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Effects of varying the expression level of recombinant human mGlu1α receptors on the pharmacological properties of agonists and antagonists

*,[1,2]Emmanuel Hermans, [1]R.A. John Challiss & [1]Stefan R. Nahorski

[1]Department of Cell Physiology and Pharmacology, University of Leicester, Medical Sciences Building, University Road, Leicester LE1 9HN, England, U.K.

1 Different expression levels of the human type 1α metabotropic glutamate (mGlu1α) receptor were obtained in transfected Chinese hamster ovary cells using an isopropyl β-D-thiogalactopyranoside (IPTG) inducible system. Expression of mGlu1α receptors could not be detected using immunoblotting or immunocytochemical approaches in non-induced cells, however, controlled expression could be induced following IPTG addition in a time- and concentration-dependent manner.

2 In induced cells (100 μM IPTG, 20 h) the agonists L-quisqualate or 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid stimulated large increases in [^{3}H]-inositol (poly)phosphate (in the presence of Li^+) and inositol, 1,4,5-trisphosphate levels.

3 Induction with 1–100 μM IPTG allowed the receptor density to be increased incrementally and this not only resulted in an increase in the maximum response to L-quisqualate, 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid and (*S*)-3,5-dihydroxy-phenylglycine, but also in an increase in the respective potencies of each agent to activate phosphoinositide hydrolysis.

4 The intrinsic activity of the partial agonist 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid dramatically increased with increasing receptor expression.

5 The activities of the competitive mGlu1α receptor antagonists (*S*)-α-methyl-4-carboxyphenylglycine and (*S*)-4-carboxy-3-hydroxyphenylglycine for inhibition of the effects of L-quisqualate or (*S*)-3,5-dihydroxyphenylglycine were found to be independent of the receptor expression level.

6 When the mGlu1α receptor was expressed at very high levels, no evidence for receptor constitutive activity could be detected, and none of the antagonists tested revealed either any intrinsic activity or negative efficacy.

7 These data demonstrate that both the potency and efficacy of mGlu1α receptor agonists are influenced by expression level, whilst mGlu1α receptor antagonist activities are independent of expression level.

Keywords: Type 1α metabotropic glutamate receptor; phosphoinositide turnover; inositol 1,4,5-trisphosphate; LacSwitch; receptor induction; IPTG; inducible expression

Abbreviations: 1*S*,3*R*-ACPD, 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid; AIDA, (*RS*)-1-aminoindan-1,5-dicarboxylic acid; CHO, Chinese hamster ovary; (*S*)-4-C3HPG, (*S*)-4-carboxy-3-hydroxyphenylglycine; (*S*)-4CPG, (*S*)-4-carboxyphenylglycine; (*S*)-3,5-DHPG, (*S*)-3,5-dihydroxyphenylglycine; GPT, glutamic-pyruvic transaminase; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; [^{3}H]-InsP, [^{3}H]-inositol (poly)phosphate; IPTG, isopropyl β-D-thiogalactopyranoside; KHB, Krebs-Henseleit buffer; (*S*)-MCPG, (*S*)-α-methyl-4-carboxyphenylglycine; mGlu receptor, metabotropic glutamate receptor; PBS, phosphate buffer saline

Introduction

The original observation that glutamate not only triggers the opening of ions channels, but also activates phospholipase C (Sladeczeck *et al*., 1985) led to the further identification of glutamate receptors coupled to G proteins (Sugiyama *et al*., 1987). Molecular cloning revealed the existence or a large family of glutamate metabotropic receptor (mGlu receptors) containing at least eight different subtypes that can be classified on the basis of their biochemical and pharmacological properties into three different groups. Group I receptors (mGlu1 and mGlu5) are preferentially coupled to the activation of phospholipase C through functional coupling to $G_{q/11}$, although mGlu1α has also been reported to activate adenylyl cyclase and to mediate arachidonic acid release. Group II (mGlu2 and mGlu3) and group III (mGlu4 and mGlu6-8) are coupled to the inhibition of adenylyl cyclase through pertussis toxin-sensitive G (G_i) proteins (see Pin & Duvoisin, 1995; Conn & Pin, 1997). Despite the large number of compounds investigated, mainly in the family of phenylglycine derivatives, the pharmacological distinction of each subtype within a group is hampered by the lack of high specific ligands. As a consequence, most studies concerning the specific interaction of putative metabotropic agonists or antagonists are performed with transfected cells expressing cloned mGlu receptors (Akam *et al*., 1997; Pickering *et al*., 1993; Thomsen *et al*., 1994a; Hayashi *et al*., 1994; Joly *et al*., 1995; Lin *et al*., 1997). Unfortunately, because of the absence of high-affinity radioligands for most of the mGlu receptors, quantitative determination of the level of expression of the receptor in these transfected cells is not possible, and interaction of compounds with expressed receptors needs to

*Author for correspondence.
[2]Current address: Laboratory of Pharmacology, Catholic University of Louvain (5410), 54 Avenue Hippocrate, 1200 Brussels, Belgium.

be investigated at the function level. In addition, stable and maintained expression of functional mGlu receptors in transfected cells has been shown to be problematic, perhaps as a consequence of regulatory processes related to the presence of glutamate in the culture medium of most cell lines (Gabellini *et al*., 1994; Desai *et al*., 1995; Lin *et al*., 1997; Carruthers *et al*., 1997).

We have previously reported a stably transfected CHO cell line in which the expression of the mGlu1α receptor is under control of an IPTG-inducible promoter (Hermans *et al*., 1998a). The use of this inducible promoter not only confers the possibility of maintaining the receptor density at very low levels during the growth of the cells and to induce its expression when required, but also allows us to manipulate the expression level of the receptor by means of varying the concentration of inducer added to the culture medium or the time of induction. In the present study, this model was used in order to study the consequences of modulating the expression level of the mGlu1α receptor on some properties of agonists and antagonists at this prototypic mGlu receptor subtype. Such an inducible system provides an appropriate model for such studies. It avoids the need to compare responses measured in different cell clones expressing different densities of receptors and constitutes an advantage in the case of this mGlu receptor, as its precise quantitation is complicated by a lack of high-affinity (antagonist) radioligands. A preliminary report of some of these data has been presented to the British Pharmacological Society (Hermans *et al*., 1998b).

Methods

Cell culture and induction

The establishment of the stably transfected Chinese hamster ovary (CHO) cell line in which the expression of the human mGlu1α receptor is controlled by the activity of a Lac-repressible promoter has been previously reported (Hermans *et al*., 1998a). These cells (CHO-Lac-hmGlu1α) were routinely cultured in Modified Eagle's medium containing glutamax, supplemented with 10% foetal calf serum, 100 μg ml^{-1} streptomycin and 10^5 u ml^{-1} penicillin in 175-cm^2 flasks in a humidified 5% CO_2 incubator maintained at 37°C. Cells were used between passage 8 and 30. The cells were seeded onto 24-well plates in 0.75 ml well^{-1} culture medium 48 h prior to signal transduction experiments. When required, IPTG was added 20 h before the experiment directly to the culture medium at the concentration indicated.

[^{3}H]-InsP determination

For [^{3}H]-inositol (poly)phosphates ([^{3}H]-InsP) determination, the cells were seeded in medium containing 1 μCi ml^{-1} [^{3}H]-inositol. Medium was replaced with 0.5 ml of the same medium supplemented with the indicated concentration of IPTG 18–20 h before the experiment. Cells were washed three times with Krebs-Henseleit buffer (KHB) (in mM) NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 1.3, $MgSO_4$ 1.2, HEPES 5 and D-Glucose (10) (pH 7.4). When glutamic-pyruvic transaminase (GPT) and pyruvate were used, these were dissolved in KHB buffer and added to the incubation buffer 15 min before any other manipulation and 30 min before agonist challenge. When antagonists were tested, these were added 15 min before agonist addition. During the last 15 min before the agonist challenge, LiCl (10 mM) was added to the incubation medium. Experiments were performed at 37°C in a final volume of 0.5 ml well^{-1} and were terminated by the addition of 0.5 ml ice-cold 1 M trichloroacetic acid. After extraction on ice for 20 min, samples (0.8 ml) were collected from the well, mixed with 200 μl EDTA (10 mM, pH 7.0) and extracted with 1 ml of a 1:1 (v v^{-1}) mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. An 800 μl sample of the aqueous extract was mixed with 50 μl $NaHCO_3$ (62.5 mM) and [^{3}H]-InsP recovered by ion-exchange chromatography on Dowex AG1-X8 (formate form) columns as previously described (Challiss *et al*., 1993).

Ins(1,4,5)P_3 determination

Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) mass was determined as described previously (Challiss *et al*., 1993). The preparation of samples for Ins(1,4,5)P_3 determination was similar to the protocol described above for [^{3}H]-InsP except that [^{3}H]-inositol was omitted from the culture medium and no LiCl was added at the incubation stage.

Immunofluorescent labelling and imaging

Cells grown for 20 h on coverslips in the absence or in the presence of IPTG (3–300 μM) were fixed for 10 min at room temperature in 2% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized for 30 min with 0.5% Triton X-100 in PBS containing 10% goat serum. After three washes with PBS, the cells were incubated for 2 h with a specific mGlu1α receptor antibody diluted 1:250 in PBS/goat serum. After washing, the cells were incubated for 90 min with a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody diluted 1:500 in PBS/goat serum. After washing, the cells were mounted on slides and examined using a Bio-Rad 600 laser scanning confocal microscope equipped with a 60× objective.

Immunoblot analysis

Cells were plated in 24-well multidishes and maintained in culture medium for 48 h before the experiment. When required, IPTG (1–100 μM) was added to the medium for the final 20 h. Plates were placed on ice and after two washes with 0.5 ml KHB, the cells were solubilized in 400 μl (Tris/HCl (125 mM), dithiothreitol (50 mM), 4% sodium dodecylsulphate, 20% glycerol, 0.01% bromophenol blue, pH 6.8. The samples were collected in centrifuge tubes and combined with a further 400 μl H_2O which was used to rinse the well. Immunoblotting was performed as previously described, using a mGlu1α receptor antiserum (100 ng ml^{-1}) and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Immunoreactive proteins were detected with enhanced chemiluminescence (ECL) reagents.

Data analysis

EC_{50} values were determined by non-linear regression analysis using the software Prism II (GraphPad, San Diego, CA, U.S.A.). Data were fitted as sigmoïdal dose-response curves with variable slope and analysed by a four parameter logistic equation. Antagonist equilibrium dissociation constants (expressed as pK_B) were calculated using the following:

$$K_B = A/(CR - 1)$$

where A is the concentration of antagonist used and CR is the ratio of EC_{50} values for the agonist measured in the presence or in the absence of the antagonist, respectively.

Materials

L-Quisqualic acid, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD), (*S*)-3,5-dihydroxyphenylglycine ((*S*)-3,5-DHPG), (*S*)-α-methyl-4-carboxyphenylglycine ((*S*)-MCPG), (*S*)-4-carboxyphenylglycine ((*S*)-4-CPG), (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) and (*S*)-4-carboxy-3-hydroxyphenylglycine ((*S*)-4-C3-HPG) were from Tocris-Cookson (Bristol, U.K.); IPTG, dithiothreitol and glutamic-pyruvic transaminase (GPT), Triton X-100, fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody, paraformaldehyde and goat serum were from Sigma Chemical Co. (Poole, U.K.); *myo*-[2-^{3}H]-inositol (70–120 Ci mmol^{-1}) and ECL reagents were from Amersham (Little Chalfort, U.K.). The antiserum raised against the C-terminus of the rat mGlu1α was from Chemicon. All cell culture media and reagents were from Gibco Life Technologies (Paisley, U.K.). LY367385 and (Clark *et al*., 1997) was kindly provided by Dr J.R. Harris (Lilly Research Laboratories, Windlesham, U.K.). All other reagents were of analytical grade and were from Fisons (Loughborough, U.K.).

Results

Immunocytochemical localization of mGlu1α receptor expression

We have previously shown that treatment of transfected CHO-Lac-hmGlu1α cells with IPTG caused a concentration- and time-dependent increase in the expression of functional mGlu1α receptors, as detected by immunoblot, using a specific mGlu1α antiserum or by measuring agonist-induced phosphoinositide hydrolysis. In the present experiments, the time of exposure of the cell to IPTG was maintained constant (20 h) whereas different levels of expression were obtained by varying the concentration of the inducer added to the culture medium. Figure 1 shows the expression of the receptor detected by immunofluorescence using a specific antiserum raised against the intracellular C-terminus of the mGlu1α receptor. When cells were grown in the absence of IPTG, no immunoreactivity could be detected. Increasing the concentration of IPTG in the culture medium from 0–300 μM resulted in a progressive increase in immunofluorescence. No specific labelling of the cells could be detected in non-transfected cells or when incubation with the specific mGlu1α receptor antiserum was omitted from the protocol. Analysis of cells induced with IPTG revealed diffuse immunoreactivity throughout the cytoplasm. Intense labelling was also observed close to the nucleus, especially in cells induced with 100 or 300 μM IPTG suggesting that a significant amount of newly synthesized receptors may be present after 20 h of induction (Figure 1). Increasing the expression of the mGlu1α receptor also resulted in marked changes in the morphology (increase in length-width ratio, data not shown) of the CHO cells, as recently reported by Kubo and colleagues (1998).

Effects of mGlu1α receptor on basal and agonist-stimulated [^{3}H]-InsP responses

Induction of mGlu1α receptor expression in the transfected CHO cells resulted in a significant increase in the basal level of [^{3}H]-InsP accumulation in the presence of Li^+ (Figure 2, inset). However, this increase most likely results from receptor activation by endogenously released glutamate, as it could be completely suppressed by addition of the glutamate metabolizing enzyme glutamic-pryuvic transaminase (GPT) and its co-substrate pyruvate. Experiments performed in the absence or presence of GPT/pyruvate indicated that the increase in the basal level [^{3}H]-InsP did not alter the response to L-quisqualate (Figure 2).

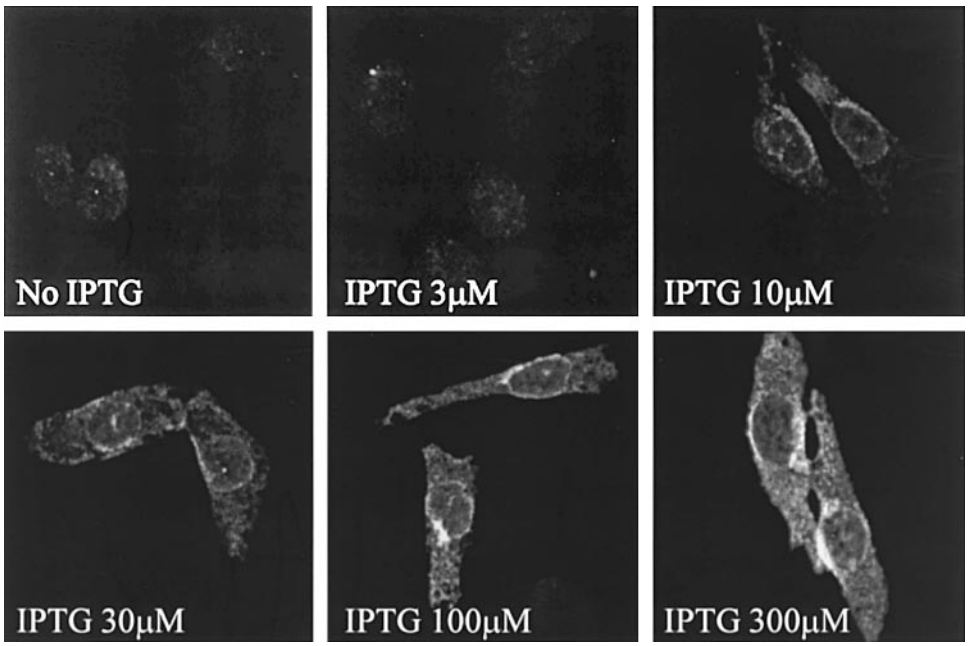

Figure 1 Fluorescence imaging of mGlu1α receptor expressed in CHO-Lac-hmGlu1α cells pre-incubated for 20 h in the absence or presence of IPTG (3, 10, 30, 100, 300 μM). Immunofluorescence staining was performed as indicated under Methods using a specific antiserum raised against the mGlu1α receptor and revealed by a fluorescein isothiocyanate-conjugated secondary antibody. Representative images corresponding to horizontal sections through the middle of the cell are shown.

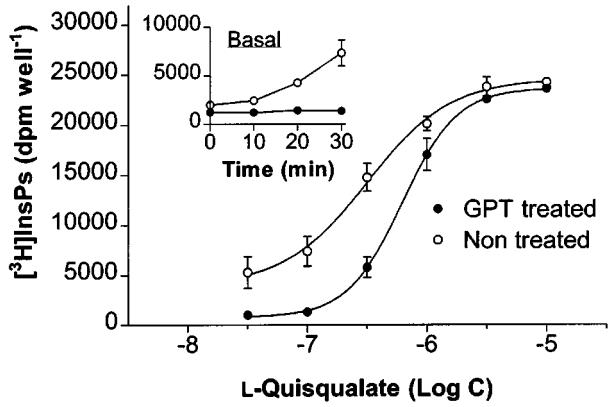

Figure 2 Effect of GPT/pyruvate on the basal and L-quisqualate-induced accumulations of [^{3}H]-InsP. After induction with IPTG (100 μM) for 20 h, CHO-Lac-hmGlu1α cells were washed and preincubated for 15 min in the presence (closed symbols) or absence (open symbols) of GPT/pyruvate before the addition of Li^+ and L-quisqualate (10 μM) (closed symbols) at the indicated concentrations. Inset: time-course of basal accumulation of [^{3}H]-InsP after addition of Li^+ alone in the presence (closed symbols) or absence (open symbols) of GPT/pyruvate. Data are means±s.e.mean from representative experiments performed three times in duplicate.

Effects of varying mGlu1α receptor expression on agonist activity

The consequence of varying mGlu1α receptor density on the properties of agonists was studied by measuring agonist-induced phosphoinositide hydrolysis in cells induced for 20 h with different concentration of IPTG. Immunoblot analysis of total cell extracts with a mGlu1α antiserum indicated that maximal expression of the receptor was obtained after induction with 30–100 μM IPTG (Figure 3C). As reported in our previous publication (Hermans *et al.*, 1998a), when analysed by immunoblotting of low percentage gels, the mGlu1α receptor migrates as two different bands (~145 and ~160 kDa). Although both bands are induced by IPTG application, and undoubtedly represent mGlu1α receptor protein, the relative expression level of each of the bands differs depending on the level of induction. At present the biochemical difference(s) between these two bands has not been characterized and we do not known whether both represent active (i.e. cell-surface located, G protein-coupled) mGlu1α receptors. This complicates the quantitation of the level of receptor expression from immunoblot analyses and therefore the variation in functional responses measured in cells expressing different densities of receptors has always been referred to the concentration of IPTG added to the culture medium.

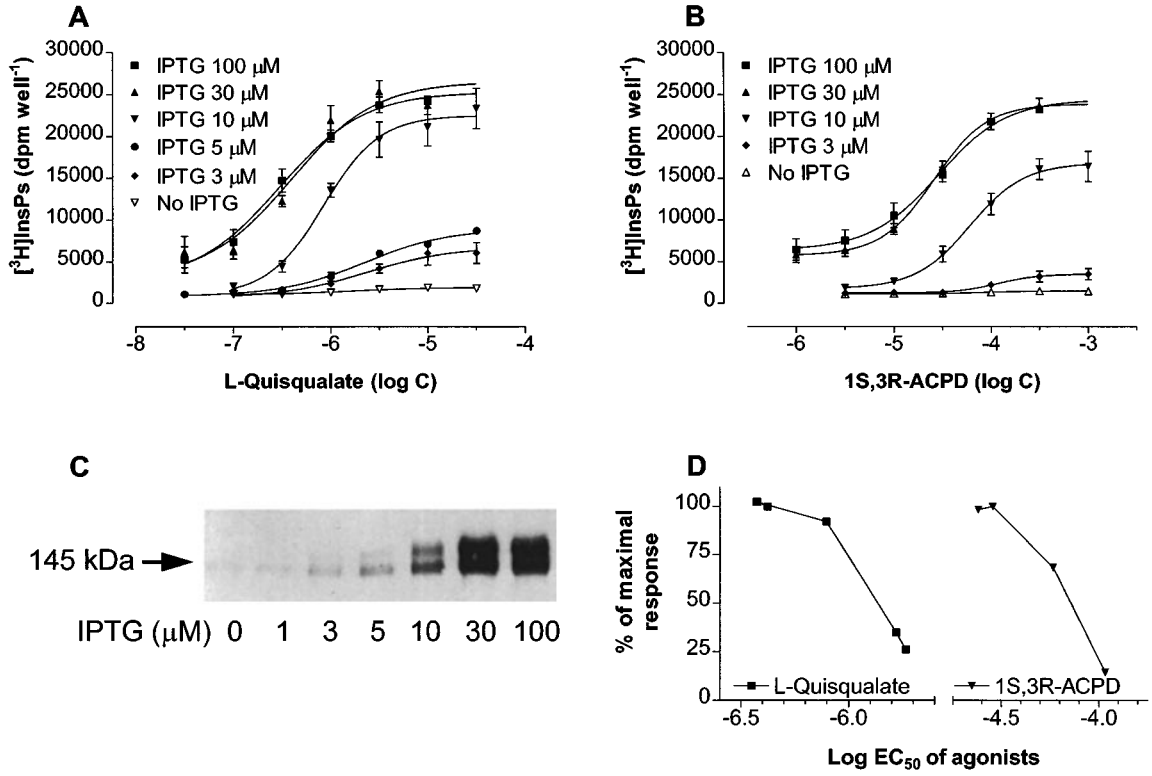

Figure 3 (A) L-quisqualate- and (B) 1*S*,3*R*-ACPD-induced accumulations of [^{3}H]-InsP in CHO-Lac-hmGlu1α cells expressing the mGlu1α receptor at different densities (obtained after pre-incubation for 20 h in the absence or presence of IPTG at different concentrations). Cells were stimulated with the agonists for 30 min in the presence of LiCl (10 mM). Data are means±s.e.mean from at least three different experiments performed in duplicate. Log EC_{50} values calculated from these curves are shown in Table 1. (C) Immunoblot analysis of total cell extracts using a specific mGlu1α receptor antiserum showing the increase in receptor expression obtained after incubation of the cells for 20 h in the presence of increasing concentrations of IPTG. (D) Shows the relationship between the potency and the efficacy of L-quisqualate and 1*S*,3*R*-ACPD measured on the cells expressing mGlu1α receptors at different densities.

Figure 3A and B show concentration-response curves to the agonists L-quisqualate and 1*S*,3*R*-ACPD in cells incubated in the absence or presence of increasing concentrations of IPTG. In non-induced cells, no significant response was detected to either agonist. Increasing the receptor density not only resulted in an increase in the maximal response to each agonist, but also in significant changes in the EC_{50} values (Table 1). For both agonists, increasing the concentration of IPTG from 3–30 μM resulted in a 4–5 fold decrease in the EC_{50} value for stimulation of [^{3}H]-InsP accumulation. Figure 3D illustrates the relationships between the increase in the maximal response and the increase in the potency for each agonist. Increasing receptor expression was found simultaneously to increase the potency and the magnitude of the response until a maximum response was obtained. As shown in the case of L-quisqualate, further increases in receptor density tended only to increase the potency of the agonist without changing the maximum response. As indicated in Table 1, Hill coefficients for agonist-induced accumulation of [^{3}H]-InsP were found to be greater than unity, possibly revealing the complex nature of the activation of phospholipase C by G-protein coupled receptors in CHO cells. Positive cooperativity may result from the amplification of the signal through the signalling cascade as well as from the positive contribution of intracellular [Ca^{2+}] in the activation of phospholipase C (Simpson *et al*., 1995).

When the concentration-response curves to L-quisqualate and 1*S*,3*R*-ACPD were evaluated for cells induced with a low concentration of IPTG (3 and 10 μM), the intrinsic activity of 1*S*,3*R*-ACPD in activating the mGlu1α receptor was significantly lower than that of L-quisqualate (being approximately 52 and 75% of the responses to L-quisqualate, respectively), confirming the partial agonist activity of 1*S*,3*R*-ACPD at this receptor subtype. However, when the receptor was expressed at a high density, the intrinsic activity of 1*S*,3*R*-ACPD approached that of L-quisqualate. The consequence of modulating the density of mGlu1α receptor on the maximal responses to L-quisqualate and 1*S*,3*R*-ACPD was further studied by measuring the accumulation of [^{3}H]-InsP induced by maximal concentrations of these two agonists (30 μM and 1 mM, respectively) on cells previously incubated with increasing concentrations of IPTG. As shown on Figure 4, although the maximal response to 1*S*,3*R*-ACPD was always lower than that for L-quisqualate, the difference between these agonist responses decreased as the concentration of IPTG was increased above 10 μM.

The pharmacological properties of L-quisqualate and 1*S*,3*R*-ACPD on the mGlu1α receptor were further investigated by measuring changes in Ins(1,4,5)P_3, the immediate second messenger product of phospholipase C activity. Contrasting with the dramate increase in [^{3}H]-InsP accumulations observed in this cell-line, agonist-induced increases in Ins(1,4,5)P_3 were relatively small. As shown on Figure 5, L-quisqualate caused a 2–3 fold increase in Ins(1,4,5)P_3 levels to a peak value at 30 s, followed by a decrease to a lower, but still elevated plateau level. 1*S*,3*R*-ACPD elicited a lower response that consisted of an essentially monophasic increase in Ins(1,4,5)P_3 to a sustained elevated plateau level (Figure 5A). Interestingly, the levels of Ins(1,4,5)P_3 obtained during the late plateau phase of the response were comparable for both agonists. Analysis of concentration-response curves for L-quisqualate and 1*S*,3*R*-ACPD performed at 30 s generated log EC_{50} (M) values of -6.11 ± 0.11 ($n=4$) and -4.49 ± 0.20 ($n=3$), respectively (Figure 5B), in broad agreement with potency estimated obtained from [^{3}H]-InsP measurements (see Table 1). The maximal increase in Ins(1,4,5)P_3 induced by each agonist were measured in cells incubated with different concentrations of IPTG (Figure 5C). A significant Ins(1,4,5)P_3 response to L-quisqualate was only obtained in cells induced with $\geqslant 5$ μM IPTG. Maximal responses obtained with 1*S*,3*R*-ACPD were found to be small as compared to the response obtained with L-quisqualate; even when tested on cells induced with high concentrations of IPTG (100–300 μM) these never achieved >45% of the responses to the full agonist. Unfortunately, the magnitude of the responses to either

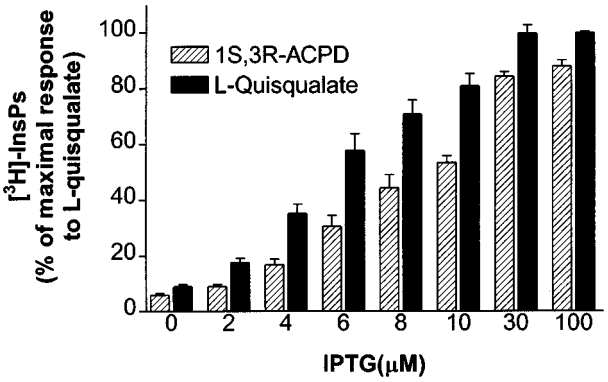

Figure 4 Maximal [^{3}H]-InsP responses to L-quisqualate (30 μM, filled bars) and 1*S*,3*R*-ACPD (1 mM, hatched bars) in CHO-Lac-hmGlu1α cells. Cells were induced for 20 h with the indicated concentrations of IPTG and the accumulations of [^{3}H]-InsP induced by the agonists were measured over a 30 min incubation in the presence of LiCl (10 mM). Results are expressed as a percentage of the response to L-quisqualate measured in cells induced with IPTG (100 μM) and are shown as means±s.e.mean from four different experiments performed in duplicate.

Table 1 Potencies of L-quisqualate and 1*S*,3*R*-ACPD for induction for phosphoinositide hydrolysis in cells expressing the mGlu1α receptor at different densities

	L-Quisqualate			*1S,3R-ACPD*		
[IPTG] (μM)	*EC_{50}* (log M) (n)	*Slope*	*E_{max}* (d.p.m. × 10^3)	*EC_{50}* (log M) (n)	*Slope*	*E_{max}* (d.p.m. × 10^3)
100	−6.38±0.10 (16)	1.66±0.35	24.5±1.9	−4.54±0.10 (4)	1.61±0.31	24.5±1.1
30	−6.43±0.05 (4)	1.82±0.21	24.6±1.1	−4.62±0.06 (4)	1.47±0.09	23.9±0.6
10	−6.10±0.12 (4)	1.52±0.30	22.5±2.5	−4.23±0.03 (4)	1.55±0.21	16.9±1.1
5	−5.78±0.06 (4)	1.81±0.34	8.6±0.3	N.D.	N.D.	N.D.
3	−5.73±0.11 (5)	1.58±0.35	6.3±0.7	−3.97±0.12 (4)	1.96±0.45	3.5±0.3

CHO-Lac-hmGlu1α cells were incubated for 20 h with different concentrations of IPTG and concentration-response curves were performed by measuring the accumulations of [^{3}H]-InsP after exposure to agonist for 30 min in the presence of LiCl (10 mM). Log EC_{50} values were estimated by non-linear regression and data are shown as means±s.e.mean from '*n*' different experiments performed in duplicate. N.D., not determined.

agonist precluded further study in cells induced with sub-optimal concentrations of IPTG.

Effects of varying mGlu1α receptor expression on antagonist activity

In addition to studying the consequence of modulating the expression of mGlu1α receptors on the properties of agonists, the effects on antagonist activities were also investigated. In order to study the effects of antagonists on the exogenously added agonists, GPT and pyruvate were added to the incubation medium of the cells. All of the mGlu1α receptor antagonists studied competitively inhibited the [^{3}H]-InsP responses to the agonists L-quisqualate, 1*S*,3*R*-ACPD and (S)-3,5-DHPG. (S)-MCPG, (S)-4-CPG, AIDA, (S)-4-C3HPG and LY367385 were found to cause parallel rightward shifts of the concentration-response curves for agonist-stimulated [^{3}H]-InsP accumulations (as shown for L-quisqualate in Figure 6). The concentration-ratios for EC_{50} values obtained in the presence compared to the absence of antagonists were used to calculate pK_B values for each antagonist. Table 2A shows that the ranking for antagonist activities was LY367385 > (*S*)-4-

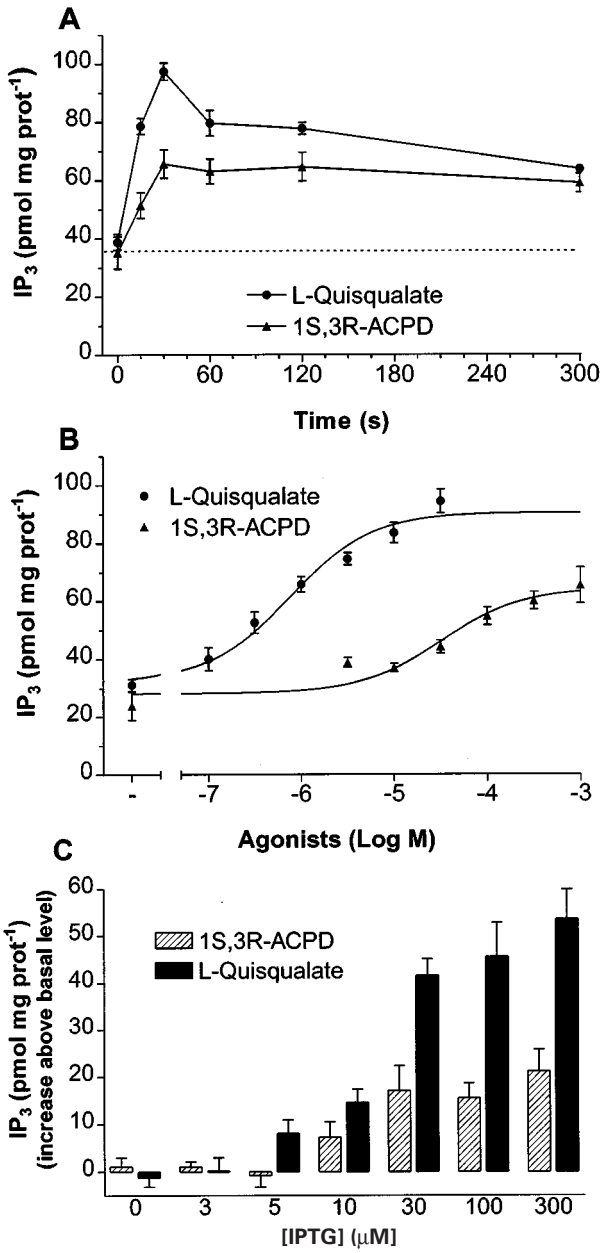

Figure 5 L-quisqualate- and 1*S*,3*R*-ACPD-induced increases in Ins(1,4,5)P_3 in CHO-Lac-hmGlu1α cells. Time-course (A) and concentration-response (B) curves for L-quisqualate- (filled circles) and 1*S*,3*R*-ACPD- (filled triangles) induced increases in Ins(1,4,5)P_3 measured in cells pre-incubated with IPTG (100 μM) for 20 h. (C) Responses to both agonists were measured on cells previously induced for 20 h with different concentrations of IPTG. In (A) and (C), concentrations of L-quisqualate and 1*S*,3*R*-ACPD were 30 μM and 1 mM, respectively. In (B) and (C), Ins(1,4,5)P_3 levels were measured after 30 s stimulation. Data are presented as means ± s.e.mean from three (A, B) and four (C) experiments performed in duplicate.

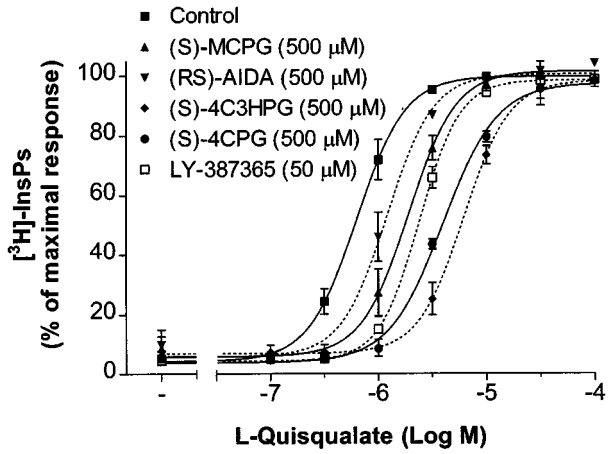

Figure 6 Antagonism of L-quisqualate-induced accumulation of [^{3}H]-InsP in CHO-Lac-hmGlu1α cells by (*S*)-MCPG, (*S*)-4-CPG, AIDA, (*S*)-4-C3HPG and LY367385. Concentration-response curves for L-quisqualate were performed in the absence or presence of each antagonist used at 500 μM, except LY367385 (50 μM) in cells previously induced by 20 h with IPTG (100 μM). Accumulations of [^{3}H]-InsP induced by L-quisqualate were measured for a 30 min incubation period in the presence of LiCl (10 mM). GPT (3 u ml^{-1}) and pyruvate (3 mM) were added to the incubation medium 30 min, and antagonists 15 min, before agonist challenge. Results are expressed as a percentage of the maximal response to L-quisqualate in the absence of antagonist. Data shown are means ± s.e.mean from at least three different experiments performed in duplicate.

Table 2 Analysis of antagonist activities at the mGlu1α receptor in CHO-Lac-hmGlu1α cells (pKb values)

Antagonist	*pK$_B$ values* L-*quisqualate*	(S)-*3,5-DHPG*	1*S*,3R-*ACPD*
(A) IPTG (100 μM)			
(*S*)-MCPG	3.73 ± 0.05 (3)	3.87 ± 0.10 (3)	3.76 ± 0.16 (4)
(*S*)-4-C3HPG	4.32 ± 0.07 (3)	4.48 ± 0.14 (3)	4.51 ± 0.12 (3)
(*RS*)-AIDA	3.40 ± 0.12 (3)	N.D.	N.D.
(*S*)-4CPG	4.08 ± 0.14 (3)	N.D.	N.D.
LY367385	4.92 ± 0.12 (4)	N.D.	N.D.
(B) IPTG (5 μM)			
(*S*)-MCPG	3.75 ± 0.05 (3)	3.66 ± 0.17 (3)	
(*S*)-4-C3HPG	4.40 ± 0.13 (3)	4.42 ± 0.15 (3)	

(A) Cells were incubated for 20 h with IPTG (100 μM) and the effects of different agonists were tested on the responses induced by L-quisqualate, (*S*)-3,5-DHPG or 1*S*,3*R*-ACPD. (B) Comparison of pK_B values for (*S*)-MCPG and (*S*)-4-C3HPG in inhibiting L-quisqualate- and (*S*)-3,5-DHPG-induced phosphoinositide hydrolysis in cells incubated for 20 h with IPTG (5 or 100 μM). Data shown are pK_B values calculated on the basis EC_{50} values for agonist-induced accumulations of [^{3}H]-InsP determined from concentration-responses curves in the absence or presence of different antagonists used at a single concentration (means ± s.e.mean from '*n*' different experiments performed in duplicate). N.D., not determined.

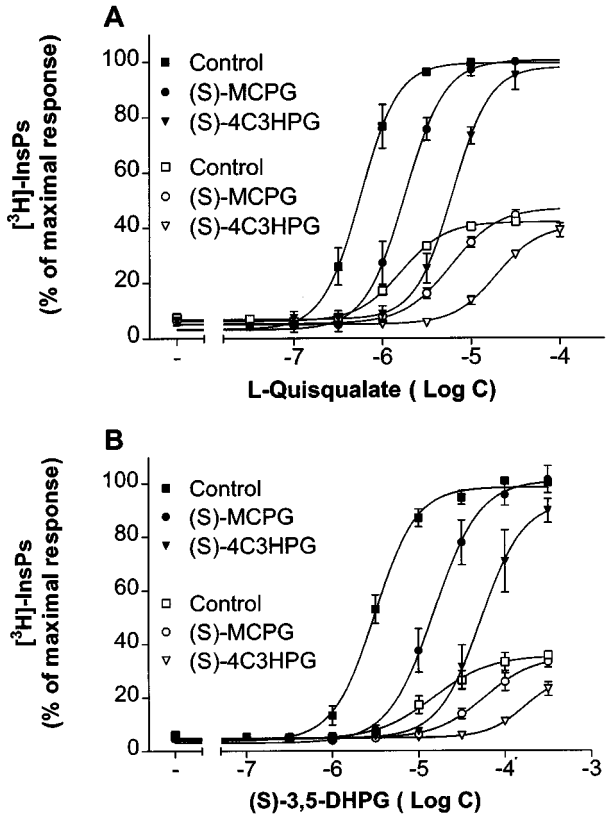

Figure 7 Antagonism of (A) L-quisqualate and (B) (*S*)-3,5-DHPG-induced accumulations of [^{3}H]-InsP in CHO-Lac-hmGlu1α cells by (*S*)-MCPG and (*S*)-4-C3HPG in cells pre-incubated for 20 h in the presence of IPTG (5 or 100 μM). Concentration-response curves for the agonists were performed in the absence or presence of antagonist (500 μM). Accumulations of [^{3}H]-InsP induced by the agonists were measured for a 30 min time-period in the presence of LiCl (10 mM). GPT (3 u ml^{-1}) and pyruvate (3 mM) were added to the incubation medium 30 min, and antagonists 15 min, before agonist challenge. Results are expressed as a percentages of the maximal responses to L-quisqualate or (*S*)-3,5-DHPG in the absence of antagonist on cells induced for 20 h with IPTG (100 μM). Data shown are means ± s.e.mean from at least three different experiments performed in duplicate. In these experiments, the log EC_{50} (M) values measured for L-quisqualate and (*S*)-3,5-DHPG in the absence of antagonist were −6.29 ± 0.11 and −5.48 ± 0.11, respectively in cells induced with IPTG (100 μM); and −5.79 ± 0.14 and −4.73 ± 0.30, respectively in cells induced with IPTG (5 μM). These values were used in the estimation of pK_B values for the antagonists shown in Table 2.

C3HPG > (*S*)-4-CPG > (*S*)-MCPG > AIDA. The pK_B values calculated for a given antagonist tested against the three agonists used were not significantly different, indicating that the activities of these antagonists were independent of the agonist utilized (shown for (*S*)-MCPG and (*S*)-4-C3HPG, see Table 2A). In the presence of GPT/pyruvate, it was not possible to characterize the effect of antagonists on the response induced by exogenously added L-glutamate, however, preliminary experiments performed in the absence of GPT/pyruvate indicated that all the antagonists tested exhibited approximately similar activities for inhibition of the L-glutamate-mediated response (data not shown).

It is important to note that when used alone, none of these antagonists were found to increase basal [^{3}H]-InsP accumulations, indicating the absence of any positive partial agonist activity, even when the receptor was expressed at the highest levels attainable in this model cell system. Likewise, no inverse agonism could be detected for any of the antagonists examined as no decrease in basal [^{3}H]-InsP levels was observed when the activity of endogenous glutamate was prevented by the presence of GPT/pyruvate. The consequences of varying the receptor expression level on the activities of antagonists were studied by measuring the inhibition of the [^{3}H]-InsP responses (stimulated by L-quisqualate or (*S*)-3,5-DHPG) by (*S*)-MCPG or (*S*)-4-C3HPG in cells expressing the mGlu1α receptor at two different levels (Figure 7). As shown above, the potencies of L-quisqualate or (*S*)-3,5-DHPG were significantly different when measured on cells previously induced with 5 or 100 μM IPTG (see legend of Figure 7). However, when this was corrected in the calculated of the pK_B values for (*S*)-MCPG and (*S*)-4-C3HPG, no significant differences were found (Table 2B). This clearly demonstrates that the activity of an antagonist does not depend upon the expression level of the mGlu1α receptor.

Discussion

A particular feature of the amino acid glutamate is that it plays a dual role as a metabolic intermediate and a neurotransmitter. As most cell-lines release significant quantities of glutamate into their culture medium, the maintenance of a stable expression of glutamate receptors in transfected cells can be problematic (Gabellini *et al*., 1994; Desai *et al*., 1995; Lin *et al*., 1997). In order to overcome this problem, we have used an IPTG-inducible expression system to develop a model of transfected CHO cells stably expressing the human mGlu1α receptor (Hermans *et al*., 1998a). A similar approach has been used previously by the group in Minneman in order to express adrenoceptors in transfected cells (Esbenshade *et al*., 1995; Zhong *et al*., 1996). Such a model of metabotropic receptor expression has proved to be useful, not only because the receptor is only expressed after induction, but also because receptor expression can be tightly controlled. In the present study, this model was used to investigate the consequences of varying the density of mGlu1α receptors expressed in CHO cells on the activities of various agonists and antagonists at this important CNS receptor subtype.

Altering the concentration of IPTG added to the culture medium allowed us to modulate the expression level in the transfected CHO-Lac-hmGlu1α cells, as shown by immunofluorescence analysis using a specific mGlu1α receptor antiserum, or by analysis of total cell extracts by immunoblots experiments. Considering the high expression level obtained despite the relatively limited duration of the induction (20 h), it was not surprising to detect the receptor widely distributed throughout the cell. When a high concentration of inducer was used, staining was particularly intense close to the nucleus, perhaps associated with newly synthesized receptors at the Golgi apparatus. Interestingly, increasing the receptor density was also found to result in changes in cell shape. Similar observations have been made recently in CHO cells stably transfected to express mGlu1α receptors and this effect was found to be dependent on the functional activation of the receptor by extracellular Ca^{2+} (Kubo *et al*., 1998). Our own experiments would suggest that mGlu1α receptor activation is enhanced in the presence of normal extracellular (1.3 mM) Ca^{2+} concentration, but also requires endogenous extracellular glutamate (Saunders *et al*., 1998).

Inducing the expression of the mGlu1α receptor in CHO cells was found to result in an increase in the basal level of phosphoinositide hydrolysis. However, this was found to be caused by glutamate release by the cells as it did not occur in the presence of the glutamate degrading enzyme GPT and its

co-substrate pyruvate. Thus, when precautions were taken to exclude the possibility of receptor activation by endogenous glutamate, the human mGlu1α receptor appeared to be devoid of any constitutive activity, even when the receptor was expressed at very high levels. This observation contrasts with previous reports indicating that the mGlu1α receptor exhibits significant constitutive activity in LLC-PK1 cells which could not be reserved by either GPT/pyruvate pre-treatment, or specific nGlu1α receptor antagonists (Brabet *et al*., 1995; Prézeau *et al*., 1996). Whether this difference reflects the cell-type and the stoichiometry of transmembrane signalling components, or a species-related (human vs rat) difference in the mGlu1α receptor, remains to be established. It is however noteworthy that previous studies have reported on the probable activation of metabotropic receptors by glutamate present in incubation medium, and the endogenous glutamate has been postulated as a possible cause of receptor desensitization or down-regulation (Thomsen *et al*., 1994b; Desai *et al*., 1995; Prézeau *et al*., 1996; Moroni *et al*., 1997; Carruthers *et al*., 1997).

When the functional coupling of the receptor was investigated by measuring agonist-induced accumulations of [^{3}H]-InsP (in the presence of Li^+), increasing the receptor expression level not only resulted in an increase in the maximal response, but also in a significant leftward shift of the concentration-response curves for L-quisqualate, (1S,3R)-ACPD and (S)-3,5-DHPG. Such an increase in agonist potency as a result of an increase in the receptor number is consistent with the involvement of a receptor reserve (see Kenakin, 1993). Classical receptor theory predicts that increasing the number of receptors will first result in an increase in the maximal responsiveness to agonists, without a change in potency until the maximal response which can be achieved in the system is reached. Thereafter, further increases in the functional receptor density should result in the development of a receptor reserve, leading to increases in the potencies of agonists without a further increase in the maximal responsiveness. As summarized in Figure 3D, although both potency and maximal response appeared to change simultaneously when the receptor level was increased, there was a direct relationship between the level of expression and the potency of agonists. Using a similar approach of controlling receptor expression, Esbenshade *et al*. (1995) and Zhong *et al*. (1996) have described changes in concentration-response relationships for agonists in cells expressing varying levels of β_1-, β_2- or α_{1B}-adrenoceptors that closely followed classical receptor theory.

Comparison of the maximal [^{3}H]-InsP responses obtained after stimulation with L-quisqualate and (1*S*,3*R*)-ACPD revealed that the relative intrinsic activity of the latter agonist was dependent on the level of receptor expression. Thus, comparable [^{3}H]-InsP responses were observed with both agonists at very high receptor density, whereas the partial agonist activity of (1*S*,3*R*)-ACPD was evident at lower receptor expression levels. This observation is in accordance with classical receptor theory that predicts that intrinsic activity of an agonist is dependent upon the extent of the receptor reserve (Hoyer & Boddeke, 1993). The relationship between receptor density and the intrinsic activity of agonists was further investigated by measuring agonist-induced Ins(1,4,5)P_3 accumulation. When the peak Ins(1,4,5)P_3 response was measured after 30 s stimulation (1*S*,3*R*)-ACPD was found to behave as a partial agonist as compared to L-quisqualate at all receptor expression levels. However, analysis of the time-course of Ins(1,4,5)P_3 accumulation CHO-LacmGlu1α cells induced with IPTG (100 μM) revealed marked differences in the responses to L-quisqualate and (1*S*,3*R*)-ACPD. Although the magnitude of the peak response measured after 30 s was significantly greater with the full agonist L-quisqualate, the level of Ins(1,4,5)P_3 accumulation measured during the plateau phase was similar for both agonists. Thus, depending upon whether Ins(1,4,5)P_3 was assessed at the peak or during the plateau phase of second messenger accumulation, (1*S*,3*R*)-ACPD could be shown to behave as either a partial or full agonist, respectively. As the plateau phase is approached within 1 min following agonist addition is it not surprising that the agonist activity of (1*S*,3*R*)-ACPD on this phase of the response closely resembles that observed for the [^{3}H]-InsP accumulation measured over a 30 min period in the presence of Li^+. The relatively small increase in Ins(1,4,5)P_3 levels over basal values during the plateau phase precluded further investigation of the difference in response obtained with L-quisqualate and (1*S*,3*R*)-ACPD at different levels of induction. However, it might be anticipated that low receptor expression levels would result in substantial differences in the magnitude of the plateau level of Ins(1,4,5)P_3 obtained with L-quisqualate and (1*S*,3*R*)-ACPD, again revealing the partial activity of the latter in activating phospholipase C through the mGlu1α receptor. Taken together, these results indicate that differences in the intrinsic activities of mGlu receptor agonists can be observed depending on the index of receptor-stimulated phosphoinositide turnover assessed. Virtually all other studies concerning the pharmacological characterization of agonists interacting with mGlu receptors coupled to the activation of phospholipase C (Group I) are based on the measurements of the accumulation of total inositol phosphates fraction in the presence of Li^+ (Hayashi *et al*., 1994; Brabet *et al*., 1995; Birse *et al*., 1993; Balázs *et al*., 1997; Joly *et al*., 1995). In the light of the present observations regarding the partial activity of (1*S*,3*R*)-ACPD, it appears that caution should be exercised when considering data obtained by measuring different indices of phosphoinositide turnover. This would support previously reported differences in the abilities of mGlu agonists to induce Ins(1,4,5)P_3 production compared to [^{3}H]-InsP accumulation in rat brain slices (Mistry & Challiss, 1996).

The activities of mGlu antagonists in this model were in accordance with the values previously reported for these compounds at the recombinant mGlu1α receptor (Conn & Pin, 1997; Moroni *et al*., 1997; Baker *et al*., 1997). However, in contrast to the results obtained with agonists, varying the density of the mGlu1α receptor in the transfected cells did not affect the activities of competitive antagonists for inhibition of agonist-induced phosphoinositide hydrolysis. This observation is consistent with classical receptor theory (Kenakin, 1993) and would further support the proposal that these competitive antagonists do not display positive or negative (inverse) agonist activity.

The identification of selective agonists and antagonists which discriminate between mGlu receptors subtypes is still a limiting step in elucidating the physiological, and possible pathophysiological roles of these receptors in glutamatergic transmission in the CNS. Some important insights have already come from the investigation of mGlu receptors in heterologous expression systems (Pickering *et al*., 1993; Hayashi *et al*., 1994; Thomsen *et al*., 1994a; Brabet *et al*., 1995; Moroni *et al*., 1997; Doherty *et al*., 1997). However, in the absence of appropriate radioligands, and therefore knowledge of relative receptor expression levels, any extrapolation of such information to native mGlu receptors with unknown receptor reserves and the potential promiscuous coupling to a variety of effectors, remains potentially hazardous. The current

study has highlighted major changes in the potency and intrinsic activity of mGlu agonists in cell expressing different levels of the mGlu1α receptor using an inducible system. This reinforces our view that any selective effects of agonists (or indeed inverse agonists) between receptor subtypes should be investigated in cells exhibiting a range of receptor expression levels and furthermore, that the inducible system described here provides a highly suitable model for such studies.

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