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Functional calcium coupling with the human metabotropic glutamate receptor subtypes 2 and 4 by stable co-expression with a calcium pathway facilitating G-protein chimera in Chinese hamster ovary cells

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Abstract

The objective of the current study was to facilitate functional calcium assays, compatible with the fluorometric imaging plate reader platform, for the human metabotropic glutamate receptor (mGluR) subtypes 2 and 4, by co-expressing each receptor with a G-protein chimera comprising Gαq with the C-terminal five amino acids replaced with those from Gαi3 (GqGi3). Transfection of GqGi3 into previously validated stable CHO cell lines expressing mGluR2 or mGluR4 allowed for the selection of new double transfectants in which application of L-glutamate and other mGluR agonists resulted in calcium coupling with a high signal:noise ratio (maximal changes in relative fluorescence units up to 20,000). The rank order of agonist potency for the stimulation of calcium mobilization in the mGluR2/GqGi3 stable cell line was LY354740 > L-CCG-I = DCG-IV > L-glutamate ≥ (2*R*,4*R*)-APDC ≥ (1*S*,3*R*)-ACPD. In the mGluR4/GqGi3 stable cell line the rank order of agonist potency was L-AP4 > L-SOP ≥ ACPT-I = L-CCG-I ≥ L-glutamate = (*R*,*S*)-PPG. By comparison, equivalent potency orders and a significant correlation in functional activities were observed when the same compounds were profiled in [^{35}S]GTPγS binding assays for each mGluR subtype. These results validate the use of functional calcium assays, amenable to high-throughput applications on the fluorometric imaging plate reader, for the mGluR2 and mGluR4 subtypes when co-expressed in stable cell lines with the GqGi3 chimera.

Keywords: Metabotropic glutamate receptor; G-protein; Chimera; FLIPR; [^{35}S]GTPγS; L-Glutamate; LY354740; L-CCG-I; L-AP4; L-SOP

1. Introduction

mGluRs are members of the superfamily of seven transmembrane spanning G-protein-coupled receptors (GPCRs) further categorized with related family three GPCRs, including the $GABA_B$, calcium sensing, and certain pheromone receptors [1–5]. Within the mGluR family eight distinct subtypes have been identified and these have been sub-classified based on sequence homologies and principal signal transduction mechanism [6]. The group I receptors include mGluRs 1 and 5 coupled primarily to Gq and the activation of phospholipase C and subsequent mobilization of intracellular calcium. Both the group II mGluRs 2 and 3, and group III mGluRs 4, 6, 7, and 8 are coupled to inhibitory G-proteins (Gi) with subsequent decreases in intracellular cAMP. L-Glutamate is the predominant excitatory neurotransmitter in the brain and dysfunction of L-glutamate transmission is implicated in both neurodegenerative, e.g. stroke, and psychiatric, e.g. schizophrenia, disorders. Since glutametergic neurotransmission is modulated by activation of mGluRs, these targets have been the focus of a number of drug discovery efforts. Consequently, assays to study the pharmacology of these receptors are important tools in the drug development process.

Functional characterization of ligands for the cloned Gi-coupled mGluRs has historically utilized forskolin stimulation of cAMP production and evaluation of compounds

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Abbreviations: (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; (2*R*,4*R*)-APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; ACPT-I, (1*S*,3*R*,4*S*)-1-aminocyclopentane-1,3,4-tricarboxylic acid; DCG-IV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine; FLIPR, fluorometric imaging plate reader; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; L-CCG-I, (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine; L-SOP, L-serine-*O*-phosphate; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; LY354740, (+)-2-aminobicyclo[3.1.0.]hexane-2,6,-dicarboxylic acid; mGluR, metabotropic glutamate receptor; (*R*,*S*)-PPG, (*R*,*S*)-4-phosphonophenylglycine.

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against this elevated level of cAMP [7,8]. Such assays are typically labor intensive and not readily amenable to high-throughput applications. Until the recent introduction of high-affinity ligands for certain mGluR subtypes [9–11], it was also not feasible to develop radioligand binding assays for the discovery and characterization of ligands acting on these receptors. Additionally, conventional agonist and antagonist radioligand binding assays do not allow for the determination of intrinsic activity at the receptor. This has led investigators to evaluate alternate means to the forskolin-stimulated adenylyl cyclase approach for profiling agonist and antagonist activities at these receptor subtypes. Two distinct approaches have proved to be valuable in the functional characterization of ligands acting, in particular, on mGluRs 2 and 4. Firstly, co-expression with promiscuous and chimeric G-proteins has successfully been used to permit coupling of these Gi-coupled receptors to Gq allowing the direct measurement of inositol phosphate accumulation [12–14]. Secondly, the use of the non-hydrolyzable form of GTP, GTPγS, has been used to study agonist-stimulated G-protein activation in the form of guanine nucleotide exchange of GDP for GTP and measurement of [^{35}S]GTPγS binding [15].

While the former studies with chimeric G-proteins have allowed for the functional characterization of both mGluRs 2 and 4, some limitations include the transient nature of receptor expression and the use of inositol phosphate accumulation measurements, not readily transferable to a high-throughput platform. With this in mind the generation of stable cell lines expressing both the mGluR subtype and promiscuous/chimeric G-protein in combination with the use of calcium-mobilization assays, compatible with the fluorometric imaging plate reader (FLIPR) formats, would represent considerable improvements in screening capabilities. A further advantage of this approach is the elimination of the use of radioactivity. In the present study, we have established stable co-expression of a GqGi3 chimera in CHO cells lines expressing either mGluR2 or mGluR4. Functional studies with these cell lines using FLIPR reveal robust agonist-stimulated calcium responses with relative potencies similar to those observed in [^{35}S]GTPγS binding assays. These results validate the use of this approach for the characterization of mGluR2 and mGluR4 ligands.

2. Methods

2.1. Transfection of GqGi3 into stable mGluR2 and mGluR4 CHO lines

The stable CHO lines expressing mGluR2 and mGluR4 used as hosts for transfection of Gq/Gi3 have been characterized previously [15]. GqGi3 expressed in pCDNA3.1(−) was co-transfected *via* electroporation and cells were selected using 800 μg/mL neomycin. Clones expressing both the receptor and chimera were selected by functional coupling to calcium using FLIPR and a single clone for each receptor subtype was selected for expansion and further characterization.

2.2. Calcium assay

Cells were plated 24 hr prior to the experiment at a seeding density of 50,000 cells/well in a 96-well plate. On the day of the assay, the confluent cell monolayers were rinsed with 1× HBSS containing 20 mM HEPES and 2.5 mM probenecid (i.e. FLIPR buffer) and were subsequently dye loaded with FLIPR buffer supplemented with 3 U/mL glutamic–pyruvic transaminase (GPT; Sigma), 3 mM pyruvic acid, 1% FBS, 0.2% pluronic acid, and 4 μM Fluo-4 AM (Molecular Probes) indicator dye for 1 hr at 37°. Following dye loading, the cells were rinsed with FLIPR buffer and maintained in FLIPR buffer containing 3 U/mL GPT and 3 mM pyruvic acid for an additional 30 min at 37° in order to facilitate removal of extracellular glutamate from the incubate. Intracellular calcium mobilization was measured by increases in fluorescence upon agonist stimulation using FLIPR following ten 1-s baseline measurements.

2.3. Measurement of [^{35}S]GTPγS binding

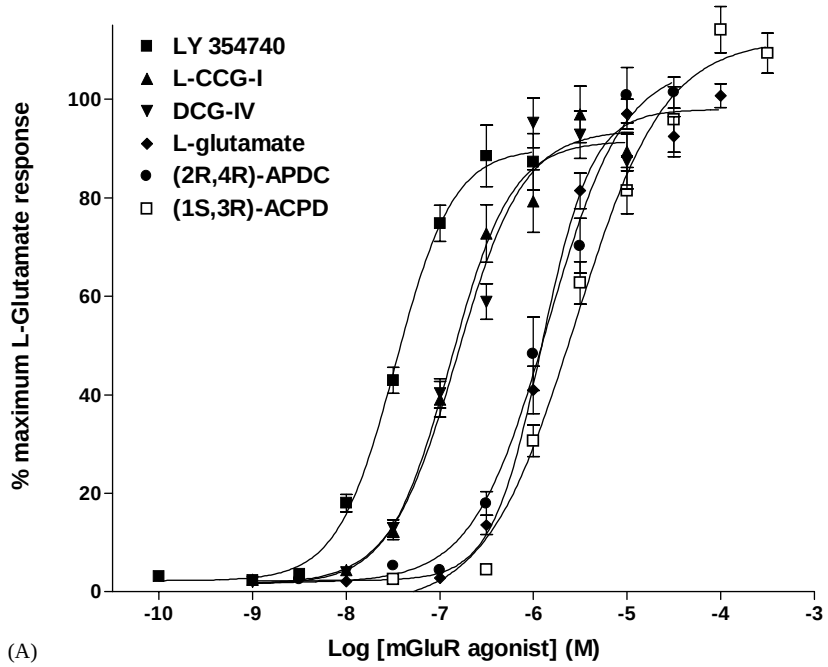
Membrane preparation and binding assay procedures were performed as described by Kowal *et al.* [15] and Lazareno and Birdsall [16].

2.4. Statistical analysis

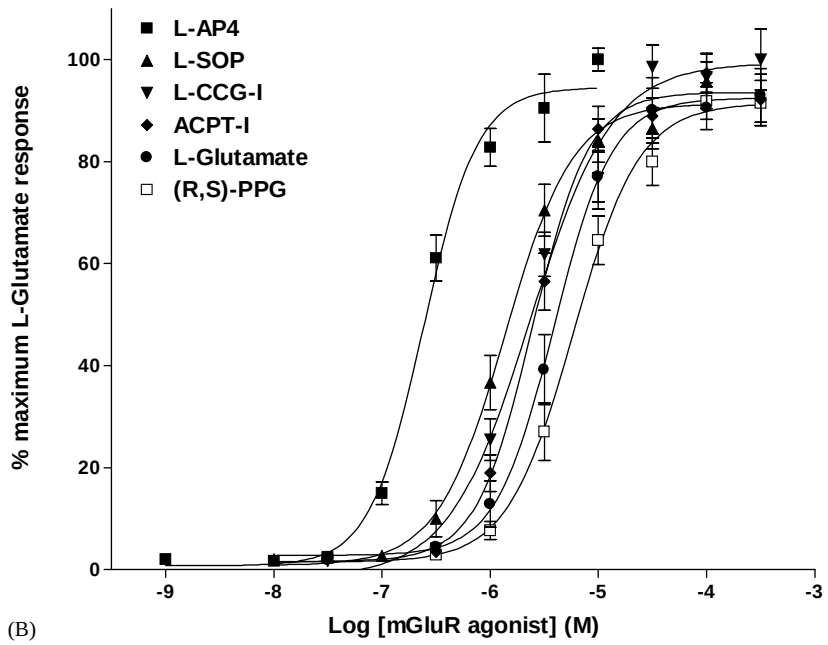
For graphical display, the data in both FLIPR and [^{35}S]GTPγS binding assays were converted to '% maximum L-glutamate response' defined by 100 and 300 μM L-glutamate for mGluRs 2 and 4, respectively. EC_{50} values were defined as the midpoint between the maximum and minimum of each individual concentration–response curve of a nonlinear regression analysis (GraphPad Prism).

3. Results and discussion

Following transfection of GqGi3 into the mGluR2 and mGluR4 expressing host cells several clones were pre-screened for the stimulation of calcium mobilization in response to L-glutamate using FLIPR and a single clone from each was further expanded for subsequent experiments. No L-glutamate-stimulated calcium responses were detected in either of the host cell lines, yet application of L-glutamate to cells co-expressing mGluR2 or mGluR4 with the GqGi3 chimera resulted in robust calcium responses with a high signal:noise with maximal changes in relative fluorescence units up to 20,000 (not shown). Detailed characterization of the mGluR2- and mGluR4-coupled calcium signaling was undertaken using established



(A)



(B)

Fig. 1. mGluR agonist concentration–response curves determined in Ca^{2+}-mobilization assays using CHO cells co-expressing mGluR2 (A) or mGluR4 (B) co-expressed with the chimeric G-protein GqGi3. Percent responses were pooled from three independent experiments performed in quadruplicate. Estimated EC_{50} values are presented in Tables 1 and 2.

mGluR agonists. All compounds tested at both receptor subtypes were highly efficacious eliciting maximum responses comparable to those observed with L-glutamate (Fig. 1A and B). The rank order of agonist potency for compounds examined on mGluR2 was LY354740 > L-CCG-I = DCG-IV > L-glutamate ≥ (2*R*,4*R*)-APDC ≥ (1*S*,3*R*)-ACPD. Agonist rank order of potency for mGluR4 was L-AP4 > L-SOP ≥ ACPT-I = L-CCG-I ≥ L-glutamate = (*R*,*S*)-PPG. For comparative purposes the same compounds used in the FLIPR assay were profiled using [^{35}S]GTPγS binding. Agonist concentration–response curves for stimulation of [^{35}S]GTPγS binding in membranes derived from mGluR2 and mGluR4 expressing cells generated essentially identical rank order of agonist potencies for either receptor subtype when compared with the calcium assay, and a high correlation between the EC_{50} estimates for stimulation of calcium mobilization and [^{35}S]GTPγS binding (Tables 1 and 2).

To examine the effect of an antagonist in the FLIPR assay the group II selective antagonist LY341495 was

Table 1
Functional activities of agonists examined in CHO cells expressing mGluR2/GqGi3 using FLIPR and in homogenate prepared from mGluR2 expressing cells (in the absence of chimeric G-protein) using [^{35}S]GTPγS binding

Agonist	FLIPR, EC_{50}, nM (95% confidence limits)	[^{35}S]GTPγS, EC_{50}, nM (95% confidence limits)
LY354740	34 (26–43)	8.2 (7.4–9.1)
L-CCG-I	130 (97–174)	70 (60–81)
DCG-IV	152 (114–202)	92 (81–103)
L-Glutamate	1231 (1043–1453)	794 (721–873)
(2*R*,4*R*)-APDC	1436 (1027–2008)	840 (706–999)
(1*S*,3*R*)-ACPD	2797 (1989–3933)	3301 (2720–4008)

Table 2
Functional activities of agonists examined in CHO cells expressing mGluR4/GqGi3 using FLIPR and in homogenate prepared from mGluR4 expressing cells (in the absence of chimeric G-protein) using [^{35}S]GTPγS binding

Agonist	FLIPR, EC_{50}, μM (95% confidence limits)	[^{35}S]GTPγS, EC_{50}, μM (95% confidence limits)
L-AP4	0.24 (0.20–0.29)	0.24 (0.18–0.32)
L-SOP	1.4 (1.1–1.8)	1.4 (1.0–2.0)
L-CCG-I	2.4 (1.7–3.2)	3.4 (2.7–4.2)
ACPT-I	2.5 (2.0–3.1)	4.5 (2.8–7.3)
L-Glutamate	3.9 (3.0–5.1)	7.7 (4.5–13)
(*R*,*S*)-PPG	5.9 (4.6–7.5)	8.1 (6.0–11)

profiled in both the mGluR2 and mGluR4 cell lines. LY341495 failed to produce a calcium response when tested alone in either cell line (not shown). The calcium response elicited by 100 nM LY354740 in the mGluR2 expressing cells was potently antagonized by LY341495 with an IC_{50} of 31 nM (25–38, 95% confidence limits), while the response to 1 μM L-AP4 in the mGluR4 expressing cells was much less sensitive to this compound producing an IC_{50} of 5.1 μM (4.2–6.3, 95% confidence limits) (Fig. 2). Finally, we examined the effect of pertussis toxin pretreatment on agonist-stimulated calcium responses in both the mGluR2 and mGluR4 cell lines co-expressing GqGi3. As illustrated in Fig. 3, a 24-hr pretreatment in the presence of 100 ng/mL pertussis toxin completely abolished the functional calcium response to L-glutamate in both receptor expressing cell lines.

Taken together, these results validate the use of a chimeric G-protein, comprising Gαq with the C-terminal five amino acids replaced with those from Gαi3, to facilitate calcium signaling assays with mGluR2 or mGluR4 following establishment of stable co-expression in CHO cells. That the coupling was facilitated by the C-terminal five amino acids of Gi3, and not simply by over-expression of Gq, was confirmed by demonstrating that agonist-stimulated responses were abolished with pertussis toxin pretreatment. Our choice of the GqGi3 chimera was based on previous success with other Gi-coupled receptors, including the serotonin 5-HT_{1A} receptor and the histamine H3 receptor, when evaluating the capacity of various Gi-family G-protein chimeras to enable coupling of these receptors to the Gq signal transduction cascade and elevation of intracellular calcium [17,18]. These studies also

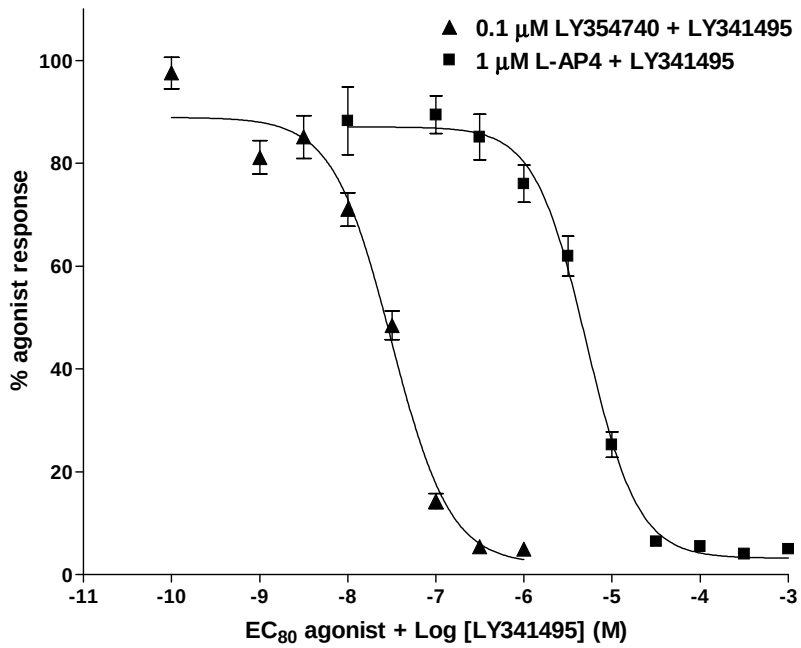


Fig. 2. Antagonism by LY341495 of the LY354740 and L-AP4 stimulated calcium mobilization in mGluR2 and mGluR4 cells, respectively, co-expressing a G-protein GqGi3 chimera. Cells were pretreated for 1 hr with LY341495 over the indicated concentration ranges before a challenge with an EC_{80} concentration of agonist. Data were calculated as percent agonist alone response and inhibition curves were constructed from the percent responses pooled from three independent experiments performed in quadruplicate.

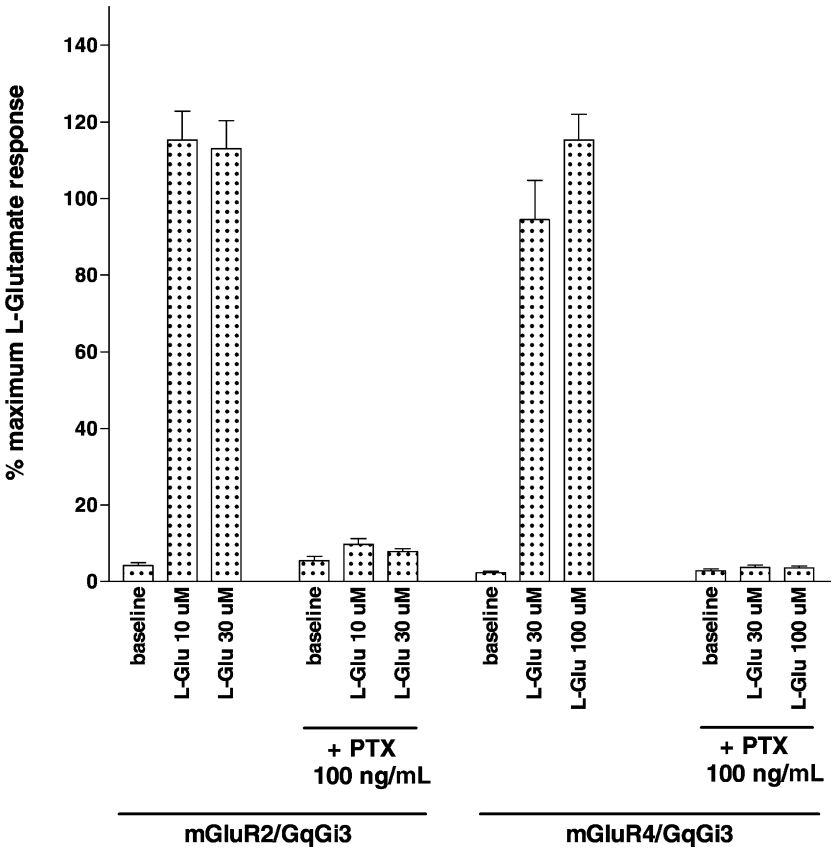


Fig. 3. Effect of pertussis toxin on the L-glutamate-stimulated calcium mobilization in mGluR2 and mGluR4 cells co-expressing a GqGi3 chimera. Cells were pretreated for 24 hr in the presence of 100 ng/mL pertussis followed by evaluation of calcium signals using FLIPR. Data from a representative experiment performed in quadruplicate are presented with identical results obtained in one other independent experiment.

took advantage of the FLIPR for the measurement of calcium responses. Advantages of the FLIPR assay are compatibility with high-throughput applications, elimination of the use of radioactivity and the ability to detect activity of agonists, antagonists, and allosteric modulators (although not addressed here) in the same assay.

Importantly, both agonist and antagonist potencies were in very good agreement with those determined previously using other assay formats [7,8,12–14,19,20]. In cases where modest differences in agonist activities were observed, these are likely attributed to several factors such as the use of different cell lines between investigators, coupling efficiency of native vs. chimeric G-proteins, and measurement of different functional endpoints. Moreover, although agonist pharmacology is faithfully reproduced in the FLIPR assay, its use should not preclude subsequent evaluation of compounds in assays reflecting endogenous G-protein coupling of the recombinantly expressed receptors and the receptors in their native environment.

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