

A Real-Time Method for Measuring cAMP Production Modulated by $G\alpha_{i/o}$ -Coupled Metabotropic Glutamate Receptors[§]

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ABSTRACT

Group II and group III metabotropic glutamate (mGlu) receptors are G protein-coupled receptors (GPCRs) that inhibit adenylyl cyclase via activation of $G\alpha_{i/o}$. The purpose of this study was to design a universal method that overcomes previous challenges in consistently measuring group II and group III mGlu-receptor (mGluR) activation in stably transfected systems. In Chinese hamster ovary (CHO) cells stably transfected with the GloSensor cAMP biosensor, we optimized conditions for simple and highly reproducible (<5% S.E.M.) measurements of cAMP in real time. The GloSensor cAMP biosensor is a recombinant firefly luciferase conjugated to a cAMP-binding domain, where cAMP binding promotes a conformational shift within the GloSensor protein, inducing luciferase activity; cAMP levels are positively correlated with light output resulting from the luciferase-mediated breakdown of D-luciferin. Each group II

and group III mGluR was then stably transfected into the CHO-GloSensor cell line, and experimental conditions were optimized for each receptor. During assay optimization, we observed ion sensitivity of several receptors and inverse agonist activity of the antagonist, LY341495 [2-[(1S,2S)-2-carboxycyclopropyl]-3-(9H-xanthen-9-yl)-D-alanine]. Although these phenomena have been previously reported, they remain poorly understood, emphasizing the GloSensor assay as an important tool with which to study group II and group III mGlu receptors. Our results highlight many advantages of using the GloSensor method for measuring activation of group II and group III mGlu receptors, and they further suggest that corresponding methods designed to measure activation of any $G\alpha_{i/o}$ - or $G\alpha_s$ -coupled GPCR will be similarly advantageous.

Introduction

Group II metabotropic glutamate (mGlu) receptors, mGlu2 and mGlu3, and group III mGlu receptors, mGlu4, mGlu6, mGlu7, and mGlu8, inhibit cAMP production via $G\alpha_{i/o}$ activation. Despite this common signaling mechanism, each receptor has unique cognitive and neurotrophic properties (Lyon et al., 2008; Caraci et al., 2011). At least in part, these differences are attributed to the unique cellular expression profile of each mGlu receptor (mGluR) within the central nervous system and to changes in expression patterns of these receptors during development (Catania et al., 1994). For example, high mGlu3-receptor expression is maintained in astrocytes through adulthood (Sun et al., 2013), whereas in the spinal cord, mGlu3-receptor expression decreases during development (Berthele et al., 1999). Unfortunately, a thorough understanding of each member's unique pharmacological, physiological, and pathophysiological role has been impeded by the lack of receptor-specific ligands.

This deficiency emphasizes the need for receptor-selective ligands as tools for characterizing the individual signaling properties of each mGluR.

Although no selective agonists exist, several nonselective mGluR agonists have entered late-stage clinical trials (Patil et al., 2007; Adams et al., 2013), but none has successfully reached the market. These clinical setbacks may be attributed to the lack of subtype selectivity, as studies suggest that even within groups, individual mGluR can play opposing physiological roles (Conn and Pin, 1997; Caraci et al., 2011). In turn, agents that preferentially target a single mGluR may demonstrate enhanced clinical efficacy compared with their nonselective counterparts. The discovery of receptor-specific ligands for each group II and group III mGluR, however, has been challenging (Niswender et al., 2008). The lack of comparability between different experimental systems limits the quantification of ligand selectivity. These systems often rely on receptors from different species, cloned into different expression vectors, transfected into different cell types, and/or characterized using different assay techniques. A clear solution to this problem would be the development of

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ABBREVIATIONS: AC, adenylyl cyclase; CHO, Chinese hamster ovary; CPPG, (RS)- α -cyclopropyl-4-phosphonophenylglycine; DCG-IV, (1R,2R)-3-[(1S)-1-amino-2-hydroxy-2-oxoethyl]cyclopropane-1,2-dicarboxylic acid; EGlu, (2S)-2-amino-2-ethylpentanedioic acid; GPCR, G protein-coupled receptor; IBMX, 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione; L-AP4, (2S)-2-amino-4-phosphonobutanoic acid; LY341495, 2-[(1S,2S)-2-carboxycyclopropyl]-3-(9H-xanthen-9-yl)-D-alanine; mGlu, metabotropic glutamate; mGluR, mGlu receptor; PDE, phosphodiesterase; PTX, pertussis toxin; RLU, relative light unit.

a single-assay paradigm to measure activation of each stably transfected $G_{\alpha_{i/o}}$ -coupled mGluR individually. To our knowledge, no such system is available, a technical insufficiency that has hindered the full pharmacological characterization of commonly used, supposedly selective ligands.

Another advantage of a universal, robust assay for measuring group II and group III mGluR activation would be the possibility of clarifying discrepancies in the literature involving the activation mechanism of each receptor. Several reports have suggested that mGluRs are directly modulated by ions (Kubo et al., 1998; Kuang and Hampson, 2006). However, few overarching trends can be extracted due to contrasting, and even contradictory, data. Although one study proposed that mGluRs are a family of calcium-sensing receptors (Kubo et al., 1998), another report shows that cations had no effect on ligand binding at mGlu3 receptors; rather, anions were required (Kuang and Hampson, 2006). With the exception of these examples, ion sensitivity at group II and group III mGluRs has not been explored.

Herein, we report the design and implementation of a single-assay paradigm with which to compare the pharmacological properties of the group II and group III mGluRs. This assay uses the GloSensor cAMP Biosensor (Promega, Madison, WI), a recombinant firefly luciferase conjugated to a cAMP-binding domain (Binkowski et al., 2011). cAMP binding promotes a conformational shift within the GloSensor protein, which induces luciferase activity. cAMP levels, which are modulated by group II and group III mGlu-receptor activation, are positively correlated with light output resulting from the luciferase-mediated breakdown of D-luciferin. We have generated mGlu2, mGlu3, mGlu4, mGlu6, and mGlu8 receptor-expressing Chinese hamster ovary (CHO)–GloSensor cell lines and characterized each receptor's coupling to cAMP inhibition using a simple, homogeneous, robust, reproducible, and real-time assay paradigm.

Materials and Methods

cDNA encoding rat mGlu2, mGlu3, mGlu4, mGlu6, and mGlu8 receptors were cloned into the pIRES2-AcGFP1 vector, digested with EcoRI (New England Biolabs, Ipswich, MA) using the In-Fusion cloning method (Clontech, Mountain View, CA). Polymerase chain reactions to amplify mGluR cDNAs (for primers, see Table 1) for insertion into the pIRES2-AcGFP1 vector were performed using the Phusion High-Fidelity DNA Polymerase Kit (New England Biolabs). The entire sequence of each mGluR construct was confirmed by sequence analysis (Genewiz, South Plainfield, NJ). The pGloSensor-22F cAMP plasmid was purchased from the Promega Corporation.

Dulbecco's modified Eagle's medium (DMEM), proline, and fetal bovine serum for cell cultures were purchased from Invitrogen (Carlsbad, CA). Receptor agonists glutamate, DCG-IV, and L-AP4, antagonists LY341495, EGlu, and CPPG, forskolin, IBMX, and pertussis toxin (PTX) were obtained from Tocris Bioscience (Ellisville, MO). D-Luciferin potassium salt was purchased from Gold Biotechnology (St. Louis, MO). [3 H]LY341495, with a specific activity of 40 Ci/mmol, was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Cell Cultures. Chinese hamster ovary-K1 cells were transfected (Lipofectamine LTX; Invitrogen) with the pGloSensor-22F plasmid and selected using 200 μ g/ml hygromycin (Invitrogen). CHO cells stably expressing the pGloSensor-22F construct (CHO-Glo) were then transfected with pIRES-AcGFP1 encoding mGlu2, mGlu3, mGlu4, mGlu6, or mGlu8 receptors and selected using 200 μ g/ml G-418 (Research Products International, Mount Prospect, IL). Stable cell lines were maintained with 0.8 μ g/ml hygromycin and 0.8 μ g/ml G-418. All cells were cultured in 6% CO₂ at 37°C in DMEM (high glucose) containing 10% fetal bovine serum, 300 μ M proline, 2 mM glutamine, and antibiotic-antimycotic (Invitrogen) and in the presence of G-418 and/or hygromycin. Cell lines were maintained in 6-cm polystyrene dishes. Cells for assay were plated on 96-well, white-walled, clear-bottom plates (Corning Life Sciences, Tewksbury, MA) and grown to confluence without G-418 or hygromycin.

Buffers. All assays were performed in either Locke's buffer or a modified Locke's buffer to replace specific ions (for concentrations of buffer substituents, see Table 2). To substitute for sodium (Locke-Na⁺), NaCl and NaHCO₃ were replaced with choline Cl and choline HCO₃, respectively. To substitute for chloride (Locke-Cl⁻), NaCl, KCl, MgCl₂, and CaCl₂ were replaced with sodium gluconate, potassium gluconate, magnesium gluconate₂, and calcium gluconate₂, respectively. Potassium (Locke-K⁺), magnesium (Locke-Mg²⁺), or calcium (Locke-Ca²⁺) was replaced with NaCl. HEPES (Locke-HEPES) was replaced with Tris and adjusted to pH 7.4 with gluconic acid. All buffer components were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of mGlu-Glo Membranes. mGlu-Glo cell membranes were prepared by harvesting adherent cells grown to confluence on 10-cm polystyrene plates. Scraped cells were resuspended in 20 ml of Tris buffer (20 mM Tris HCl, pH 7.4) and centrifuged at 1000g for 5 minutes at 4°C. The supernatant was discarded, and the pelleted cells were frozen at -80°C overnight. The membrane pellets were resuspended in 20 ml of fresh Tris buffer, homogenized for 20 seconds with a Brinkman Polytron homogenizer, and centrifuged at 35,000g for 10 minutes at 4°C. After discarding the supernatant, this protocol was repeated once. The final pellet was resuspended in fresh Tris buffer and kept on ice until the start of the binding assay. Protein concentration was determined using a Bradford protein assay.

Radioligand Binding. Membrane homogenates were incubated with the indicated concentrations of [3 H]LY341495 in Tris buffer, in the absence or presence of the indicated concentrations of glutamate, to measure total and nonspecific binding, respectively. After incubating

TABLE 1
Primers used to insert individual mGluRs into EcoRI-cut pIRES2-AcGFP1

Primer Name	Direction	Sequence (5'–3')
mGlu2F	Forward	GACTCAGATCTCGAGGGGGCCATGGAATCACTGCTTGGGTTTCTG
mGlu2R	Reverse	AATTTCGAAGCTTGAGTCAAAGCGACGACGTTGTTGAGTC
mGlu3F	Forward	GACTCAGATCTCGAGGGATTTCATGAAGATGTTGACAAGACTACAA
mGlu3R	Reverse	AATTTCGAAGCTTGAGTCACAGAGATGAGGTGGTGGAGTC
mGlu4F	Forward	CGTCAGATCCGCTAGTCCGAAATGTCCGGGAAG
mGlu4R	Reverse	GTCCGGTAGCGCTAGCTAGATGGCATGGTTGGT
mGlu6F	Forward	CGTCAGATCCGCTAGGAGCAGATGGGGAGGCTC
mGlu6R	Reverse	GTCCGGTAGCGCTAGCTACTTGGCGTCTCTGCTGC
mGlu8F	Forward	CGTCAGATCCGCTAGGAGAAAATGGTATGCGAG
mGlu8R	Reverse	GTCCGGTAGCGCTAGTCAGATCGAATGATTACT

R, reverse; F, forward.

TABLE 2

Components of buffers used to examine specific ion's effects on mGluR activation

Component	Locke	Locke-Na ⁺	Locke-Cl ⁻	Locke-K ⁺	Locke-Ca ²⁺	Locke-Mg ²⁺	Locke-HEPES
	<i>mM</i>						
NaCl	154	—	—	154	156.6	156	154
KCl	5.6	5.6	—	—	5.6	5.6	5.6
CaCl ₂	1.3	1.3	1.3	1.3	—	1.3	1.3
MgCl ₂	1	1	1	1	1	—	1
NaHCO ₃	3.6	—	3.6	3.6	3.6	3.6	3.6
Glucose	5.6	5.6	5.6	5.6	5.6	5.6	5.6
HEPES	20	20	20	20	20	20	—
Choline Cl	—	154	—	5.6	—	—	—
Choline HCO ₃	—	3.6	—	—	—	—	—
Sodium gluconate	—	—	154	—	—	—	—
Potassium gluconate	—	—	5.6	—	—	—	—
Tris	—	—	—	—	—	—	20

for 1 hour on ice, samples were collected on S and S30 filters (Schleicher & Schuell, Keene, IL) by vacuum filtration. Radioactivity captured on the filters was measured by liquid scintillation counting (Beckman LS6500; Beckman Coulter, Brea, CA), and specific [³H]LY341495 binding was calculated as the difference between total and nonspecific binding.

GloSensor cAMP Assay, Data Analysis, and Statistics. All GloSensor assays were performed using live cells, in a 96-well format on sterile white-walled, clear-bottom plates. Culture medium was aspirated and replaced with 100 μ l of Locke's buffer containing 450 μ g/ml D-luciferin (Locke-Luc). To equilibrate the cells with substrate, plates were preincubated in the dark at room temperature for 1 hour. Bioluminescence was quantified using the EnVision Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences, Waltham, MA) using a 1-second integration time. Before drug addition, each plate was read five times at 2-minute intervals to establish basal bioluminescence levels from each well. The average of these five prereadings was used to normalize each well's response to account for differences in GloSensor expression and cell density. After the five prereadings, a final concentration of 1 μ M forskolin with the appropriate final concentration of agonist, and/or antagonist was added in 50 μ l of Locke-Luc buffer (150 μ l final volume). Measurements were taken every 2 minutes for 18 minutes after drug addition. The bioluminescence measured at 16 minutes was used for "end-point" experiments.

For the real-time competition experiments (Fig. 5), a second treatment (vehicle, agonist, or antagonist) was applied at 18 minutes in 50 μ l of Locke-Luc buffer (200 μ l final volume). To account for the volume increase, forskolin and the first treatment drug were also added to maintain initial concentrations.

In the experiments with PTX, 1 μ g/ml of PTX was added to each well 24 hours prior to assay. For "end-point" assays, statistical significance was assessed using Student's *t* test. Concentration-response curves were fitted to data points by nonlinear regression using a four-parameter logistic equation. All calculations were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Results

The GloSensor Assay Measures Forskolin-Stimulated cAMP Production in CHO-Glo Cells. A new assay for measuring group II and group III mGluR activity was designed to use the GloSensor cAMP biosensor (Promega). In this system, increased cAMP levels are directly proportional to increased luminescence, which results from the enzymatic breakdown of the substrate, D-luciferin. CHO cells were transfected with the GloSensor plasmid (Binkowski et al., 2011), and multiple clones were tested for maximal

luminescence in response to 10 μ M forskolin. The clone with the largest increase in luminescence in response to forskolin was selected, and a single cell line (CHO-Glo) was established. Untransfected CHO cells lacking the GloSensor construct did not produce a detectable signal, whereas treatment of CHO-Glo cells with the adenylyl cyclase (AC) activator forskolin caused a concentration-dependent increase in relative light units (RLU) over time (Fig. 1A). The concentration dependency of cAMP production is shown using data measured 16 minutes after the addition of forskolin (Fig. 1B). The EC₅₀ for forskolin was calculated in this system to be 9.44 ± 0.60 μ M, which is comparable to the reported forskolin EC₅₀ of 5–10 μ M (Seamon et al., 1981). From these data, 1 μ M forskolin was chosen as a concentration sufficient to stimulate cAMP production with minimal potential for substrate depletion and a reliable signal-to-noise ratio. Hence, this concentration was used throughout the study. The group II and group III mGluR ligands used in this study were tested on CHO-Glo cells to ensure that these compounds would not affect cAMP levels in the absence of transfected mGluR. Several group II and/or group III mGluR agonists (glutamate, DCG-IV, and L-AP4) and antagonists (LY341495, EGlu, and CPPG) were assayed at saturating concentrations. Because of the lack of endogenous mGluRs, none of the drugs used in this study significantly altered cAMP production in CHO-Glo cells (Fig. 1C).

The GloSensor Assay Measures Glutamate-Mediated Decreases in cAMP Production in mGlu-Glo Cells. CHO-Glo cells were transfected with individual group II or III mGluR cDNAs, previously cloned into pIRES2-AcGFP1. Stable clones of CHO-Glo cells expressing individual mGluR types (mGlu2-Glo, mGlu3-Glo, mGlu4-Glo, mGlu6-Glo, mGlu7-Glo, and mGlu8-Glo) were selected based on AcGFP fluorescence and maximal light output in response to forskolin treatment. In Locke buffer, all mGlu-Glo cell lines, except for mGlu3-Glo, showed significant decreases in forskolin-stimulated cAMP production in response to 100 μ M glutamate (Fig. 2). Since several studies have demonstrated that mGluR activation is sensitive to ions (Kubo et al., 1998; Kuang and Hampson, 2006), we examined whether a buffer constituent was precluding mGlu3-Glo cells from inhibiting forskolin-stimulated cAMP production in response to glutamate treatment. Each buffer constituent was individually replaced with a structurally distinct ion of like charge (Table 2). Sodium was replaced with choline (Locke-Na⁺). Potassium,

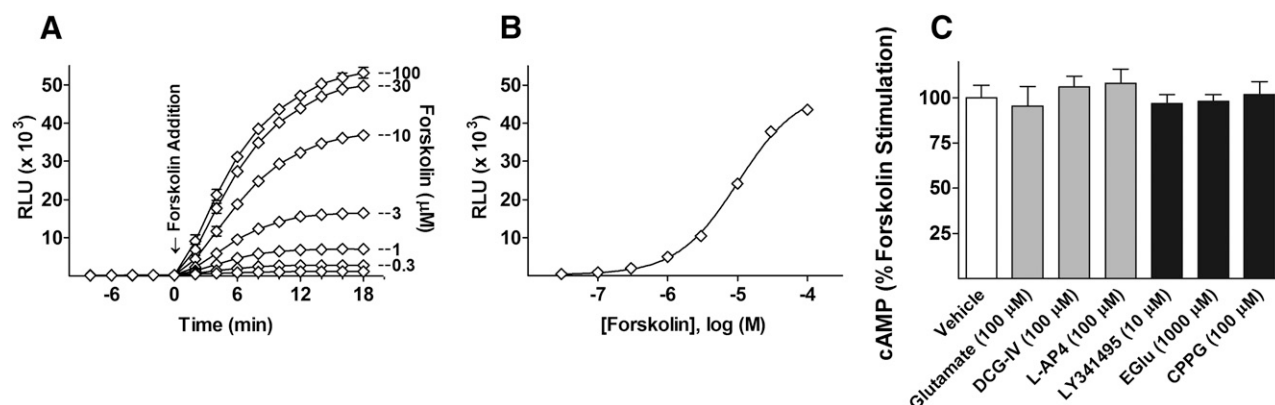


Fig. 1. Forskolin-mediated cAMP production in stably transfected CHO cells. (A) CHO cells, stably transfected with the GloSensor cAMP biosensor (CHO-Glo), show concentration-dependent increases in RLU over time when treated with increasing concentrations of the adenylyl cyclase activator forskolin. The reading from each well was normalized to its own basal luminescence, determined before forskolin addition. (B) The forskolin concentration-response curve was generated from data obtained 16 minutes after forskolin treatment. (C) Group II and group III mGluR agonists and antagonists do not affect forskolin-mediated cAMP production in CHO-Glo cells (lacking a transfected receptor). Data are means $\pm$ S.E.M. of three individual experiments performed in triplicate.

magnesium, or calcium was replaced with sodium (Locke-K⁺, Locke-Mg²⁺, Locke-Ca²⁺). Chloride, replaced with gluconate, was reduced from 164.2 to 4.6 mM (Locke and Locke-Cl⁻, respectively). HEPES was replaced with Tris, and the pH was adjusted to 7.4 with gluconic acid (Locke-HEPES). The removal of K⁺, Mg²⁺, or Ca²⁺ had no significant effect on cAMP production in any mGlu-Glo cell line. Locke-Na⁺ or Locke-

HEPES showed nonspecific decreases in cAMP inhibition at all mGlu-Glo cells (Fig. 2). mGlu7-Glo cells did not respond to glutamate under any of these conditions.

In low chloride buffer (Locke-Cl⁻), glutamate significantly inhibited forskolin-stimulated cAMP production in mGlu3-Glo cells (Fig. 2, mGlu3-Glo). In contrast, the removal of chloride decreased glutamate efficacy at mGlu4-Glo cells (Fig. 2, mGlu4-Glo), a result consistent with a previous report (Kuang and Hampson, 2006). Chloride removal showed no significant effect at any other mGlu-Glo cell line. Because this finding allowed reliable measurements of mGlu3-receptor activation, all subsequent mGlu3 receptor GloSensor assays were performed in Locke's buffer containing 4.6 mM chloride (Locke-Cl⁻), whereas all mGlu2, mGlu4, mGlu6, and mGlu8

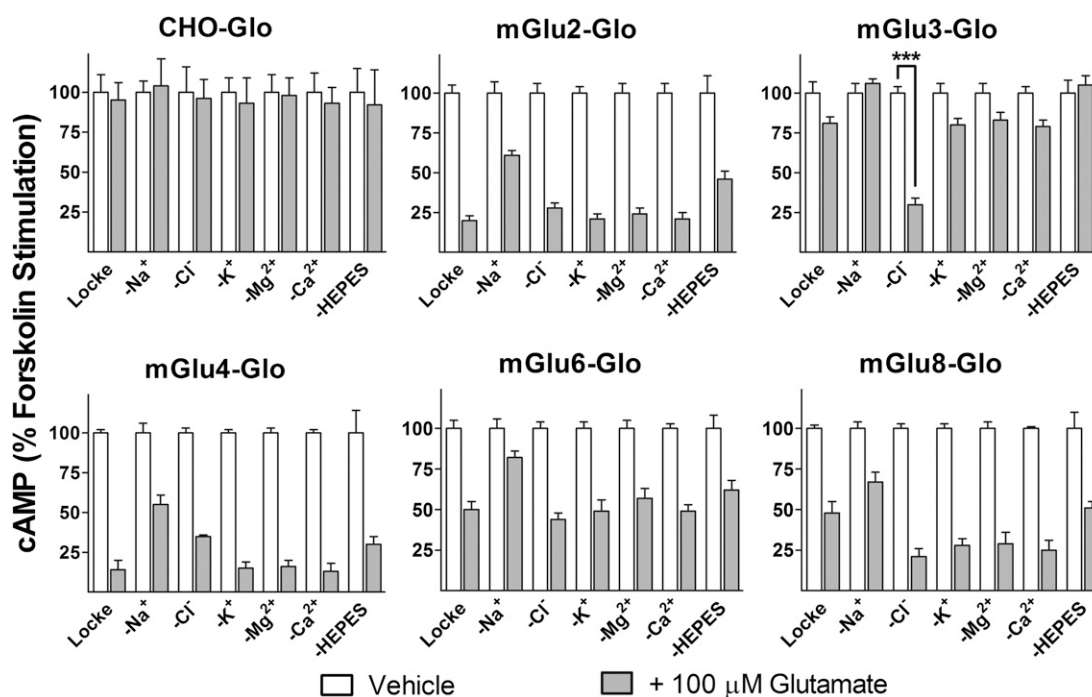


Fig. 2. Ion sensitivity of glutamate-mediated group II and group III mGluR activation. CHO-Glo or mGlu-Glo cells were treated with vehicle (white bars) or 100 μ M glutamate (gray bars) in a series of modified Locke's buffers (Table 2). In each buffer, data were normalized as a percent of vehicle treatment. Glutamate-mediated decreases in cAMP production were statistically significant ($P < 0.01$) at all mGlu-Glo cell lines, with the exception of mGlu3-Glo. Glutamate activation of mGlu3 receptors was significant only when Locke-Cl⁻ was used as the assay buffer. Statistical differences in the data were assessed by a Student's *t* test. Data are mean $\pm$ S.E.M. of three individual experiments performed in triplicate. *** $P < 0.001$.

receptor GloSensor assays were performed using Locke's buffer (Table 2). Nearly all reports of mGlu receptor modulation of cAMP production used end-point assays in the presence of IBMX to inhibit phosphodiesterases (PDEs). In this study, experiments were performed to compare the efficacy of glutamate in the absence or presence of IBMX at all mGlu-Glo cell lines. As expected, in the presence of IBMX, total luminescence was increased (Supplemental Fig. 1A), although no difference in glutamate efficacy was observed at any mGlu-Glo cell line (Supplemental Fig. 1B).

Radioligand-Binding Studies Using Membranes from mGlu-Glo Cell Lines. Radioligand binding studies using [3 H]LY341495 were conducted both to estimate the levels of receptor expression in all mGlu-Glo cell lines and to determine whether mGlu7-Glo cells were unresponsive due to insufficient receptor expression. To approximate levels of receptor expression, K_d concentrations of [3 H]LY341495 determined for human mGluRs (Wright et al., 2000) were used with membranes (50 μ g of protein) from each mGlu-Glo cell line. Only membranes from mGlu2-, mGlu3-, mGlu7-, and mGlu8-Glo cells demonstrated reliable specific binding. To our knowledge, the K_d values of [3 H]LY341495 at rat mGluRs have not been reported; therefore, B_{max} values were calculated using reported K_d values for human mGluRs (Wright et al., 2001). Calculated B_{max} values ranged from 0.26 to 1.5 pmol/mg (Supplemental Table 1).

The GloSensor Assay Provides a Real-Time Measurement of Concentration-Dependent Agonist-Stimulated $G\alpha_{i/o}$ Activation in mGlu-Glo Cell Lines. When assayed in Locke-Luc at mGlu2-, mGlu4-, mGlu6-, and mGlu8-Glo and Locke-Cl $^-$ at mGlu3-Glo cells, all cell lines showed glutamate-mediated, concentration-dependent decreases in forskolin-stimulated cAMP production in real time (Fig. 3). By 8 minutes after drug addition, glutamate-mediated decreases

in cAMP production were proportional at all subsequent time points. Data from the 16-minute time point showed the best signal-to-noise ratio and were used for all concentration-response curves (Fig. 4).

To ensure that effects on cAMP levels were entirely mediated by mGluR activation of $G\alpha_{i/o}$ proteins, the ability of glutamate to inhibit cAMP production was measured in the presence of pertussis toxin. PTX is a bacterial exotoxin that inactivates $G\alpha_{i/o}$ proteins via ADP-ribosylation of $G\alpha_{i/o}$ subunits. As previously demonstrated, 100 μ M glutamate was sufficient to significantly reduce cAMP levels at all mGlu-Glo cell lines (Fig. 2). However, in cells pretreated overnight with 1 μ g/ml PTX, glutamate failed to inhibit cAMP production (Fig. 4). PTX treatment had no effect on forskolin-stimulated cAMP production in CHO-Glo cells (data not shown). These results indicate that the inhibition of cAMP production in mGlu-Glo cell lines is receptor-mediated and entirely modulated by $G\alpha_{i/o}$ proteins.

To further characterize these cell lines, the potencies of two agonists were determined at each receptor. All cell lines were assayed with the endogenous mGluR agonist glutamate. Additionally, mGlu2- and mGlu3-Glo cell lines were assayed with the group II mGluR-selective agonist DCG-IV; mGlu4-, mGlu6-, and mGlu8-Glo cell lines were assayed with the group III mGluR-selective agonist L-AP4 (Fig. 4). Both DCG-IV and L-AP4 were more potent than glutamate at their respective receptors (Conn and Pin, 1997). As expected, DCG-IV and L-AP4 were inactive up to 100 μ M at group III and group II mGluRs, respectively. EC_{50} values calculated for glutamate, DCG-IV, and L-AP4 are summarized in Table 3.

The GloSensor Assay Measures Real-Time Reversibility of Agonists and Antagonists in mGlu-Glo Cell Lines. To ensure that decreases in cAMP mediated by agonists were attributed to receptor-selective, orthosteric

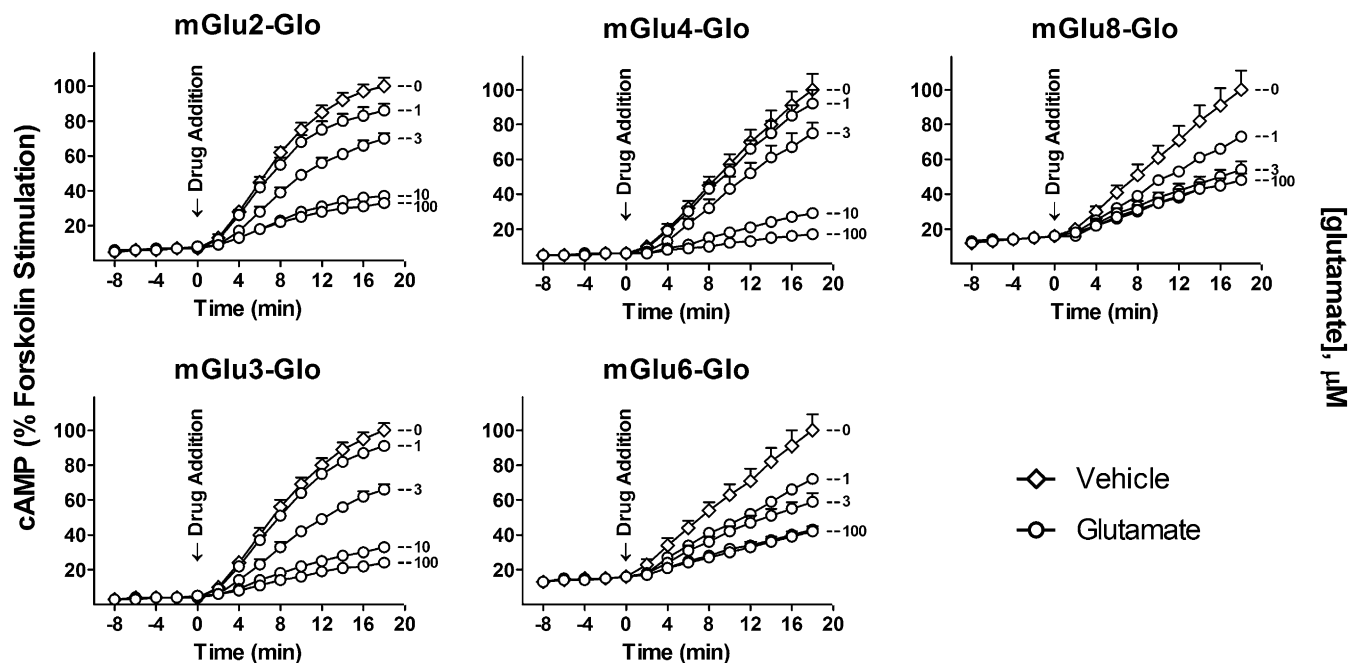


Fig. 3. mGluR-mediated inhibition of cAMP production in stably transfected CHO cells. mGlu-Glo cell lines were treated with 1 μ M forskolin and increasing concentrations of glutamate. Concentration-dependent decreases in RLUs were observed at all cell lines. Data from each treatment was normalized to its own basal luminescence. Data are mean $\pm$ S.E.M. of three individual experiments performed in triplicate.

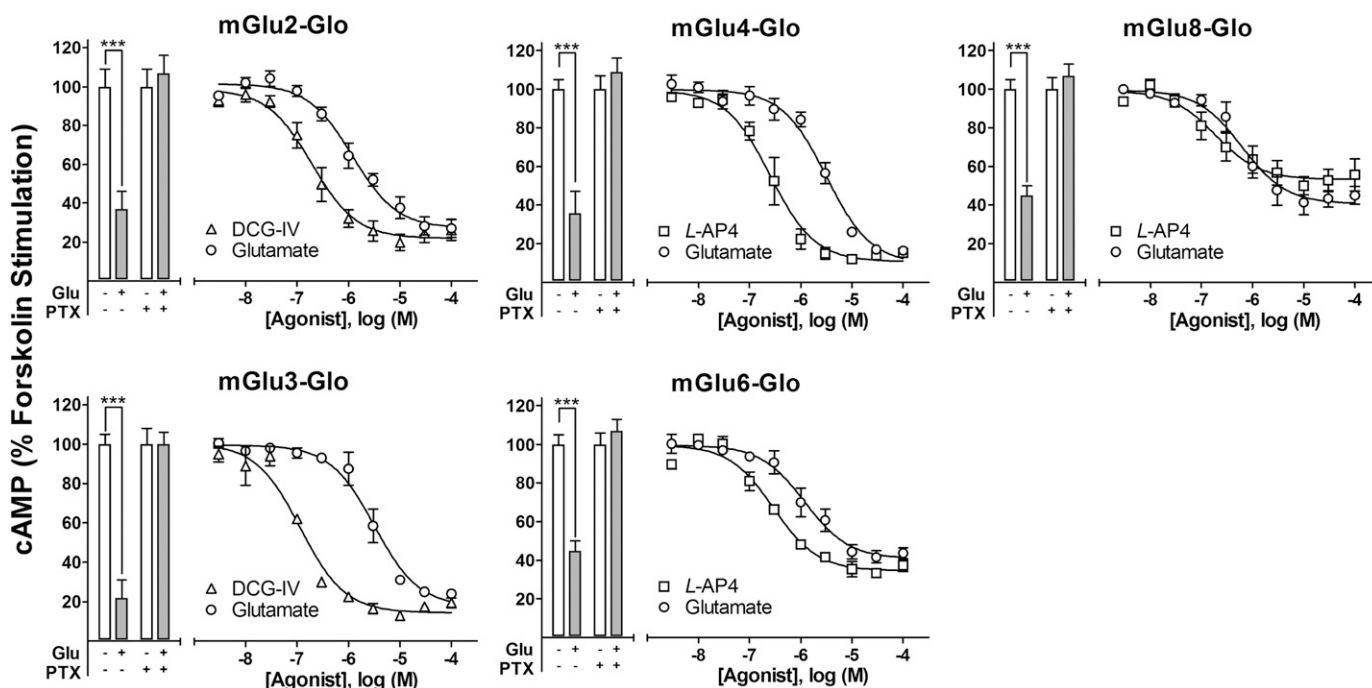


Fig. 4. mGluR agonists decrease cAMP production in mGlu-Glo cell lines in a PTX-sensitive, concentration-dependent manner. mGlu-Glo cells were pretreated with (or without) PTX before each assay. Vehicle (white bars) or 100 μ M glutamate (gray bars), $\pm$ PTX, were normalized to their respective vehicle-treated values. mGlu-Glo cell lines were treated with vehicle or increasing concentrations of mGluR agonists, glutamate, or either DCG-IV or L-AP4. Statistical significance was assessed by a Student's *t* test. Data for concentration-response curves were normalized to vehicle-treated values. Data are mean $\pm$ S.E.M. of three individual experiments performed in triplicate. ****P* < 0.001.

interactions, competition assays were performed to determine whether the competitive antagonist LY341495 would reverse glutamate-mediated inhibition of cAMP production. Vehicle, glutamate (100 μ M), or LY341495 (1 μ M) was added to mGlu-Glo cells and light output was measured every 2 minutes for 18 minutes. A second drug treatment (vehicle, 1 mM glutamate, or 10 μ M LY341495) was applied 18 minutes after the first addition, and light output was measured every 2 minutes for 18 minutes. (Fig. 5). Tenfold higher concentrations of drug were used during the second treatment to ensure full competition with the prior treatment. mGlu3-Glo cells were assayed using Locke-Cl⁻. During the first addition (time = 0–18 minutes), glutamate prevented cAMP production in all mGlu-Glo cell lines, but not in CHO-Glo cells. Notably, during the first addition, treatment with the antagonist LY341495 showed inverse efficacy, increasing cAMP production above vehicle treatment in mGlu4-, mGlu6-, and mGlu8-Glo cell lines, suggesting its action as an inverse agonist. After the second addition (time = 18–36 minutes),

glutamate inhibited cAMP production in all vehicle-treated and LY341495-treated mGlu-Glo cells, but not in CHO-Glo cells. Additionally, during the second addition, glutamate-treated mGlu-Glo cells challenged with LY341495 not only reversed glutamate-mediated inhibition of cAMP production but also showed inverse agonism at all cell lines (Fig. 5). Interestingly, inverse agonism was present in mGlu2- and mGlu3-Glo cell lines only when LY341495 was added to agonist treated cells, whereas mGlu4-, mGlu6-, and mGlu8-Glo cell lines showed inverse agonism in both the presence and absence of agonist. After the second treatment, non-specific, transient decreases in RLU signal, probably because of the volume change in the wells, were observed at all cell lines, including the control CHO-Glo cell line. Decreases in luminescence were also observed after 30 minutes, possibly because of PDE activity, desensitization of adenylyl cyclase, or cellular exhaustion.

The GloSensor Assay Accurately Approximates Antagonist Affinity. Our data show that the GloSensor assay

TABLE 3

EC₅₀ (μ M) values calculated from the GloSensor assay are comparable to reported values EC₅₀ values calculated from data shown in Fig. 4 using the three-parameter logistic equation

Errors are reported as confidence interval (CI).

	Glutamate			DCG-IV			L-AP4		
	EC ₅₀	95% CI	Reported	EC ₅₀	95% CI	Reported	EC ₅₀	95% CI	Reported
mGlu2 Receptor	2.9	1.8–4.7	4–20 ^a	0.91	0.50–1.7	0.3 ^a	>100	—	>100
mGlu3 Receptor	3.2	2.4–4.3	4–5 ^a	0.87	0.79–0.96	0.2 ^a	>100	—	>100
mGlu4 Receptor	3.9	2.2–6.9	3–20 ^a	>100	—	>100	0.26	0.16–0.42	0.4–1.2 ^a
mGlu6 Receptor	1.2	0.67–2.1	16 ^a	>100	—	>100	0.28	0.19–0.42	0.9 ^a
mGlu8 Receptor	0.62	0.35–1.1	0.02 ^a	>100	—	>100	0.19	0.06–0.56	0.4 ^a

^aData from Conn PJ and Pin JP (1997).

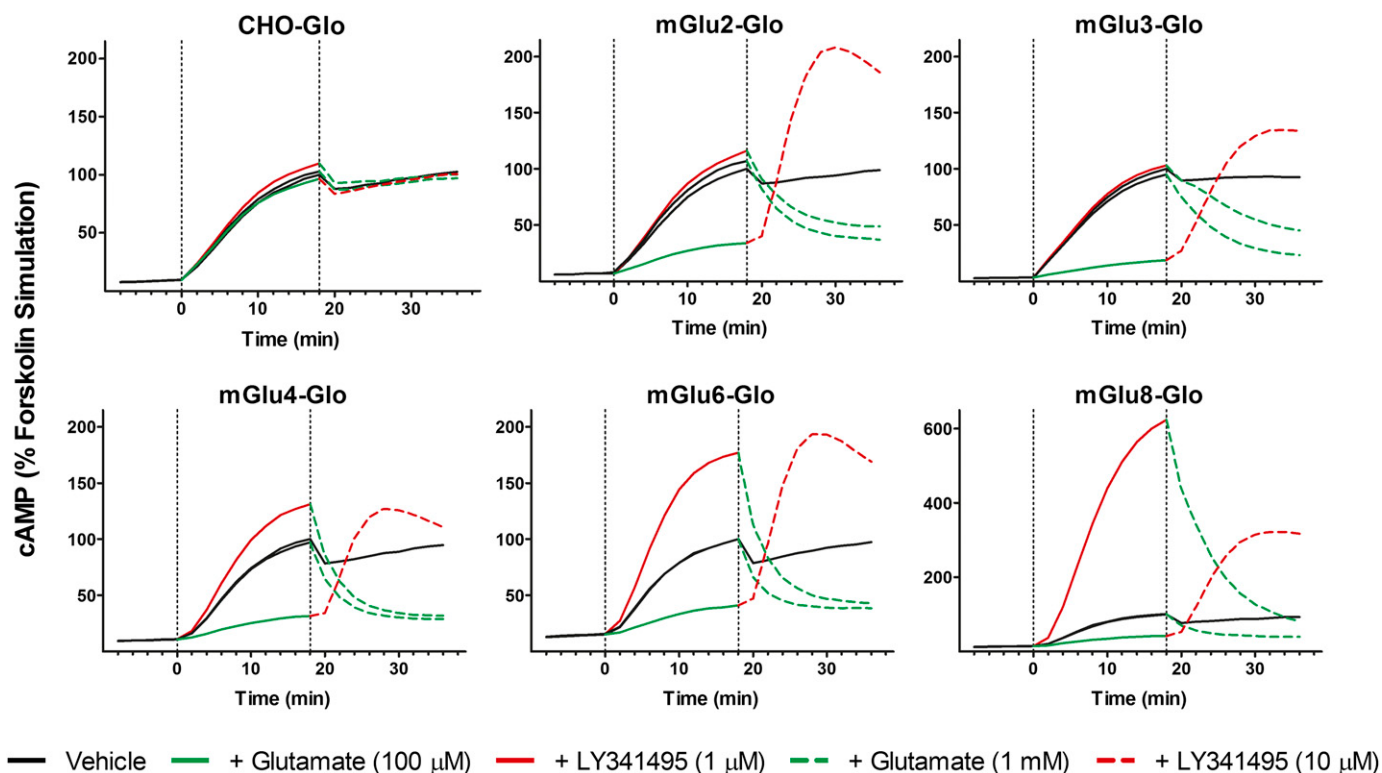


Fig. 5. Real-time reversibility of agonists and antagonists effects on cAMP production. CHO-Glo or mGlu-Glo cells were pre-equilibrated with the substrate in Locke's buffer (Locke 4.6 at mGlu3-Glo), and basal luminescence was measured as before. Vehicle (black line), glutamate (100 μ M, green dashed line), or LY341495 (1 μ M, red dashed line) was added first. A second treatment of vehicle (black line), glutamate (1 mM, green, solid line), or LY341495 (10 μ M, red solid line) was added at 18 minutes. For the sake of clarity, S.E.M. is not shown, but averaged 8% through the experiment. Data represent the means of three individual experiments performed in triplicate.

effectively measures the concentration dependency of agonist-mediated cAMP inhibition. To further examine the behavior of antagonists, LY341495 was used at increasing concentrations to shift the concentration-response curve of glutamate at mGlu3 receptors (Fig. 6). As expected for competitive antagonists, a rightward shift in the glutamate concentration-response curve was observed with increasing concentrations of LY341495. Each curve was normalized to its own maximal RLU in the absence of glutamate, and glutamate EC_{50} values were determined at each concentration of LY341495. On the basis of these calculations, a Schild plot was constructed, yielding a K_b of LY341495 at rat mGlu3 receptors of 1.8 nM. This estimate is consistent with the K_d values of 0.75 nM determined empirically for LY341495 at transfected human mGlu3 receptors (Johnson et al., 1999).

Discussion

Group II and group III mGluRs have been implicated as therapeutic targets for treating a variety of cognitive and neurodegenerative diseases, including schizophrenia, stress and anxiety disorders, Parkinson's disease, and Alzheimer's disease (Calabresi et al., 1999; Niswender and Conn, 2010; Caraci et al., 2011). Although the group II and group III mGluRs share a common signaling mechanism, selective activation of just one receptor subtype can have unique physiologic consequences (Corti et al., 2007). Unfortunately, a thorough understanding of these differences has been hindered, as few subtype-selective ligands have been identified. A universal, robust, and reproducible method to evaluate

and compare the individual pharmacological properties of each receptor subtype would provide an essential tool for the discovery of subgroup-selective drugs. Although a variety of techniques for measuring mGluR-induced $G\alpha_{i/o}$ activity have been successful, few groups have measured group II and

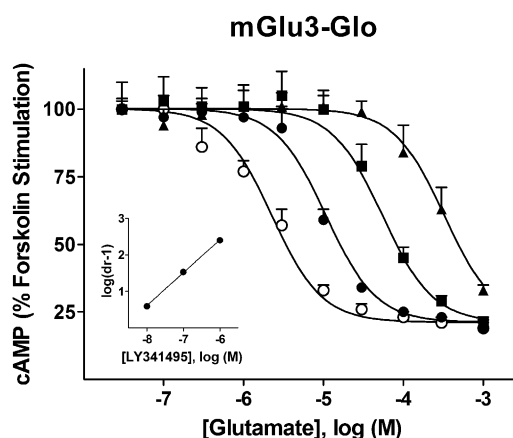


Fig. 6. The GloSensor assay measures antagonist activity. mGlu3-Glo cells were pre-equilibrated with substrate in Locke-Cl⁻. Increasing concentrations of glutamate were added, without or with several concentrations of LY341495 (0, white circles; 10^{-8} , black circles; 10^{-7} , black squares; 10^{-6} , black triangles). The K_b values were calculated using GraphPad Prism software. (Inset) A Schild plot is shown where dr indicates dose ratio and the values of $dr-1$ are plotted versus antagonist concentration. Data are mean $\pm$ S.E.M. of three individual experiments performed in triplicate.

group III mGluR activation with the same method in the same system. This may be attributed to the challenge of engineering a method to reliably measure $G\alpha_{i/o}$ -coupled mGluR activation. Groups have resorted to measuring noncanonical $G_{\beta/\gamma}$ activation (Malherbe et al., 2001; Niswender et al., 2008) using complicated Förster resonance energy transfer (FRET) assays that require artificial receptor constructs (Yanagawa et al., 2011), engineering chimeric receptors (Wroblewska et al., 1997), or the use of cell-free radioligand binding assays (Schweitzer et al., 2000). Herein, we report a novel, comprehensive method that reliably measures group II and III mGluR-mediated inhibition of AC. A schematic of the GloSensor method is presented in Fig. 7.

As others have reported, our group also faced difficulties measuring mGluR-mediated $G\alpha_{i/o}$ activation (Niswender et al., 2008). While establishing that the GloSensor assay to measure mGlu2-, mGlu4-, mGlu6-, and mGlu8-receptor activation was surprisingly straightforward, we encountered challenges establishing an assay for mGlu7 and mGlu3 receptors. Surprisingly, mGlu7-Glo cells showed significant specific binding of the radioligand [3 H]LY341495, whereas no measurable specific binding was observed at mGlu4-Glo or mGlu6-Glo cell lines. Previous reports indicate that [3 H]LY341495 is not suitable for binding studies using human mGlu4 receptors, probably because of a high K_d value (Wright et al., 2000), and this may be true for rat mGlu4 receptors. Likewise, although the mGlu6-Glo cell line is functional, differences between the K_d values of [3 H]LY341495 at human versus rat mGlu6 receptors could explain the lack of specific binding observed in this study. Finally, the calculated B_{max} values indicate that mGlu2-, mGlu3-, mGlu7-, and mGlu8-Glo cell lines have a lower receptor density than previously reported for human mGluR-expressing cell lines (Wright et al., 2000) and group II mGluRs in rat brain tissue (Wright et al., 2001).

Although a functional mGlu7-Glo cell line remains elusive, our data suggest that the reason measuring mGlu3-receptor

signaling via $G\alpha_{i/o}$ has proven difficult is that chloride prevents measurements of agonist-mediated mGlu3-receptor activation. This finding is consistent with other studies that have suggested that mGluRs are directly modulated by ions. For example, one study proposed that mGluRs are a family of calcium-sensing receptors, where $CaCl_2$ was shown to activate mGlu3 receptors (Kubo et al., 1998). Although Ca^{2+} did not affect any mGluR activation in our system, Cl^- removal from the assay media greatly improved mGlu3 receptor-mediated responses to agonists. In agreement with our findings, a separate study showed that, although cations had no effect, small anions affected ligand binding at mGlu3 receptors (Kuang and Hampson, 2006). This report complements our data showing that agonist activation of the mGlu3 receptor is affected specifically by chloride. Although reducing chloride concentration allows for reproducible measurements of mGlu3 receptor activation, further work is required to describe the mechanism of mGlu3 receptor chloride sensitivity.

Data obtained using saturating concentrations of the antagonist LY341495 revealed an increase in cAMP production relative to vehicle treatment (Fig. 5). At mGlu2- and mGlu3-Glo cell lines, this phenomenon was only observed after agonist treatment. This finding supports other reports of $G\alpha_{i/o}$ -coupled G protein-coupled receptor (GPCR) activation supersensitizing AC, and it suggests that group II mGluR activation may supersensitize AC as well (Watts, 2002; Watts and Neve, 2005). At the group III mGluRs, LY341495 mediated increases in cAMP production in both the absence and presence of agonist. These observations suggest that, in mGlu4-, mGlu6-, and mGlu8-Glo cell lines, LY341495 was either competing with an unidentified agonist that had also supersensitized AC or LY341495 was acting as an inverse agonist by inhibiting constitutive receptor activity. Although these data are consistent with other reports of antagonist-mediated increases in cAMP production at many of the $G\alpha_{i/o}$ -coupled mGluRs in transfected cells (Suzuki et al., 2007), it is

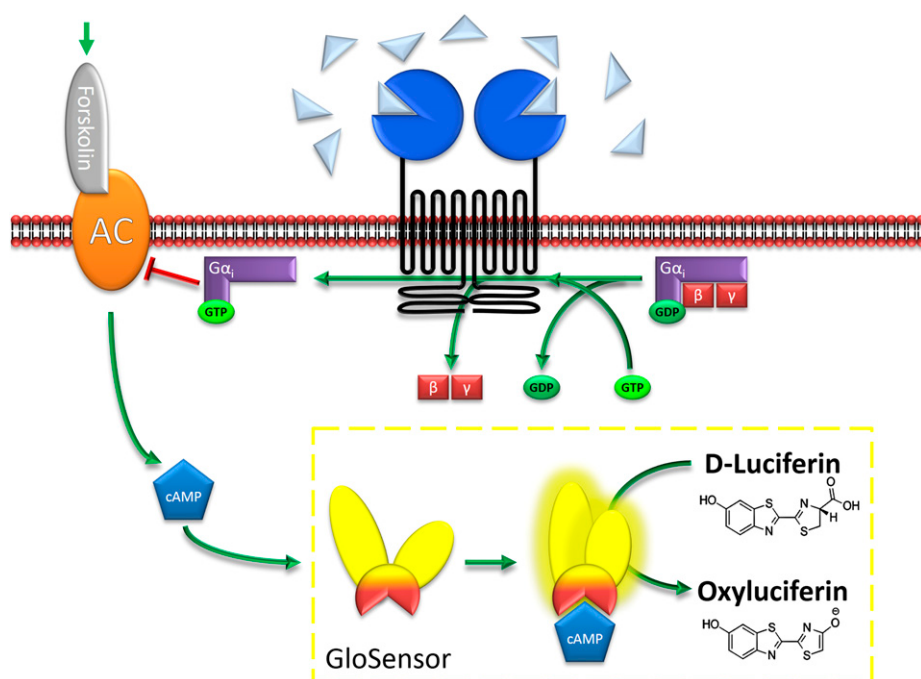


Fig. 7. Schematic representation of the GloSensor assay. Forskolin stimulates cAMP production via adenylyl cyclase (AC). cAMP binding to the GloSensor construct causes a conformational shift that increases luciferase activity, whereby the D-luciferin substrate is oxidized to produce oxyluciferin and light. Agonist stimulation of group II or group III mGluRs activates heterotrimeric $G_{i/o}$ proteins, where the liberated α -subunit then inhibits AC activation. Agonist activity is measured as decreased light output relative to vehicle treatment.

not clear whether this phenomenon is present in native systems. Further investigation is required to determine the mechanism and physiologic relevance of this inverse agonism.

The GloSensor cAMP biosensor provides many advantages over other methods for measuring mGluR activation: (1) every assay to date that has measured mGluR-mediated $G\alpha_{i/o}$ activity has been an endpoint assay, requiring IBMX to inhibit PDEs. In contrast, the GloSensor method measures cAMP production in real time. This novelty allows us to stimulate and inhibit receptor activity on the same cells in the same assay, demonstrating the competitive and reversible nature of orthosteric ligands acting at the mGluRs. Furthermore, this real-time system has the capability to expose kinetic differences (i.e., efficiency of coupling to G proteins, ligand on-off rates, deactivation profiles, effects on PDEs, etc.) between any $G\alpha_{i/o}$ -coupled receptors. (2) The GloSensor method allows direct, functional measurements of the classic signaling pathway modulated by group II and group III mGluRs. Although competition binding assays allow for measurements of ligand affinity, they do not address any functional properties associated with the ligand, namely, whether a ligand is an agonist, partial agonist, antagonist, or inverse-agonist. Furthermore, Förster resonance energy transfer-based methods, although useful, do not use wild-type receptors. Other methods measure noncanonical $G_{\beta/\gamma}$ -mediated potassium flux as an alternative indicator for receptor activation. (3) In contrast to many electrophysiological and biophysical methods, the GloSensor method does not require expensive equipment, as all measurements can be performed with any luminescence detector. The substrate is inexpensive, and we have performed experiments using several luciferin salts, all of which produced similar results. Set-up and assay times are particularly short with the GloSensor method, making it an efficient assay paradigm that can facilitate rapid and reliable measurements of ligand activity in a high-throughput screening format. Although a homogeneous assay system for measuring $G\alpha_{i/o}$ activation mediated by the group II and III mGluRs will address many discrepancies in the literature, the emergence of GPCR-mediated G protein-independent signaling suggests that using just one assay system to investigate drug-receptor interactions will provide incomplete information; rather, it is necessary to integrate data from several distinct functional assays, ligand affinity experiments, and structural studies to provide a comprehensive ligand-receptor profile (Shoichet and Kobilka, 2012).

In summary, we developed a new method to measure $G\alpha_{i/o}$ -coupled mGluR activity in stably transfected cells. This assay has already helped to clarify a discrepancy in the literature showing that chloride, but not calcium, affects mGlu3-receptor signaling. Together with LY341495-mediated inverse agonism, these results demonstrate that the GloSensor method is a useful tool for investigating properties of mGluR signaling. The GloSensor method provides a cost-effective, efficient, real-time approach for measuring the canonical signaling mechanism of group II and group III mGluRs, and it should prove equally applicable to any $G\alpha_{i/o}$ - or $G\alpha_s$ -coupled GPCR.

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Authorship Contributions

Participated in research design: DiRaddo, Miller, Wroblewska, Wolfe, Wroblewski.

Conducted experiments: DiRaddo, Hathaway, Grajkowska, Wroblewska.

Contributed new reagents or analytic tools: DiRaddo, Miller, Liotta, Wroblewski.

Performed data analysis: DiRaddo, Hathaway, Wroblewski.

Wrote or contributed to the writing of the manuscript: DiRaddo, Miller, Hathaway, Wroblewska, Wolfe, Liotta, Wroblewski.

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