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Ecdysone-Based System for Controlled Inducible Expression of Metabotropic Glutamate Receptor Subtypes 2, 5, and 8

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Stable and inducible expression of human metabotropic glutamate receptor types 2, 5, and 8 was achieved in HEK293 cells using the ecdysone inducible system. Treatment of the respective cell lines with ponasterone A resulted in time- and concentration-dependent induction of receptor expression. In all cases, the functional activation of receptors was determined by measuring increases in intracellular calcium. The physiologically $G\alpha_i$-coupled receptors mGluR2 and mGluR8 were successfully coupled to phospholipase C activation using the chimeric G protein $G\alpha_{q/o}$. The pharmacological properties of recombinant receptors were characterized and proved to be similar to native receptors. Our data suggest that the ecdysone system has a number of characteristics that make it well suited for expressing mGluRs and that the combined use of this system and chimeric G proteins allows receptors to be characterized using a rapid and straightforward Ca^{2+} assay. (*Journal of Biomolecular Screening* 2005:841-848)

Key words: metabotropic glutamate receptors, inducible expression, chimeric G proteins

INTRODUCTION

GLUTAMATE IS GENERALLY RECOGNIZED as being the most important excitatory neurotransmitter in the mammalian CNS. It is intimately involved in a wide range of neuronal and glial processes, by the activation of both ionotropic and metabotropic (mGluR) receptor subtypes.[1] These latter receptors are G protein–coupled receptors (GPCRs) and belong to a group of receptors that includes the Ca^{2+} sensing receptor, the $GABA_B$ receptors, and a large number of putative olfactory, pheromone, and taste receptors.[2] The mGlu receptors are widely distributed throughout the CNS, and there is now substantial evidence for an important role of mGluRs in normal brain functions as well as in neurological and psychiatric disorders.

The metabotropic glutamate receptors form a family of 8 subtypes that are subdivided into 3 groups based on their sequence homology, pharmacology, and transduction mechanisms. Group I receptors (mGluR1 and mGluR5) couple through $G\alpha_{q/11}$ proteins to the activation of phospholipase C, resulting in phosphoinositide hydrolysis, the release of calcium from intracellular stores and the activation of protein kinase C. Groups II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7, and mGluR8) are negatively coupled to adenyl cyclase (AC) through $G\alpha_i/G\alpha_o$ proteins, thereby inhibiting cyclic AMP (cAMP) formation and cAMP-dependent protein kinase activation.[3] Group I receptors usually act postsynaptically to increase neuronal excitability, whereas the group II and III receptors most often act presynaptically to reduce neurotransmitter release, including glutamate.[4] Excessive glutamatergic neurotransmission has been shown to underlie many CNS diseases and to play an important role in the pathophysiology of diseases such as depression, anxiety, and chronic pain, which represent key areas of interest for drug development. Accordingly, mGluR group I antagonists or group II/III agonists could represent useful agents for the treatment of these conditions. Indeed, group I antagonists have been shown to possess antidepressant activity in preclinical models[5] and to be very effective agents in the treatment of prolonged and chronic pain.[6-8] Studies using specific group II agonists suggest that such compounds may be useful in the treatment of inflammatory pain,[9,10] chronic pain,[11] and anxiety.[12] Less is known about the role of group III mGluRs, principally due to the lack of potent and selective ligands. However, studies using knockout mice show that animals lacking mGluR8 display a marked increase in anxiety-related behavior, possibly suggesting that mGluR8 agonists may also produce anxiolysis.[13] In support of this notion, specific group III agonists have recently been shown to produce anxiolytic and antidepressant-like effects.[14] The metabotropic glutamate receptors thus represent a potential

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goldmine of drug targets for the treatment of many CNS disorders, and their involvement in depression, anxiety and chronic pain makes them particularly appealing targets.

An important prerequisite in the search for selective mGluR modulators is the development of specific clonal cell lines for each receptor type. This has become even more important since the discovery of allosteric modulatory sites located in the 7 TM region of mGluRs. These sites are less conserved among groups and subtypes and thus make it possible to identify new chemical entities with improved selectivity within the family of metabotropic glutamate receptors. Functional cell-based assays for single mGluRs permit chemical libraries to be screened in the search for potent agonists and antagonists as well as the more selective allosteric modulators.

One of the problems in isolating cell lines that express glutamate receptors is the fact that glutamate is released from cells into the culture medium and hence causes desensitization and/or down-regulation of the receptor.[15] To circumvent this problem, we used an inducible system, the ecdysone system. This system is based on the ability of ecdysone, an insect steroid, to potently induce transcription via the activation of a heterodimeric receptor composed of the ecdysone receptor (EcR) and the ultraspiracle protein (USP).[16] Responsiveness to ecdysone in mammalian cells is recreated by transfection of EcR, the retinoid X receptor (RxR; the mammalian homologue of USP), an ecdysone-responsive reporter, and application of ecdysone or a synthetic analogue such as ponasterone A. Expression of a given cDNA can be placed under the control of the EcR/RxR heterodimer, which activates transcription in the presence of the exogenous inducing agent. Our results suggest that this system is very well suited to the development of functional mGluR receptor assays and, in combination with the use of chimeric $G\alpha_{q/o}$ proteins, allows the use of a single assay platform to analyze all receptor types.

MATERIALS AND METHODS

Molecular biology

cDNA was synthesized from human hippocampal mRNA (Clontech, Mountain View, CA) using the superscript kit according to the manufacturer's (Invitrogen, Paisley, UK) instructions. The coding sequences of mGluR2 and mGluR5b were amplified from hippocampal cDNA by PCR using a proofreading enzyme Pfu_{Turbo} (Stratagene, La Jolla, CA). A plasmid pCMV6-XL5-mGluR8 encoding the human mGluR8a cDNA was acquired from Origene (Rockville, MD), and the hmGluR8a coding sequence was amplified from this plasmid by PCR. All amplified products were completely sequenced on both DNA strands to make sure that sequence errors had not been introduced in the amplification process. The mGluR coding sequences were subcloned into the expression vector pIND (Invitrogen) using appropriate restriction enzymes. A chimeric G protein $G\alpha_{q/o}$ constructed by replacing the last 5 amino acids of the $G\alpha_q$ alpha subunit with those of the $G\alpha_o$ protein as previously described[17] was a kind gift of Dr. F. Monsma (Schering-Plough Research Institute, Kenilworth, NJ). The chimeric $G\alpha_{q/o}$ coding sequence was subcloned into pCDNA3-Hygro using appropriate restriction enzymes. A pIND vector expressing mGluR8a and the $G\alpha_{q/o}$ as a single transcript was constructed by inserting an IRES sequence between mGluR8a and $G\alpha_{q/o}$. The pIRESneo2 vector plasmid (Clontech) was digested with *XhoI* and *SmaI* to remove the Neo^r coding sequence and polyA sequence; they were replaced by $G\alpha_{q/o}$ cDNA, obtained from a pCDNA3.1-$G\alpha_{q/o}$ plasmid through *XhoI-SmaI* cleavage. Then, the IRES-$G\alpha_{q/o}$ cassette was subcloned into a pIND-mGluR8a plasmid as a NotI fragment, just downstream from the receptor cDNA previously cloned into pIND as a *HindIII-NotI* fragment. The mGluR5b and mGluR8a sequences were identical to those previously reported,[18,19] whereas the amplified mGluR2 cDNA was identical to the variant reported by Kowal and others.[20]

Isolation of clonal cell lines

The HEK-293EcR cell line (Invitrogen), stably expressing the heterodimeric ecdysone receptor from the pVgRXR plasmid, was maintained in DMEM high-glucose medium, supplemented with 10% fetal bovine serum (FBS), 10,000 U/mL penicillin, 10 mg/mL streptomycin, 2 mM GlutaMAX™ (Invitrogen), and 400 µg/mL Zeocin™ (Invitrogen) at 37° C, in an atmosphere containing 5% CO_2. Stable transfections with mGluRs and chimeric G protein–coding vectors were performed using calcium phosphate according to standard protocols. Stably transfected clones were obtained after selection with 550 µg/mL Geneticin® (Invitrogen) (mGluR5b and mGluR8a-$G\alpha_{q/o}$ cell lines) and 550 µg/mL Geneticin® (Invitrogen) and 200 µg/mL hygromycin B (mGluR2-$G\alpha_{q/o}$ cell line). All cell culture media and reagents were from Invitrogen (Paisley, UK). The sensitivity to DMSO was tested for all cell lines; no signs of toxicity were observed when DMSO was added up to a final concentration of 1%.

Western blotting

Cells were plated in 6-well dishes in complete DMEM and induced by adding various amounts of a 1 mM stock of ponasterone A (Invitrogen). Ponasterone A was dissolved in 100% ethanol, and ethanol levels were compensated in all induced cultures. After a 24-h induction period, cells were washed with ice-cold phosphate-buffered saline, detached from the dishes using a scraper, and resuspended in lysis buffer (NaCl 150 mM, Tris-HCl 50 mM, Triton 1%, NP40 1%, SDS 0.2%, EDTA 2 mM, PMSF 1 mM, NaF 50 mM, and protease inhibitors). Samples were collected on ice and centrifuged at 10,000*g* for 15 min at 4° C and were subsequently stored at –80° C. Immunoblotting was performed using a polyclonal anti-mGluR5 antibody (Upstate, Waltham, MA) 0.5 µg/mL and a monoclonal anti-GAPDH antibody (Chemicon, Temecula, CA) 1 µg/mL, following standard procedures.

Measurement of intracellular calcium transients

Cells from clonal cell lines were seeded into black, clear-bottom, 96-well plates at a density of 60,000 cells/well, in DMEM high glucose supplemented with 5% dialysed FBS, 3 U/mL glutamic-pyruvic transaminase (Sigma, St. Louis, MO), 5 mM sodium pyruvate, 5 μM ponasterone A. Following 24-h incubation, the cells were loaded with a fluorescent calcium indicator, either Calcium 3® or FLIPR® calcium assay kit, both supplied by Molecular Devices (Sunnyvale, CA). The dyes were dissolved in the assay buffer, which consisted of Hanks' balanced salt solution (HBSS; Gibco Life Technologies, Carlsbad, CA) buffered with 20 mM Hepes solution (Sigma). To avoid dye bleaching, 2.5 mM probenecid (Sigma) was added to the calcium indicator solution. Cells were loaded with the dye for 2 h at 37° C in a CO_2 incubator. A fluorometric imaging plate reader (FLIPR 384; Molecular Devices) was used to measure intracellular calcium by increases in fluorescence upon agonist stimulation following 30-s baseline measurement or 10 min antagonist administration.

Compounds

Agonists. (1S,3R-ACPD), 1-aminocyclopentane-1S,3R-dicarboxylic acid; (2R,4R-APDC), (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylic acid; (L-AP4), L(+)-2-amino-4-phosphono butyric acid; (L-CCG 1), (2S,3S,4S)-α-(carboxycyclopropyl) glycine; (CHPG), (*RS*)-2-Chloro-5-hydroxyphenylglycine; (DCG-IV), (2S2′R,3′R)-2-(2′3′-dicarboxycyclopropyl) glycine; (DCPG), (S)-3-4-Dicarboxyphenylglycine; (DHPG), 3,5-dihydrophenylglycine; L-Glutamate; (NAAG), N-acetylaspartylglutamate; (PPG), (R,S)-4-phosphonophenylglycine; quisqualic acid.

Antagonists. (LY341495), (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; (MPEP), Methylphenylethynylpyridine; (MTEP) 3-[(2-methyl-1,3-thiazol-4-yl)ethyl]pyridine.

All agonists and antagonists were purchased from Tocris Cookson (Bristol, UK). Compounds were dissolved in HBSS or DMSO depending on their solubility. The final concentration of DMSO in all samples was compensated.

Data analysis

EC_{50}/IC_{50} values were determined by nonlinear regression analysis using the software Prism 4.0 (Graphpad, San Diego, CA). Data were fitted as sigmoidal concentration-response curves and analyzed by a 4-parameter logistic equation. The Z′ factor,[21] a direct measure of assay quality, was measured using the following equation:

$$Z' = 1 - \frac{(3\sigma_{c^+} + 3\sigma_{c^-})}{|\mu_{c^+} - \mu_{c^-}|},$$

where σ_{c+} and σ_{c-} are the standard deviations of positive and negative control samples, respectively, and μ_{c+} and μ_{c-} are the means of positive and negative controls, respectively. We tested the robustness of our mGluR5 assay in its ability to demonstrate both agonism and antagonism. Calculating the Z′ in the agonism assay, the positive control was vehicle + quisqualate (100 nM), whereas the negative control was vehicle only. We used 48 wells of a 96-well plate for the positive and negative controls, respectively, with 3 replicate plates per day, and we performed the assay on 3 consecutive days. In determining the Z′ for the antagonism assay, we used a similar protocol but used vehicle + quisqualate (100 nM) as the positive control and vehicle + MPEP (50 nM) + quisqualate (100 nM) as the negative control; again, we used 3 half-plates for positive and negative controls, respectively, with 3 replicate plates per day, and we performed the assay over 3 consecutive days. The final Z′ for the assay is the average of the antagonism and agonism determinations.

RESULTS

The ecdysone system was used to establish heterologous inducible expression of representative mGlu receptors from all 3 groups, mGluR5b (group I), mGluR2 (group II), and mGluR8a (group III). In this system, transgene expression is under the control of a minimal heat shock promoter, which contains 5 copies of the ecdysone/glucocorticoid responsive element (5X E/GRE).[16] The level of transcription of the gene of interest can be simply modulated by the addition of the inducer ponasterone A, a synthetic analogue of ecdysone. Optimal 293_{EcR} clonal cell lines expressing each mGlu receptor were obtained and pharmacologically and biochemically characterized.

mGluR5 inducible cell line

mGluR5b is a $G\alpha_q$-coupled receptor; its activation thus leads to an increase in intracellular calcium, which can be measured using a FLIPR. Clonal cell lines expressing mGluR5 were initially prescreened for the stimulation of calcium mobilization in response to quisqualate following 24-h induction with 5 μM ponasterone A. A single clonal cell line referred to as 2A4 was chosen for further study. To determine how receptor density can be modulated in this system, cells were induced for 24 h with increasing concentrations (1, 3, and 5 μM) of ponasterone A, and mGluR5 protein immunoreactivity was determined by Western blotting. The mGluR5 protein could not be detected in uninduced cells, indicating near total absence of transcription, but it is visible after incubation with 1 μM ponasterone A, and the amount of immunoreactive signal progressively increases with increasing concentrations of inducer (see Fig. 1, inset). The functional coupling of the receptor was tested by measuring quisqualate-induced Ca_i^{2+} mobilization after ponasterone A induction. As shown in Figure 1, quisqualate (0.001-3 μM) elicited a concentration-dependent increase in Ca_i^{2+}, with maximal changes in relative fluorescence units (RFUs) of 8100 ± 210, $10{,}200 \pm 47$, and $11{,}700 \pm 308$ ($n = 3$)

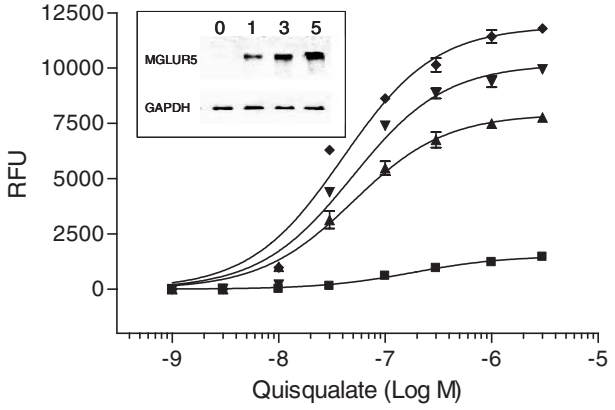

FIG. 1. Concentration response curves (mean ± SEM, $n = 3$) for the effect of quisqualate on calcium mobilization assay using 293_{EcR} cells expressing mGluR5b without induction (■) and induced with 1 μM (▲), 3 μM (▼), and 5 μM (◆) concentrations of ponasterone A. The inset shows Western blot analysis, displaying the extent of immunoreactive mGluR5b protein 24 h after induction with 0, 1, 3, and 5 μM concentrations of ponasterone A.

after 1, 3, and 5 μM ponasterone, respectively. Corresponding agonist EC_{50} (log M) values of –7.38 at 1 μM, –7.40 at 3 μM, and –7.51 at 5 μM inducer concentration were obtained. In the absence of induction by ponasterone A, no significant functional response was detected following quisqualate stimulation, indicating tight regulation of recombinant receptor expression. A small amount of signal was noted, however, at higher quisqualate concentrations; we believe that this is probably due to a low level of unregulated transcription as no signal was observed in our nontransfected cell lines when challenged with equivalent concentrations of quisqualate.

A detailed characterization of the pharmacological properties of the hmGluR5b receptor was undertaken using reference agonists and antagonists. As shown in Figure 2a, all characterized agonists were highly efficacious, eliciting maximum responses comparable to those observed with the endogenous agonist L-glutamate. The rank order of agonist potency for examined compounds was quisqualate > L-glutamate > DHPG > 1S-3R-ACPD > CHPG, in agreement with that determined for the native rat receptor[22] and the recombinant human receptor.[23] EC_{50} values were calculated as reported in Table 1. To examine the effect of antagonists, cells were pretreated with the noncompetitive mGluR5 selective antagonists MPEP and MTEP and the competitive antagonist LY 341495. All antagonists produced a concentration-dependent inhibition of quisqualate-induced calcium mobilization, as shown in Figure 2b. The following rank order of potency was determined: MPEP > MTEP > LY341495; IC_{50} values were calculated against an EC_{80} concentration of quisqualate and are reported in Table 1.

Calcium-coupled group II and III inducible cell lines

To use intracellular calcium transients as an assay readout for group II and III receptors, we cotransfected hmGluR2 and hmGluR8a receptors with the chimeric G protein $G\alpha_{q/o}$, which has been shown to effectively couple with all $G\alpha_{i/o}$-coupled mGlu receptors, at least in transient transfections.[24,25] Because stable overexpression of the chimeric $G\alpha_{q/o}$ protein could potentially cause alterations in normal cell physiology, we tried 2 different experimental approaches. In our mGluR2 cell line, we constitutively expressed the chimeric G protein but kept the metabotropic receptor under the control of the inducible promoter, whereas with our mGluR8 cell line, both receptor and G protein were inducible. In the latter case, inducible expression of both the mGlu receptor and chimeric G protein was achieved by inserting an IRES sequence between the receptor and the G protein coding sequences, thus ensuring that they were expressed as a single transcript under the control of the inducible promoter.

Table 1. Functional Activities of Agonists and Antagonists Examined in 293_{EcR} Cell Lines 2q/o, 2A4, and 8q/o Expressing mGluR2, mGluR5, and mGluR8, Respectively

	EC_{50} μM		
Agonist	*mGluR2*	*mGluR5*	*mGluR8*
L-glutamate	1.6 (1.1-2.4)	1.5 (1.1-2.2)	8.8 (5.8-11)
Quisqualate	>1000	0.03 (0.02-0.05)	>1000
DHPG		4.2 (2.8-5.7)	
DCG-IV	0.13 (0.08-0.18)		
1S,3R-ACPD	3.1 (2.2-4.2)	15.7 (11.6-21.2)	
2R,4R-APDC	1.3 (0.9-1.8)		
L-CCG I	0.14 (0.08-0.2)		2.8 (1.6-4.9)
L-AP4			1.6 (0.9-2.9)
PPG			4.7 (2.7- 8.1)
DCPG			0.8 (0.4-1.6)
NAAG	>1000		
Antagonist	*IC_{50} μM at 300nM DCG-IV*	*IC_{50} μM at 100 nM Quisqualate*	*IC_{50} μM at 14 μM Glutamate*
MPEP	>100	0.02 (0.01-0.03)	>100
MTEP		0.09 (0.06-0.14)	
LY341495	0.0012 (0.0009-0.0015)	35.3 (22.4-48.2)	0.13 (0.08-0.16)

A description of the abbreviations used for all agonists and antagonists can be found in the Materials and Methods section under Compounds. Data are the mean EC_{50} values of agonists and IC_{50} values of antagonists determined from 3 independent experiments performed in quadruplicate. Values are given with corresponding 95% confidence limits. Each dose-response curve consisted of 8 different drug concentrations. All antagonist IC_{50} values were determined at agonist concentrations corresponding to the EC_{80} of the specific agonist, 300 nM DCG-IV for mGluR2, 100 nM quisqualate for mGluR5, and 14 μM L-glutamate for mGluR8.

mGluR2-inducible cell line

Following cotransfection of mGluR2 and $G\alpha_{q/o}$, clonal cell lines were prescreened for DCG-IV-induced Ca_i^{2+} mobilization following induction with ponasterone A (5 μM). A single clone, referred to as 2q/o, which revealed agonist-dependent Ca_i^{2+} mobilization when induced with negligible background levels in the absence of induction, was expanded and characterized further. Clone 2q/o gave very robust calcium signals, with maximal changes in RFUs in response to DCG-IV of up to 45,000 and a calculated EC_{50} value of 130 nM. This cell line showed excellent signal-to-

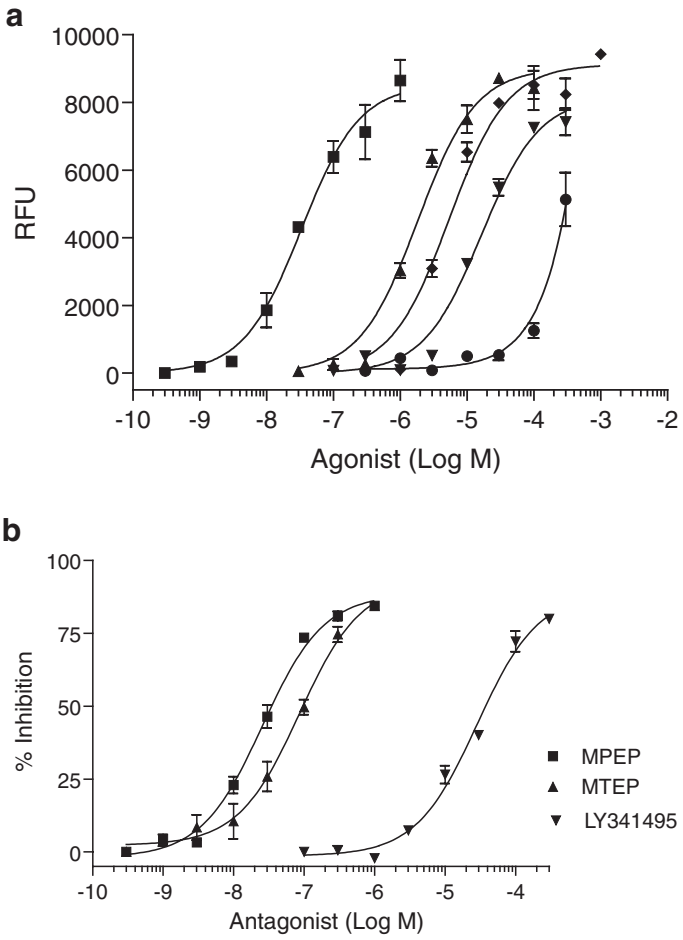

FIG. 2. (**a**) Effect of various agonists on Ca^{2+} mobilization in cell line 2A4 expressing mGluR5b. Agonists are quisqualate (■), L-glutamate (▲), DHPG (◆), 1S,3R-ACPD (▼) and CHPG (●). Values are means ± SEM of quadruplicate determinations from a typical experiment. (**b**) Antagonism by MPEP (■), MTEP (▲), and LY341495 (▼) of the agonist-stimulated calcium mobilization in the mGluR5b expressing cell line 2A4. Cells were pretreated for 10 min with the various antagonists over the indicated concentration ranges before a challenge with an EC_{80} (100 nM) concentration of quisqualate. Inhibition curves were constructed from the percentage responses pooled from 3 independent experiments performed in quadruplicate. Values are means ± SEM.

background ratio, as no significant DCG-IV-stimulated calcium responses were detected in uninduced cells, as shown in the inset in Figure 3. To show that the pharmacology of the receptor is unaltered in this system where the receptor couples through an exogenous chimeric G protein, mGluR2-coupled calcium signaling was measured in response to a number of reference agonists for metabotropic glutamate receptors. As shown in Figure 3, the group II selective agonist DCG-IV as well as all the group II preferred agonists including L-CCG-I, 2R,4R-APDC, and 1S,3R-APDC were highly efficacious, eliciting maximum responses comparable to those observed with the endogenous agonist L-glutamate. In contrast, the addition of the group I agonist quisqualate or the

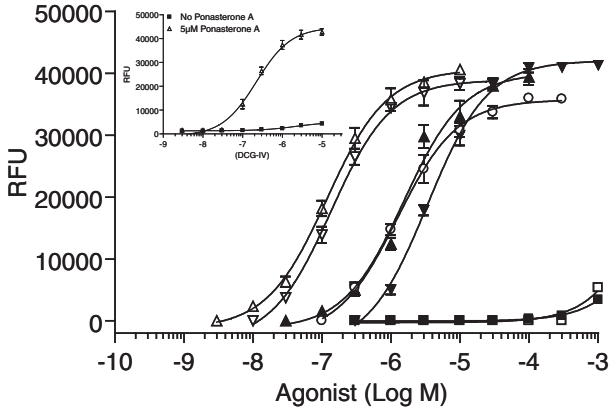

FIG. 3. Effect of various agonists on Ca^{2+} mobilization in cell line 2q/o expressing mGluR2 and the chimeric G protein $G\alpha_{q/o}$. Agonists are △ DCG-IV (group II selective), ▽ L-CCGI (group II preferred), ▲ L-glutamate, ○ 2R,4R-APDC (group II preferred), ▼ 1S,3R-ACPD (group II preferred), □ NAAG (mGluR3 selective), and ■ quisqualate (group I selective). Values are means ± SEM of quadruplicate determinations from a typical experiment. Inset concentration-response curves (mean ± SEM, $n = 3$) for the effect of DCG-IV on calcium mobilization using cell line 2q/o expressing mGluR2 and the chimeric G protein $G\alpha_{q/o}$, pretreated for 24 h in the absence (■ or presence △ of ponasterone A (5 μM).

mGluR3-specific agonist NAAG did not elicit an increase in intracellular calcium. The rank order of agonist potency in the recombinant cell line expressing hmGluR2 and the chimeric $G\alpha_{q/o}$ protein was DCG-IV ~ L-CCG-I > L-glutamate ~ 2R,4R-APDC > 1S,3R-APDC. Calculated EC_{50} values for each agonist at the recombinant hmGluR2 receptor are summarized in Table 1. These data are in agreement with previous work undertaken on the native rat receptor[26] as well as the physiologically coupled recombinant hmGluR2 where cAMP or GTPγS assays were used.[20,27] We also tested the reference compound LY341495, which acts as an antagonist at all mGluRs but has the highest affinity for the group II receptors. The compound tested in the presence of an EC_{80} concentration of DCG-IV resulted in a concentration-dependent inhibition of agonist-induced Ca_i^{2+} mobilization, as shown in Figure 4, giving an estimated IC_{50} of 1.2 nM.

mGluR8-inducible cell line

Following transfection of the 293_{EcR} cell line with the inducible mGluR8a-$G_{q/o}$ construct, clonal cell lines were isolated and characterized for L-glutamate-dependent Ca_i^{2+} mobilization after induction with 5 μM ponasterone A. A single clone referred to as 8q/o was chosen for further studies. When induced, clone 8q/o gave robust concentration-dependent intracellular calcium increases after being stimulated with L-glutamate and undetectable agonist-induced signal in the absence of induction (Fig. 5, inset). A detailed pharmacological analysis was performed on the recombinant cell line using a number of well-characterized agonists, and the rank order of potency was determined as DCGP > L-AP4 > L-CCG-I > PPG>L-glutamate, as depicted in a representative experi-

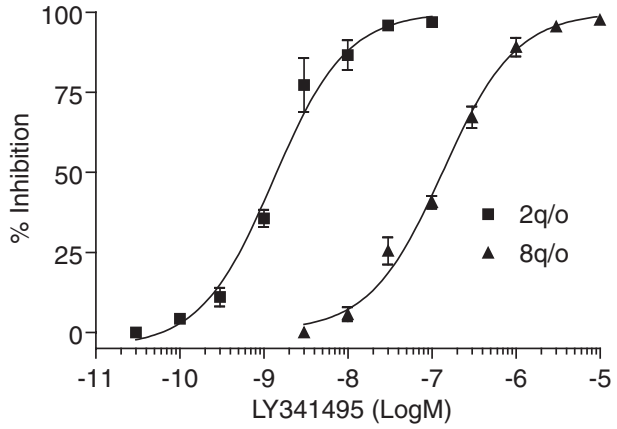

FIG. 4. Antagonism by LY341495 of the DCG-IV and L-glutamate stimulated calcium mobilization of cell lines 2q/o and 8q/o, respectively. Cells were pretreated for 10 min with LY341495 over the indicated concentration ranges before a challenge with an EC_{80} concentration of agonist. Inhibition curves were constructed from the percentage responses pooled from 3 independent experiments performed in quadruplicate. Calculated IC_{50} values are presented in Table 1.

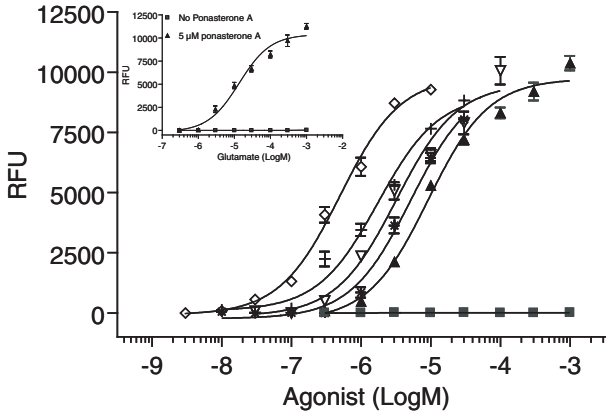

FIG. 5. Effect of various agonists on CA_i^{2+} mobilization in cell line 8q/o expressing mGluR8 and the chimeric G protein $G\alpha_{q/o}$. Agonists are (S)-3,4-DCPG (◇; mGluR8 selective), + L-AP4 (+; group III selective), L-CCG-I (▽; group II-III selective), RS-PPG (*; mGluR8 selective), L-glutamate (▲), and quisqualate (■; group I selective). Values are means ± SEM of quadruplicate determinations from a typical experiment. (Inset) Concentration-response curves (mean ± SEM, $n = 3$) for the effect of L-glutamate on calcium mobilization using cell line 8q/o expressing mGluR8 and the chimeric G protein $G\alpha_{q/o}$, pretreated for 24 h in the absence (■) or presence (▲) of ponasterone A (5 µM).

ment in Figure 5. These results are in agreement with data obtained from hmGluR8a-expressing cell lines using cAMP assays[19,28] or radioligand binding assays.[29] Agonist EC_{50} values for our hmGluR8a-expressing cell line were calculated and are summarized in Table 1. L-glutamate-induced Ca_i^{2+} release was tested in the presence of the competitive antagonist LY341495. When an agonist concentration corresponding to an EC_{80} value was used to stimulate the receptor, addition of the reference antagonist resulted in a concentration-dependent inhibition of glutamate-elicited calcium signal with a calculated IC_{50} of 130 nM. As expected, LY341495 was more potent on hmGluR2 than hmGluR8 (Fig. 4).

Inducible cell line stability and assay reproducibility

To test the functional stability of the mGluR-expressing inducible cell lines, the biological response and the pharmacological properties of the recombinant receptors were tested at increasing passage number. We tested cell lines up to 30 passages, and no significant changes in the efficacy or potency of agonists to induce Ca_i^{2+} mobilization after ponasterone A induction was observed. In addition, no significant response to agonists in uninduced cells was shown with increasing passage number. To determine the robustness of the assay, we determined the Z′ for the mGluR5 cell line 2A4; this was calculated as 0.7, showing that the assay is very reproducible. The ecdysone-inducible system would thus appear to be sufficiently robust to allow it to be used in screening assays in which very high reproducibility is essential.

DISCUSSION

Pharmacologically active compounds that are able to modulate the activity of a specific metabotropic glutamate receptor subtype may be effective in the treatment of a wide range of CNS diseases. However, it has been traditionally very difficult to find small molecules acting at the agonist-binding site that achieve true subtype selectivity. The generation of high-throughput screening has made it possible to search for compounds that interact with allosteric sites rather than the historically targeted orthosteric site. The first such compounds described for the mGluRs were MPEP and CPCCOet, which are negative allosteric modulators selective for mGluR5 and mGluR1, respectively.[30,31] Recently, positive allosteric modulators selective for mGluR1, mGluR2, mGluR4, and mGluR5 have also been described.[32-35] The availability of functional cell-based assays for all members of the mGluR family makes it possible to screen chemical libraries in the search for potent agonists, antagonists, and allosteric modulators. Equally important, clonal cell lines can be used to counterscreen active compounds to determine if their activities are indeed receptor specific. This is particularly important since the revelation that the noncompetitive mGluR5 antagonist MPEP also acts as a positive modulator of mGluR4[36] and the discovery of PHCCC, a compound highly related to CPCCOet that predominantly acts as a positive modulator of mGluR4 but is also a noncompetitive inhibitor of mGluR1.[34]

We have used the ecdysone system to functionally express the $G\alpha_q$ coupled mGluR5 and in combination with the chimeric $G\alpha_{q/o}$ protein to functionally express mGluR2 and mGluR8. In all of our cell-based assays, the receptors displayed a pharmacological profile for both agonists and antagonists that faithfully represented that of the native receptors. Our results show that the ecdysone system allows expression to be tightly controlled, producing barely detectable background levels and high signal-to-background ratios. It also allowed us to develop assays that show long-term sta-

bility, showing both consistent EC_{50} values and RFUs even at high passage numbers. Assay reproducibility was also good, and our mGluR5 assay has a calculated Z′ value of 0.7, showing remarkable across-plate uniformity. The combined use of the ecdysone system and the chimeric G protein $G\alpha_{q/o}$ not only avoids receptor desensitization and time- and passage-dependent receptor downregulation but also allowed us to use the same fluorescent assay format for all mGlu receptors. Having a single assay format that can be used to evaluate all mGluRs should accelerate both screening and counterscreening activities. Here, we present data from representative receptors from each group, but the same approach should be applicable to all mGlu receptors. To express hmGluR2 and hmGluR8, we employed 2 distinct strategies: In our 2q/o cell line, the chimeric G protein was constitutively expressed, whereas in our 8q/o cell line, the expression of the chimeric G protein was inducible. Both strategies were successful, suggesting that overexpression of a chimeric G protein is not particularly deleterious for the cell. However, as the $G\alpha_{q/o}$ protein can couple to any $G\alpha_i$/$G\alpha_o$-coupled cellular receptor, limiting its expression temporally to that of its desired target should, at least in theory, reduce any potentially undesired effects.

To date, there are very few reports of the ecdysone system being used to express GPCRs. However, in 1 notable exception, different 5HT receptors were expressed using the Tet on and the ecdysone systems; although the authors reported higher overall expression levels with the Tet on system, the ecdysone system gave much better induction ratios with effectively undetectable background levels.[37] Other groups have used the expression of reporter genes to compare different inducible systems, and their results also demonstrate that the ecdysone system produces very high induction ratios with negligible background expression.[38,39] Our results not only show that the ecdysone system allows expression to be tightly controlled but also demonstrate that the system can produce very reproducible assays. We believe that the ecdysone system is an excellent system for functional receptor studies and that it can produce sufficiently robust assays to be used in screening campaigns in which reproducibility and assay stability are of paramount importance.

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