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Functional selectivity induced by $mGlu_4$ receptor positive allosteric modulation and concomitant activation of G_q coupled receptors

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ABSTRACT

Metabotropic glutamate receptors (mGlus) are a group of Family C Seven Transmembrane Spanning Receptors (7TMRs) that play important roles in modulating signaling transduction, particularly within the central nervous system. $mGlu_4$ belongs to a subfamily of mGlus that is predominantly coupled to $G_{i/o}$ G proteins. We now report that the ubiquitous autacoid and neuromodulator, histamine, induces substantial glutamate-activated calcium mobilization in $mGlu_4$-expressing cells, an effect which is observed in the absence of co-expressed chimeric G proteins. This strong induction of calcium signaling downstream of glutamate activation of $mGlu_4$ depends upon the presence of H_1 histamine receptors. Interestingly, the potentiating effect of histamine activation does not extend to other $mGlu_4$-mediated signaling events downstream of $G_{i/o}$ G proteins, such as cAMP inhibition, suggesting that the presence of G_q coupled receptors such as H_1 may bias normal $mGlu_4$-mediated $G_{i/o}$ signaling events. When the activity induced by small molecule positive allosteric modulators of $mGlu_4$ is assessed, the potentiated signaling of $mGlu_4$ is further biased by histamine toward calcium-dependent pathways. These results suggest that $G_{i/o}$-coupled mGlus may induce substantial, and potentially unexpected, calcium-mediated signaling events if stimulation occurs concomitantly with activation of G_q receptors. Additionally, our results suggest that signaling induced by small molecule positive allosteric modulators may be substantially biased when G_q receptors are co-activated.

This article is part of a Special Issue entitled 'Metabotropic Glutamate Receptors'.

1. Introduction

Seven Transmembrane Spanning/G-Protein-Coupled Receptors (7TMRs/GPCRs) represent the majority of drug targets currently used in clinical practice. Much interest has recently been placed on the discovery and characterization of allosteric modulators for these receptors due to several potential advantages over traditional orthosteric ligands in terms of drug development (reviewed in (Keov et al., 2011)). For example, many natural endogenous ligands are peptides or small amino acids which possess limitations in pharmacokinetic properties, preventing their development as drug candidates. Additionally, the orthosteric agonist binding sites of many 7TMRs are highly conserved across family members, making selectivity for a particular receptor within one group difficult to achieve. Due to their interaction with the receptor at a site distinct from the orthosteric site, allosteric ligands often possess very high receptor selectivity. Allosteric modulators also have the ability to provide a more subtle and physiologically-relevant approach to increasing or decreasing target activity because receptor regulation will occur only in the presence of the endogenous ligand (Bridges and Lindsley, 2008; Conn et al., 2009). Furthermore, allosteric potentiators, or positive allosteric modulators (PAMs), may, in some cases, avoid receptor desensitization and/or downregulation that can occur after chronic administration of an orthosteric agonist (Bridges and Lindsley, 2008; Conn et al., 2009). As allosteric modulators function by exerting either positive (PAMs) or negative (NAMs) cooperativity with the orthosteric ligand, mechanistically they will exhibit a "ceiling" effect (i.e., maximal receptor occupancy may not translate to maximal effect on receptor activation), which may avoid target/mechanism-mediated side effects that could arise from accidental overdose.

While allosteric modulators of 7TMRs provide potential advantages/distinctions over orthosteric ligands, these compounds also greatly complicate our understanding of receptor pharmacology. In recent years, there has been a growing appreciation of the ability of a single 7TMR to simultaneously regulate multiple signaling cascades (Kenakin, 2005), some of which are G protein-

Abbreviations: mGlu receptor, metabotropic glutamate receptor; CHO, Chinese Hamster Ovary; DHFR(−), Dihydrofolate Reductase; DMSO, Dimethyl Sulfoxide; GPCR, G-Protein-Coupled Receptor; 7TMR, Seven Transmembrane Spanning Receptor; CNS, Central Nervous System.

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independent, such as β arrestin-regulated pathways. This phenomenon, now well established for orthosteric ligands, has been termed "functional selectivity", "biased signaling", or "ligand directed trafficking" (Urban et al., 2007; Keov et al., 2011); we will refer to this phenomenon as functional selectivity in the present manuscript. There are now also clear examples of 7TMR allosteric modulator-mediated functional selectivity (Mathiesen et al., 2005; Marlo et al., 2009). While this behavior introduces complexity into ligand development, it is anticipated that capitalizing on functionally selective effects will provide exciting opportunities to tailor new drug therapy to specifically regulate coupling of 7TMRs to beneficial signaling pathways but not others, potentially reducing adverse effects.

There are numerous mechanisms by which functionally selective pharmacology can be induced by allosteric ligands. For example, 7TMRs have the ability to adopt multiple structural conformations, any of which might be stabilized by an allosteric modulator. This can translate into the ability of a modulator to preferentially regulate some pathways and not others based on the particular conformation they stabilize. Receptor activity is also regulated by other cellular proteins, such as G proteins, arrestins, or scaffolding proteins, which also act in an allosteric fashion to affect receptor conformations. In this case, compound pharmacology can be altered depending on the context in which a receptor is expressed (e.g., (Niswender et al., 2010)), presumably due to the different proteins or cellular components interacting with the receptor in various cell types.

An alternate possibility that may affect the outcome of functional selectivity would be convergent signaling pathways that are activated (or inhibited, or simply absent) in a certain temporal or spatial context. It has previously been demonstrated that activation of the $G_{i/o}$-coupled $GABA_B$ receptor, in conjunction with the G_q-coupled metabotropic glutamate 1 ($mGlu_1$) receptor, produces a signaling convergence at the level of phospholipase C β3 ($PLC_{\beta3}$) to induce potentiated calcium mobilization (Pin et al., 2009; Rives et al., 2009). In these studies, this phenomenon was not due to heterodimerization/oligomerization of the receptors, was generalizable to other receptor pairs, and was demonstrated to exhibit functional relevance in cerebellar Purkinje cells and cultured cortical neurons where these two receptors are co-expressed (Rives et al., 2009). In the present manuscript, we extend these findings to explore potentially functionally selective effects induced by this type of signaling convergence. We describe that, as for the $GABA_B$ and $mGlu_1$ receptor combination, activation of a G_q coupled histamine receptor, the H_1 receptor, dramatically potentiates the ability of the $G_{i/o}$-coupled metabotropic glutamate 4 ($mGlu_4$) receptor to induce intracellular calcium mobilization. However, histamine does not potentiate the ability of $mGlu_4$ activation to modulate other "common" $G_{i/o}$-regulated signaling cascades, such as cAMP inhibition. These results suggest that H_1 co-activation biases $mGlu_4$-mediated signaling events toward certain signaling pathways. Furthermore, when small molecule PAMs of $mGlu_4$, are included in these assays, the potentiated signaling of $mGlu_4$ is further biased by histamine toward calcium-dependent pathways. Our results suggest that convergence of these signaling pathways may result in substantial, and potentially unexpected, increases in calcium responses downstream of $mGlu_4$ activation, particularly when receptor activity is potentiated using positive allosteric modulators.

2. Materials and methods

2.1. Cell line establishment and cell culture

Establishment and culture of the human $mGlu_4$ ($hmGlu_4$)/G_{qi5}/CHO-DHFR(−) has been described in (Niswender et al., 2008). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted.

Guinea pig H_1 (gp H_1)/CHO-K1 cells were obtained by stable transfection of CHO-K1 cells with guinea pig H_1 receptor in pcDNA3.1 vector (a generous gift of Mike Zhu, Ohio State University). Single G418-resistant clones were isolated and screened for H_1-mediated calcium mobilization as described below. Monoclonal gpH_1/CHO-K1 cells were cultured in 90% Dulbecco's modified Eagle's medium (DMEM), 10% dialyzed fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 μg/mL proline (Sigma-Aldrich, Inc., St. Louis, MO) and 400 μg/mL G418 sulfate (Mediatech, Inc., Herndon, VA).

Rat $mGlu_4$/CHO-K1 cells, rat $mGlu_2$/CHO-K1 cells, rat $mGlu_4$/H_1/CHO-K1 cells, and rat $mGlu_2$/H_1/CHO-K1 cells were obtained by stable transfection of either CHO-K1 cells or gpH_1/CHO-K1 cells with rat $mGlu_4$ or $mGlu_2$ receptor in a pIRESpuro3 vector (Invitrogen). Polyclonal cells were cultured in 90% DMEM, 10% dialyzed FBS, 100 U/mL penicillin/streptomycin, 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 μg/mL proline (Sigma-Aldrich, Inc., St. Louis, MO), 20 μg/mL puromycin (Sigma-Aldrich, Inc., St. Louis, MO) without or with 400 μg/mL G418 sulfate (for H_1 expressing cell lines, Mediatech, Inc., Herndon, VA).

Rat $mGlu_4$/M_1/CHO-K1 cells were generated by stable transfection of rat $mGlu_4$/CHO-K1 cells with rat M_1 muscarinic receptor DNA in pcDNA3.1 vector. Polyclonal cells were cultured in 90% DMEM, 10% dialyzed FBS, 100 U/mL penicillin/streptomycin, 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 μg/mL proline (Sigma-Aldrich, Inc., St. Louis, MO), 400 μg/mL G418 sulfate (Mediatech, Inc., Herndon, VA) and 20 μg/mL puromycin (Sigma-Aldrich, Inc., St. Louis, MO).

2.2. Calcium mobilization assays

For assays performed using the Flexstation (Molecular Devices, Sunnyvale, CA), cells were seeded at a density of 60,000 cells/100 μL/well in Costar 96-well tissue culture-treated plates. For assays performed using the Hamamatsu FDSS 6000 or 7000 (Hamamatsu, Japan), cells were seeded at 30,000 cells/20 μL/well in Greiner 384-well clear-bottomed, tissue culture–treated plates. Cells were incubated in assay medium (90% DMEM, 10% dialyzed FBS, 20 mM HEPES and 1 mM sodium pyruvate) overnight at 37 °C/5% CO_2 and assayed the following day.

Fluo-4/acetoxymethyl ester (Invitrogen) was dissolved as a 2.3 mM stock in DMSO and mixed in a 1:1 ratio with 10% (w/v) Pluronic acid F-127 and diluted in assay buffer (Hanks' balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid; Sigma-Aldrich) to a final concentration of 2 μM. Cells were dye-loaded for 45 min at 37 °C; dye was then removed and replaced by appropriate volume of assay buffer. For single-add experiments, a series of different concentrations of glutamate or histamine were diluted into assay buffer as 2× stock. For histamine fold-shift and potency experiments, histamine was diluted as 2× stock and added at the first add. After 150 s, the appropriate volume of a 5× glutamate stock was added in a second addition. For experiments using antagonists or PAMs, compounds were added at a 2× final concentration in the first addition followed by the desired concentration of agonist in the second addition.

2.3. Total RNA isolation, reverse transcription and polymerase chain reaction (RT–PCR)

CHO-K1 cells and $mGlu_4$/G_{qi5}/CHO-DHFR(−) cells were seeded in 10 cm cell culture dishes one day before the experiment. On the second day, cells were harvested and total mRNA from each cell line was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was quantified by Nanodrop and 0.5 ug was reversely transcribed into cDNA by iScript cDNA Synthesis Kit (Bio-Rad, Philadelphia PA) according to the manufacturer's protocol. Reactions were carried out both in the presence and in the absence of reverse transcriptase (as negative controls). One tenth of each yielded cDNA sample was used to perform polymerase chain reaction (PCR) using primers for histamine H_1 receptor, which were designed to match the conserved sequence for human, rat and mouse H_1:

H_1 Forward: CTCAACCTGCTGGTGCTGTA
H_1 Reverse: GAAGTCTGTCTCACACTTGTC

pcDNA3.1-gpH_1 (guinea pig H_1 receptor) plasmid was used as positive controls for H_1, while water was used as a negative control for PCR reactions. The amplification protocol was 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min. The final extension step was at 72 °C for 5 min. The yielded PCR products were then electrophoresed on a 1% agarose gel containing ethidium bromide in parallel with 1 Kb Plus DNA Ladder (Invitrogen).

2.4. Phosphoinositide hydrolysis assays

$mGlu_2$/H_1/CHO-K1 cells were plated in 24-well plates at a density of 100,000 cells/well/0.5 mL in growth medium two days before the assay. On the following day, growth media was removed and replace with 0.5 mL/well assay media containing 0.5 μCi/mL [^{3}H]inositol. Cell plates were incubated at 37°C/5% CO_2 overnight and assayed on the third day. For stimulation of phosphoinositide hydrolysis, the [^{3}H] inositol-containing assay medium was first aspirated from wells and replaced with 200 μL of assay buffer (HBSS supplemented with 20 mM HEPES and 30 mM LiCl).