak-corrected multiple comparisons following one- or two-way ANOVA. All statistical analysis was performed using GraphPad Prism 6 software.

3. Results

3.1. Glutamate increases viability of granule cells in K5 conditions via activation of $mGlu_1$ receptors

To maximize cell viability, primary cultures of cerebellar granule neurons are maintained in media supplemented with high potassium (K25) (Gallo et al., 1987). Decreasing potassium to 5 mM (K5) reduced granule cell viability and after 16 h the amount of viable cells in K5 cultures was approximately 35% of that in K25 cultures (Fig. 1). Addition of glutamate to K5 media caused a concentration-dependent increase in granule cell viability, with an EC_{50} of 76 μM