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Characterization of [^{3}H]-(2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine ([^{3}H]-DCG IV) binding to metabotropic mGlu$_2$ receptor-transfected cell membranes

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1 The binding of the new selective group II metabotropic glutamate receptor radioligand, [^{3}H]-(2**S**,2′**R**,3′**R**)-2-(2′,3′-dicarboxycyclopropyl)glycine ([^{3}H]-DCG IV), was characterized in rat mGlu$_2$ receptor-transfected CHO cell membranes.

2 [^{3}H]-DCG IV binding was pH-dependent, but was not sensitive to temperature. Saturation analysis showed the presence of a single binding site, with a K_d value of 160 nM and a B_{max} value of 10 pmol mg^{-1} protein. Binding was not sensitive to Na^+-dependent glutamate uptake blockers or Cl^--dependent glutamate binding inhibitors. Furthermore, up to concentrations of 1 mM, the glutamate ionotropic receptor agonists, N-methyl-D-aspartic acid (NMDA), (**S**)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate, did not affect [^{3}H]-DCG IV binding.

3 Of the compounds observed to inhibit [^{3}H]-DCG IV binding, the most potent were the recently described selective group II agonist, (+)-2-aminobicyclo-[3.1.0]hexane-2,6-dicarboxylate (LY 354740; K_i value 16 nM) and antagonist, 2-amino-2-(2-carboxycyclopropan-1-yl)-3-(dibenzopyran-4-yl) propanoic acid (LY 341495; K_i value 19 nM). As expected, for a G-protein-coupled receptor, guanosine-5′-O-(3-thiotriphosphate) (GTPγS) inhibited [^{3}H]-DCG IV binding in a concentration-dependent manner, with an IC_{50} value of 12 nM.

4 A highly significant correlation was observed between the potencies of compounds able to inhibit [^{3}H]-DCG IV binding and potencies obtained for agonist activity in a GTPγ^{35}S binding functional assay. In addition, these studies identified a number of compounds with previously unknown activity at mGlu$_2$ receptors, including L(+)-2-amino-3-phosphonopropionic acid (L-AP3), L(+)-2-amino-5-phosphonopentanoic acid (L-AP5), 3-((**RS**)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (**R**-CPP), N-acetyl-L-aspartyl-L-glutamic acid (NAAG) and (**RS**)-α-methylserine-O-phosphate (MSOP).

Keywords: Metabotropic glutamate receptor; [^{3}H]-DCG IV; mGlu$_2$ receptor; radioligand binding

Introduction

The G-protein-coupled metabotropic glutamate (mGlu) receptors play important roles as modulators of glutamatergic and other major neurotransmitter systems in the brain (for review, see Conn & Pin, 1997). At present, at least eight receptors have been cloned and, on the basis of their sequence similarities, signal transduction and agonist rank order of potencies, these receptors have been sub-divided into three groups. Group I mGlu receptors, comprising mGlu$_1$ and mGlu$_5$, are coupled to phosphoinositide (PI) hydrolysis; groups II (mGlu$_2$ and mGlu$_3$) and III (mGlu$_4$, mGlu$_6$, mGlu$_7$ and mGlu$_8$) mGlu receptors are negatively coupled to adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation, but have distinct pharmacological profiles (for review see Conn & Pin, 1997).

Many investigations into the pharmacology and second messenger transduction mechanisms of mGlu receptors have been carried out in cell lines transfected with mGlu receptor clones. However, due to the absence of radiolabelled ligands with sufficient affinity to measure receptor expression, these experiments have lacked a detailed pharmacological characterization. Not only can variations in levels of receptor expression in different cell lines be the cause of pharmacological discrepancies, but can also result in differences in coupling to second messenger transduction mechanisms (Cottechia *et al*., 1990). Therefore, in order to characterize further the pharmacology of mGlu receptors, we have radiolabelled the group II mGlu receptor-selective agonist (2**S**,2′**R**,3′**R**)-2-(2′,3′-dicarboxycyclopropyl) glycine (DCG IV). DCG IV has previously been shown to inhibit forskolin-stimulated cyclic AMP formation with potencies of 300 and 200 nM for Chinese hamster ovary (CHO) cells expressing mGlu$_2$ and mGlu$_3$ receptors, respectively (Hayashi *et al*., 1993). Furthermore, DCG IV was without agonist activity for both mGlu$_1$ and mGlu$_4$ (Hayashi *et al*., 1993).

The high affinity of DCG IV for group II mGlu receptors has enabled us to develop a [^{3}H]-DCG IV binding assay using membranes of mGlu$_2$-expressing CHO cells. In addition, we have compared the activities of various compounds able to inhibit [^{3}H]-DCG IV binding, with their effects in functional assays: guanosine-5′-O-(3-thiotriphosphate) (GTPγ^{35}S) binding and inhibition of forskolin-stimulated cyclic AMP production in mGlu$_2$-expressing CHO cells.

Transfection

cDNA encoding the rat mGlu$_2$ receptor protein in pBluescript II was obtained from Prof. S. Nakanishi (Kyoto, Japan), and subcloned into the eukaryotic expression vector pcDNA I-amp from Invitrogen (NV Leek, The Netherlands). This vector construct (pcD1mGR2) was co-transfected with a psvNeo plasmid encoding the gene for neomycin resistance, into CHO cells by a modified calcium phosphate method described by

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Chen & Okayama (1988). The cells were maintained in Dulbecco's Modified Eagle's medium with reduced L-glutamine (2 mM final concentration) and 10% dialysed foetal calf serum from Gibco BRL (Basel, Switzerland). Selection was made in the presence of G-418 (1000 μg ml^{-1} final). Clones were identified by reverse transcription of 5 μg total RNA, followed by PCR with mGlu$_2$ receptor specific primers 5′-atcactgcttgggtttctggcactg-3′ and 5′-agcatcactgtgggtggcatag-gagc-3′ in 60 mM Tris HCl (pH 10), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 25 units ml^{-1} Taq polymerase with 30 cycles annealing at 60°C for 1 min, extension at 72°C for 30 s and 1 min 95°C denaturation.

Cyclic AMP measurement

Cells were seeded in 96-well plates and grown for 3 days in a DMEM medium containing 10% dialysed foetal calf serum, 1 mM glutamine and the neomycin analogue G-418 (300 μg ml^{-1}). Agonist activity was tested by the addition of the compound in the presence of 30 μM forskolin. Putative antagonists were tested by incubation with the compound in the presence of 10 μM 1**S**,3**R**-ACPD and 30 μM forskolin. Following washing and 15 min preincubation in PBS buffer, cells were incubated at 37°C for 30 min in the presence of compounds. The reaction was terminated by the addition of 120 μl absolute ethanol and the plate frozen at −80°C. The liquid phase was evaporated in an oven at 50°C, and cyclic AMP levels in the wells were determined by use of a commercially available enzymeimmunoassay kit (RPN 225, Amersham, Zürich, Switzerland) without acetylation.

Membrane preparation

Cells, cultured as above, were harvested and washed three times with cold PBS and frozen at −80°C. The pellet was resuspended in cold 20 mM HEPES-NaOH buffer containing 10 mM EDTA (pH 7.4), and homogenized with a polytron (Kinematica, AG, Littau, Switzerland) for 10 s at 10 000 r.p.m. After centrifugation for 30 min at 4°C, the pellet was washed once with the same buffer, and once with cold 20 mM HEPES-NaOH buffer containing 0.1 mM EDTA (pH 7.4). Protein content was measured by the Pierce method (Socochim, Lausanne, Switzerland) with bovine serum albumin used as standard.

[^{3}H]-DCG IV binding

After being thawed, the membranes were resuspended in cold 50 mM Tris-HCl buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$ (pH 7) (binding buffer). The final concentration of the membranes in the assays was 25 μg protein ml^{-1}. Association experiments were performed by incubating 50 nM [^{3}H]-DCG IV with membranes, at room temperature, for different time periods before filtration. Saturation isotherms were determined by incubating various [^{3}H]-DCG IV concentrations (0.3–1000 nM) at room temperature, for 1 h. For inhibition experiments, membranes were incubated with 50 nM [^{3}H]-DCG IV at room temperature, for 1 h, in the presence of various concentrations of inhibitors. Following the incubations, membranes were filtered onto Whatmann GF/C glass fibre filters and washed 5 times with cold binding buffer. Non-specific binding was measured in the presence of 1 μM LY 354740. After transfer of the filters into plastic vials containing 10 ml of Ultima-gold scintillation fluid (Packard, Zürich, Switzerland), the radioactivity was measured by liquid scintillation in a Tri-Carb 2500 TR counter (Packard, Zürich, Switzerland).

GTPγ^{35}S binding

After being thawed, the membranes were washed once and resuspended in cold 20 mM HEPES-NaOH buffer containing 10 mM $MgCl_2$ and 100 mM NaCl (pH 7.4). Wheatgerm agglutinin SPA beads (RPNQ0001, Amersham) were suspended in the buffer containing 1 mM dithiothreitol (40 mg of beads ml^{-1}). The membranes and the beads were mixed (beads: 13 mg ml^{-1}, membranes: 200 μg protein ml^{-1}) and incubated with 2 μM GDP at room temperature for 1 h, under mild agitation. GTPγ^{35}S binding was performed in 96-well microplates (picoplate, Packard, Zürich, Switzerland) in a total volume of 180 μl with 15 μg membrane protein and 0.3 nM GTPγ^{35}S. Non specific binding was measured in the presence of 10 μM cold GTPγS. To study the effect of antagonists, GTPγ^{35}S binding was stimulated with 10 μM 1**S**,3**R**-ACPD. Plates were sealed and incubated at room temperature for 2 h, under agitation. The beads were allowed to settle and the plate counted in a Top-Count (Packard, Zürich, Switzerland) with quench correction.

Materials

L-Aspartate-β-hydroxamate, L-cystine, GTPγS and N-methyl-D-aspartic acid (NMDA) were obtained from Sigma (Buchs, Switzerland). D(−)-2-amino-5-phosphonopentanoic acid (D-AP5), L-glutamate, 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK 801), 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX) and quisqualate were from RBI (Zürich, Switzerland). (**S**)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (1**R**,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid (1**R**,3**R**-ACPD), (1**S**,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid (1**S**,3**R**-ACPD), (1**S**,3**S**)-1-aminocyclopentane-1,3-dicarboxylic acid (1**S**,3**S**-ACPD), (**S**)-4-carboxy-3-hydroxy phenylglycine (**S**-4C3HPG), (**S**)-4-carboxyphenylglycine (**S**-4CPG), 3-((**RS**)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (**R**-CPP), dihydrokainic acid (DHKA), (**S**)-3,5-dihydroxyphenylglycine (**S**-DHPG), 2(**S**)-α-ethylglutamic acid (EGLU), (**S**)-3-hydroxyphenylglycine (**S**-3HPG), ibotenate, kainate (KA), L(+)-2-amino-3-phosphonopropionic acid (L-AP3), L(+)-2-amino-4-phosphonobutyric acid (L-AP4), L(+)-2-amino-5-phosphono pentanoic acid (L-AP5), 2**S**,1′**S**,2′**S**-2-(2′-carboxycyclopropyl)glycine (L-CCGI), (2**S**,1′**S**,2′**R**)-2-(carboxycyclopropyl)glycine (L-CCG III), (**S**)-2-amino-2-methyl-4-phosphonobutanoic acid (MA-P4), (2**S**,3**S**,4**S**)-2-methyl-2-(carboxy cyclopropyl)glycine (MC-CG), (**RS**)-α-methyl-3-carboxy-4-hydroxyphenyl glycine (M3-C4HPG), (**RS**)-α-methyl-3-carboxymethylphenylglycine (M3-CMPG), (+)-α-methylcarboxyphenylglycine ((+)- MCPG), (**RS**)-α-methyl-4-phosphonophenyl glycine (MPPG), (**RS**)-α-methylserine-O-phosphate (MSOP), (**RS**)-α-methyl-4-sulphonophenylglycine (MSPG), (**RS**)-α-methyl-4-tetrazolyl-phenylglycine (MTPG), N-acetyl-L-aspartyl-L-glutamic acid (NAAG), and L(−)-threo-3-hydroxyaspartic acid (L-THA) were purchased from Tocris Cookson (Bristol, U.K.). Forskolin,7-deacetyl-7-(O-N-methylpiperazino)-γ-butyryl dihydrochloride was obtained from Calbiochem (Lucerne, Switzerland). GTPγ^{35}S (specific activity 1000 Ci mmol^{-1}) was from Amersham (Zürich, Switzerland). (2**S**,2′**R**,3′**R**)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG IV), (+)-2-aminobicyclo-[3.1.0]hexane-2,6-dicarboxylate (LY 354740) and 2-amino-2-(2-carboxycyclopropan-1-yl)-3-(dibenzopyran-4-yl) propanoic acid (LY 341495; as a mixture of 4 diastereoisomeres) were synthesized at Hoffmann-La Roche Ltd. by Drs J. Wichmann, G. Adam and T. Woltering, respectively. [^{3}H]-DCG IV

(specific activity 17.5 Ci mmol^{-1}) was synthesized at Hoffmann-La Roche Ltd. by Dr P. Huguenin according to a procedure developed by Dr J. Wichmann. Aniracetam was also synthesized at Hoffmann-La Roche Ltd.

Data analysis

The inhibition or activation curves were fitted with a four parameter logistic equation giving EC_{50} and IC_{50} values, and Hill coefficients by use of Deltagraph (Deltapoint, Monterey, CA, U.S.A.). K_i values were calculated with the Cheng and Prusoff equation: $K_i = (IC_{50})/(1 + ([A]/K_d))$, where [A] is the concentration and K_d the dissociation constant of [^{3}H]-DCG IV (Cheng & Prusoff, 1973). Saturation experiments were analysed with the iterative non linear curve fitting software LIGAND (G.A. McPherson, Elsevier-Biosoft, U.K.). Fractional receptor occupancy was calculated from the fitted concentration-response curves for the stimulation of GTPγ^{35}S binding and a derivation of the law of mass action: fractional receptor occupancy = [A]/(K_d + [A]), making the approximation that $K_d = K_i$ (Ruffolo, 1982).

Results

[^{3}H]-DCG IV binding

No specific [^{3}H]-DCG IV binding was observed in membranes prepared from the parental non-transfected CHO cells. However, with mGlu$_2$ receptor-transfected cells, [^{3}H]-DCG IV specific binding was linearly dependent on membrane protein concentration between 5 and 40 μg (data not shown). Binding was not sensitive to temperature (data not shown), but was affected by an increase in pH from 6, with a maximal specific binding at pH 7, and with a decrease to almost zero at pH 10 (Figure 1). Association experiments showed that nearly 20% of the maximum was already bound less than 2 min after the addition of 50 nM of the ligand (Figure 2). With 50 nM [^{3}H]-DCG IV, equilibrium was almost achieved after 1 h (Figure 2).

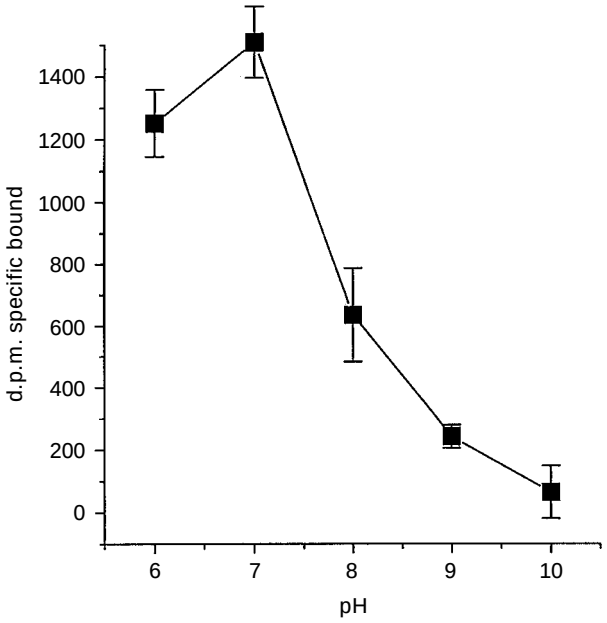

Figure 1 Effect of pH on [^{3}H]-DCG IV binding on mGlu$_2$ receptor-transfected CHO cell membranes. Data are expressed in d.p.m. specific bound and represent mean of three experiments performed in triplicate; vertical lines show s.d.

The dissociation was fast, as more than 90% of the [^{3}H]-DCG IV, specifically bound after 1 h of incubation, was dissociated in less than 1 min (Figure 2).

Saturation analyses were performed at equilibrium with concentrations of [^{3}H]-DCG IV from 0.3 to 1000 nM (Figure 3). The analysis of the curve, by use of the computer programme LIGAND, indicated the presence of a single binding site with a K_d value of 160 ± 19 nM. The maximal

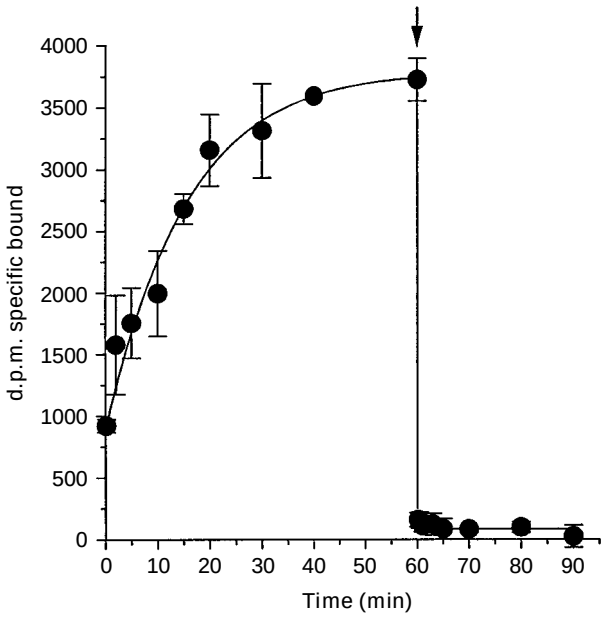

Figure 2 Association and dissociation kinetics of 50 nM [^{3}H]-DCG IV on mGlu$_2$ receptor-transfected CHO cell membranes at room temperature. The dissociation was initiated by the addition of 10 μM cold DCG IV (arrow). Data are expressed as d.p.m. specific bound and are mean of three experiments, performed in triplicate. Vertical lines show s.d.

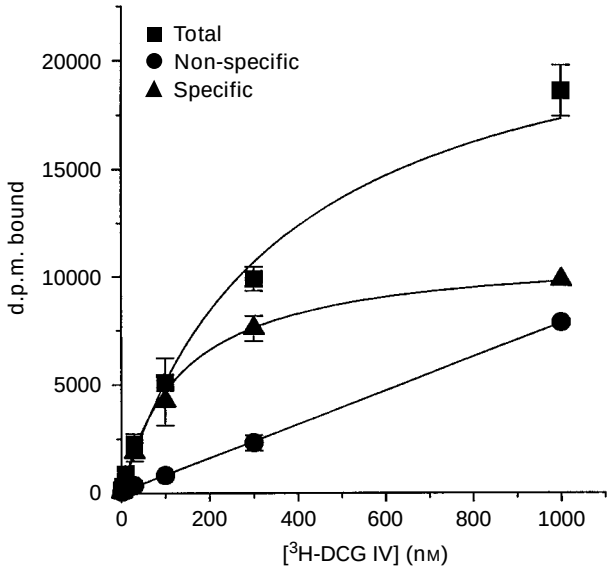

Figure 3 Saturation analysis of [^{3}H]-DCG IV binding on rat mGlu$_2$ receptor-transfected cell membranes. Membranes were incubated at room temperature for 1 h with various concentrations of [^{3}H]-DCG IV. Total bound, non specific bound, measured in presence of 1 μM LY 354740, and specific bound in d.p.m. are the mean of three individual experiments, performed in triplicate. Vertical lines show s.d.

number of binding sites (B_{max}) was equal to 10 ± 3 pmol mg^{-1} protein.

In addition to the determination of the binding properties of [^{3}H]-DCG IV on mGlu$_2$ receptor-transfected cell membranes, the effects of an extensive number of other compounds were tested on [^{3}H]-DCG IV binding (see Table 1 and Figure 4 for selected compounds). The Na^+-dependent glutamate uptake blockers, L-aspartate-β-hydroxamate, L-THA and DHKA all had K_i values greater than 1 mM. However, L-CCG III, another potent Na^+-dependent glutamate uptake blocker, inhibited binding with a K_i value of 30 μM (see Table 1).

NMDA and 1**R**,3**R**-ACPD, two NMDA receptor agonists, did not affect [^{3}H]-DCG IV binding up to concentrations of 1 mM. Furthermore, AMPA, NBQX and KA had K_i values higher than 1 mM and the AMPA receptor modulator, aniracetam, had no effect on the binding, up to a concentration of 100 μM. Finally, the selective group I mGlu receptor agonists, **S**-3HPG and **S**-DHPG, failed to inhibit [^{3}H]-DCG IV binding, at concentrations of 1 mM.

The most potent inhibitors of [^{3}H]-DCG IV binding were the new, selective group II agonist, LY 354740 (K_i value 16 nM) and antagonist, LY 341495 (K_i value 19 nM) (see Table 1 and Figure 4). Other compounds that potently inhibited [^{3}H]-DCG IV binding were the mGlu receptor agonists, DCG IV (K_i value 100 nM) and L-CCGI (K_i value 111 nM). All compounds observed to be active in the [^{3}H]-DCG IV binding assays, inhibited binding by the same extent. The activities of various putative mGlu receptor antagonists were also tested. The K_i values for the following compounds ranged from approximately 5 to 100 μM with the following rank order of potency: MSOP = MCCG > MPPG > (+)MCPG > MSPG > -MTPG. In addition, **RS**-M3CMPG and **RS**-M3C4HPG were also able to inhibit [^{3}H]-DCG IV binding, but with K_i values greater than 100 μM. Two other mGlu receptor ligands, L-AP3 and L-AP4, were observed to be active on the binding, with K_i values of 17 and 59 μM, respectively. Interestingly, a number of compounds were found to be active on [^{3}H]-DCG IV binding which were previously unknown as mGlu receptor agonists, i.e. the NMDA receptor ligands L-AP5 (K_i value 34 μM) and **R**-CPP (K_i value 320 μM). Furthermore, as expected for a G-protein-coupled receptor, guanosine-5′-O-(3-thiotriphosphate) (GTPγS) inhibited [^{3}H]-DCG IV binding (maximal 70% inhibition) in a concentration-dependent manner, with an IC_{50} value of 12 nM and Hill coefficient of 0.6 (data not shown).

Table 1 K_i values and Hill coefficients for the inhibition of [^{3}H]-DCG IV binding on rat mGlu$_2$ receptor-transfected CHO cell membranes

Compound	K_i (μM)	*Hill*
LY 354740	0.016 ± 0.004	0.82 ± 0.1
LY 341495	0.019 ± 0.007	1.0 ± 0.06
DCG IV	0.098 ± 0.02	0.85 ± 0.07
L-CCG I	0.111 ± 0.03	0.82 ± 0.06
L-Glutamate	3.8 ± 0.9	1.1 ± 0.2
MSOP	5.3 ± 2	0.85 ± 0.2
MCCG	6.4 ± 2	0.9 ± 0.08
1**S**, 3**R**-ACPD	10 ± 1	1 ± 0.3
MPPG	13 ± 3	0.76 ± 0.07
S-4C3HPG	14 ± 3	0.94 ± 0.1
L-AP3	17 ± 2	1 ± 0.2
(+)-MCPG	26 ± 5	1 ± 0.1
L-CCG III	29 ± 0.8	0.9 ± 0.1
1**S**, 3**S**-ACPD	29 ± 13	1 ± 0.1
L-AP5	34 ± 6	1 ± 0.09
EGLU	42 ± 6	0.9 ± 0.1
MSPG	58 ± 18	0.7 ± 0.1
L-AP4	59 ± 17	0.8 ± 0.05
Quisqualate	103 ± 14	1 ± 0.1
MTPG	112 ± 46	0.8 ± 0.07
MAP4	114 ± 24	0.98 ± 0.3
NAAG	134 ± 55	0.8 ± 0.03
M3CMPG	154 ± 32	1.2 ± 0.2
M3C4HPG	164 ± 26	1 ± 0.02
R-CPP	320 ± 40	0.96 ± 0.1

Values are mean ± s.d. of the K_i values and Hill coefficients calculated from 3 individual experiments, performed in duplicate.

GTPγ^{35}S binding

Compounds active in the [^{3}H]-DCG IV binding studies were also tested on basal or 1**S**,3**R**-ACPD-stimulated GTPγ^{35}S binding in similar membranes to those used for [^{3}H]-DCG IV binding studies. The agonist rank order of potency of a selection of the compounds tested was: LY 354740 > L-CCG I > L-glutamate = 1**S**,3**R**-ACPD > quisqualate > **R**-CPP (see Table 2, and, for selected compounds, Figure 5a). Even though the maximal effect evoked by 1**S**,3**R**-ACPD was similar to that elicited by LY 354740, the maximal stimulations by L-CCG I, L-glutamate and quisqualate were significantly lower than that of LY 354740 (Student's *t* test) (Figure 5a). Figure 5b shows concentration-response curves for some of the compounds tested on GTPγ^{35}S binding stimulated by 1**S**,3**R**-ACPD (10 μM). The rank order of potency for a selection of the antagonists tested was: LY 341495 > MSOP > L-AP3 > L-AP5 > MSPG > L-AP4 (see Table 3).

A correlation between the EC_{50} values of the agonists able to stimulate GTPγ^{35}S binding and their K_i values for the inhibition of [^{3}H]-DCG IV binding is shown in Figure 6. The correlation coefficient was 0.97 and the slope of the line was unity. For LY 354740, 1**S**,3**R**-ACPD, L-glutamate and L-CCG I, effect versus receptor occupancy curves were determined as described in Methods. Figure 7 shows that almost 50% of mGlu$_2$ receptors must be occupied by LY 354740 or L-CCG I in order to obtain a response 50% of maximum. However, fractional receptor occupation by L-glutamate and 1**S**,3**R**-

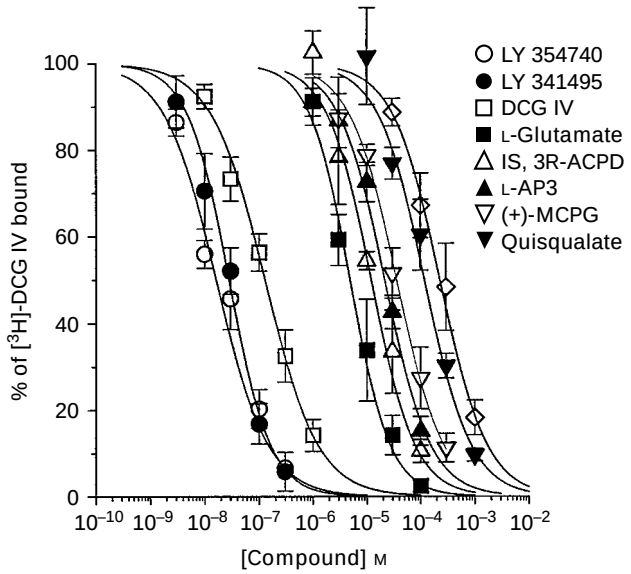

Figure 4 Inhibition of [^{3}H]-DCG IV binding on rat mGlu$_2$ receptor-transfected cell membranes by: LY 354740, LY 341495, DCG IV, L-glutamate, 1**S**,3**R**-ACPD, L-AP3, (+)-MCPG and quisqualate. Results are expressed as % of [^{3}H]-DCG IV specific bound and are the mean of three individual experiments, performed in triplicate. Vertical lines show s.d.

ACPD need only be 30 and 15%, respectively, to elicit the same magnitude of response.

Forskolin-stimulated cyclic AMP formation

Some of the compounds observed to inhibit [^{3}H]-DCG-IV binding and found to be active on GTPγ^{35}S binding were previously unknown as mGlu receptor ligands. Therefore, to confirm further their activity at mGlu$_2$ receptors, we tested a selection of these compounds in another functional assay: forskolin-stimulated cyclic AMP production in mGlu$_2$ receptor-transfected CHO cells.

DCG IV evoked potent, complete inhibition of forskolin (30 μM)-stimulated cyclic AMP formation in mGlu$_2$ receptor-transfected CHO cells, with an IC$_{50}$ value of 88$\pm$9 nM (Figure 8). Interestingly, NAAG also elicited full inhibition of the forskolin cyclic AMP response in mGlu$_2$ receptor-transfected CHO cells, despite being over three orders of magnitude less potent than DCG IV (IC$_{50}$ value, 205$\pm$73 μM; Figure 8). Furthermore, the NMDA antagonist, **R**-CPP, also acted as an agonist in the cyclic AMP functional assay. **R**-CPP completely inhibited the forskolin cyclic AMP response with an IC$_{50}$ value of 162$\pm$35 μM (Figure 8).

The putative mGlu receptor antagonist, L-AP3, and the NMDA receptor antagonist, L-AP5, were observed to be potent inhibitors of [^{3}H]-DCG IV binding and 1**S**,3**R**-ACPD-stimulated GTPγ^{35}S binding. In addition, both compounds reversed the inhibition of the forskolin response evoked by 10 μM 1**S**,3**R**-ACPD, in a concentration-dependent manner. At concentrations of 1 mM, L-AP3 and L-AP5 inhibited the effect of 1**S**,3**R**-ACPD by 42 and 31%, respectively (data not shown). However, despite being active in [^{3}H]-DCG IV and GTPγ^{35}S binding experiments, L-AP4 failed to affect inhibition of the forskolin response by 10 μM 1**S**,3**R**-ACPD in mGlu$_2$ receptor-expressing CHO cells. L-AP4 was without effect up to concentrations of 3 mM, even in the presence of the Na^+-dependent glutamate uptake blocker, L-THA (10μM) (data not shown).

Discussion

In recent years, a number of groups have used [^{3}H]-glutamate to study the radioligand binding properties of group II mGlu receptors in a variety of preparations. For example, Laurie *et al.* (1995) characterized the binding of [^{3}H]-glutamate to membranes of mGlu$_3$ receptor-transfected HEK cells. In addition, [^{3}H]-glutamate, in the presence of ionotropic glutamate receptor agonists, has been used to investigate native group II receptors in rat brain membranes (Schoepp & True, 1992; Wright *et al.*, 1994). However, the binding of glutamate to several non-receptor sites, and its active uptake by several membrane transporters, can result in ambiguous results when [^{3}H]-glutamate is used as a radioligand. The use of a selective group II mGlu receptor ligand would be preferable when binding to these receptors is being investigated. In contrast to the endogenous ligand, glutamate, at concentrations below 1 μM, DCG-IV is without activity at group I and III mGlu, and ionotropic glutamate receptors (Hayashi *et al.*, 1993) and is therefore, an ideal candidate for a selective group II mGlu receptor radioligand.

Table 2 EC$_{50}$ values, Hill coefficients and maximum effect for the stimulation of GTPγ^{35}S binding on rat mGlu$_2$ receptor-transfected cell membranes

Compounds	*EC$_{50}$ (μM)*	*Hill*	*Maximum (%)*
LY 354740	0.02$\pm$0.01	0.91$\pm$0.06	114$\pm$9
L-CCG I	0.08$\pm$0.02	1.17$\pm$0.26	92$\pm$11
DCG IV	0.2$\pm$0.004	0.92$\pm$0.06	78$\pm$10
L-Glutamate	1$\pm$0.4	0.9$\pm$0.1	95$\pm$7
1**S**, 3**R**-ACPD	1.3$\pm$0.4	1$\pm$0.3	106$\pm$12
1**S**, 3**S**-ACPD	5$\pm$1	1.3$\pm$0.3	118$\pm$2
L-CCG III	12$\pm$5	1.3$\pm$0.4	118$\pm$3
Quisqualate	22$\pm$9	2$\pm$0.6	35$\pm$3
NAAG	68$\pm$30	1$\pm$0.2	120$\pm$5
R-CPP	150$\pm$35	1.3$\pm$0.3	113$\pm$3

Values are mean$\pm$s.d. of the EC$_{50}$ values, Hill coefficients and maximum effect calculated from 3 individual experiments, performed in quadruplicate.

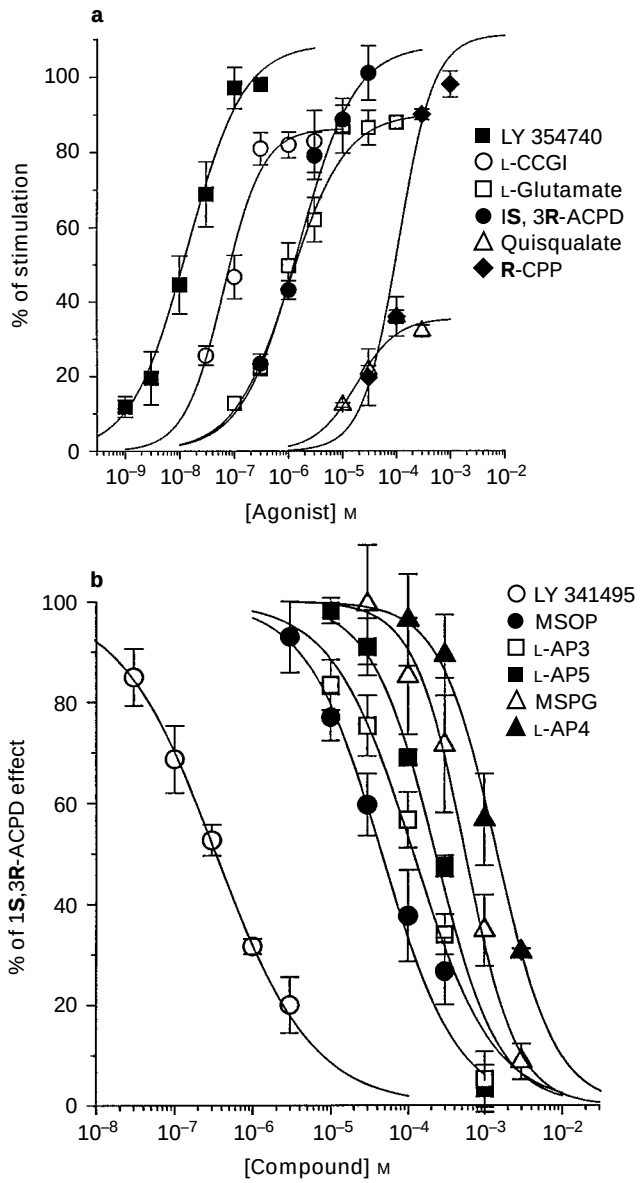

Figure 5 (a) Concentration-dependent stimulation of GTPγ^{35}S binding on rat mGlu$_2$ receptor-transfected cell membranes by LY 354740, L-CCG I, L-glutamate, 1**S**,3**R**-ACPD, quisqualate and **R**-CPP. Results are expressed as % above basal and are mean of three individual experiments, performed in quadruplicate. (b) Concentration-dependent inhibition of 1**S**,3**R**-ACPD (10 μM)-stimulated GTPγ^{35}S binding on rat mGlu$_2$ receptor-transfected cell membranes by LY 341495, MSOP, L-AP3, L-AP5, MSPG and L-AP4. Results are expressed as % of 1**S**,3**R**-ACPD-stimulated GTPγ^{35}S binding and are the mean of three individual experiments, performed in quadruplicate. Vertical lines show s.d.

Table 3 K_i values and Hill coefficients for the inhibition of 1**S**, 3**R**-ACPD (10 μM)-stimulated GTPγ^{35}S binding on rat $mGlu_2$ receptor-transfected cell membranes

Compounds	K_i (μM)	*Hill*
LY 341495	0.03±0.006	0.6±0.03
MSOP	7±2	0.7±0.2
L-AP3	11±3	0.8±0.04
MPPG	12±2	0.89±0.2
MAP4	15±3	1.3±0.2
(+)MCPG	15±2	1±0.3
L-AP5	22±1	1.3±0.2
MCCG	23±4	1.5±0.3
MTPG	41±14	1.4±0.4
MSPG	58±10	1.3±0.7
M3CMPG	64±22	1.5±0.3
EGLU	94±3	0.7±0.07
M3C4HPG	108±9	1.8±0.2
L-AP4	143±18	1.5±0.05

Values are mean±s.d. of the K_i values and Hill coefficients calculated from 3 individual experiments, performed in quadruplicate.

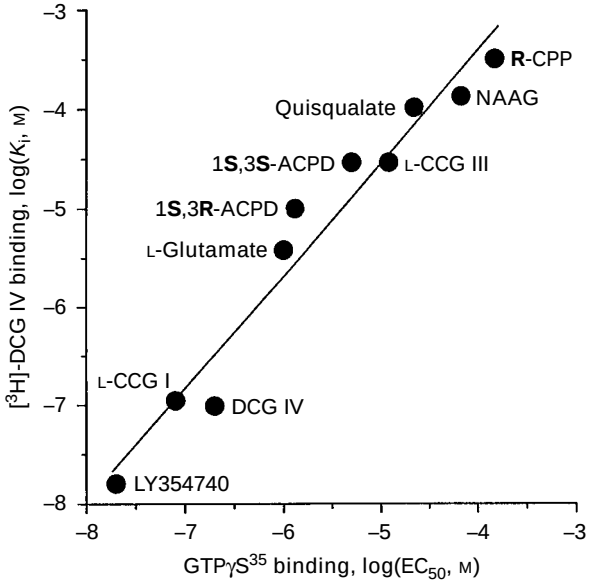

Figure 6 Correlation between agonist EC_{50} values to stimulate GTPγ^{35}S binding and K_i values to inhibit [^{3}H]-DCG IV binding on $mGlu_2$ receptor-transfected CHO cell membranes. Values were taken from Tables 1 and 2 and converted to their logarithm.

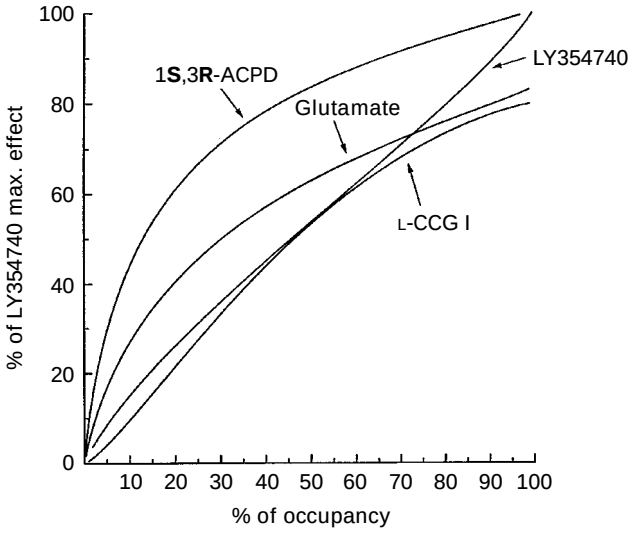

Figure 7 The relationship between % receptor occupancy and effect (as a % of LY 354740 maximal effect) for LY 354740, 1**S**,3**R**-ACPD, L-glutamate and L-CCG-I. % occupancy was calculated by use of the law of mass action and K_i values derived from [^{3}H]-DCG IV binding studies.

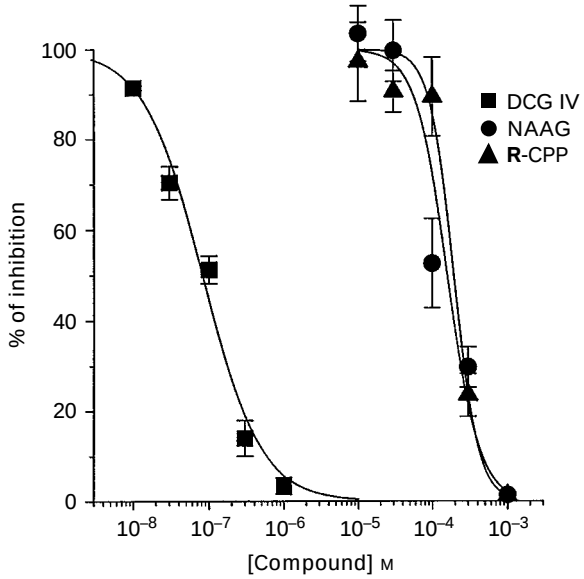

Figure 8 Inhibition by DCG IV, NAAG and **R**-CPP of forskolin-stimulated cyclic AMP production in $mGlu_2$ receptor-transfected CHO cells.

This study is the first to show [^{3}H]-DCG IV radioligand binding and indicates that the ligand is well suited for use in the characterization of group II mGlu receptor pharmacology. [^{3}H]-DCG IV binding was insensitive to the ionotropic glutamate receptor agonists, NMDA, AMPA and kainate, and to the Na^+-dependent uptake blockers, L-THA and DHKA, and Cl^--dependent glutamate binding inhibitors. Furthermore, binding was inhibited by GTPγS, confirming that, in $mGlu_2$ receptor-transfected CHO cells, [^{3}H]-DCG IV binding is to the G-protein-coupled $mGlu_2$ receptor. In contrast, Laurie *et al.* (1995) failed to obtained an effect of GTPγS on [^{3}H]-glutamate binding to membranes from $mGlu_3$ receptor-transfected HEK cells, the lack of effect of GTPγS possibly being due to the non-selectivity of [^{3}H]-glutamate binding. As the inhibition evoked by GTPγS in our study was incomplete, further experiments are necessary in order to determine if GTPγS causes a shift in affinity of [^{3}H]-DCG IV.

With regard to the agonists tested, there was a good correlation between the potencies of the agonists in both [^{3}H]-DCG IV binding and stimulation of GTPγ^{35}S binding. However, there was a difference in the potency of quisqualate, but this might be due to the partial agonist effect of the compound in the GTPγ^{35}S binding assay. Analysis of the receptor occupancy versus effect curves of a selection of the agonists suggests that 1**S**,3**R**-ACPD needs to occupy a smaller number of receptors in order to elicit the same size response. Therefore, in comparison with other agonists tested, 1**S**,3**R**-ACPD has a larger receptor reserve in this model (Figure 7). Being dependent on the total number of receptors, receptor reserve would be expected to vary between different expression systems. Furthermore, it would be interesting to compare the

results of the [^{3}H]-DCG IV binding experiments with those in which a radiolabelled antagonist is used.

Novel mGlu$_2$ receptor agonists were also identified by [^{3}H]-DCG IV and GTPγ^{35}S binding. In comparison with DGC IV, NAAG and **R**-CPP were much less potent, but evoked almost complete inhibition of the forskolin cyclic AMP response. Interestingly, Wroblewska *et al*. (1997) have recently claimed that NAAG is a selective agonist at mGlu$_3$ receptors. However, the results presented in this study, from functional tests of GTPγS binding and inhibition of the forskolin cyclic AMP responses, clearly show agonist activity of NAAG at the mGlu$_2$ receptor.

In agreement with a recent study by Sekiyama *et al*. (1996), these results demonstrate that the new phenylglycine derivatives, **RS**-M3CMPG and **RS**-M3C4HPG are both antagonists at mGlu$_2$ receptors, nonetheless, the compounds lack potency as their calculated K_i values for [^{3}H]-DCG IV and GTPγ^{35}S binding were greater than 100 μM. However, the K_i values of the putative mGlu receptor antagonist, MSOP, obtained from both [^{3}H]-DCG IV binding and GTPγ^{35}S binding studies, indicate that MSOP is a potent antagonist of mGlu$_2$ receptors. This high potency of MSOP at group II mGlu receptors is particularly interesting as Thomas *et al*. (1996) recently showed that MSOP reversed 1**S**,3**S**-ACPD-induced depression of the monosynaptic excitation of neonatal rat motoneurones, with a K_D value greater than 700 μM.

With the exception of MCCG, MAP4 and L-AP4, the K_i values for all the antagonists tested were found to be very similar in the functional GTPγ^{35}S assays in comparison with [^{3}H]-DCG IV binding. However, recent studies have indicated that MCCG and MAP4 also have agonist properties and are able to inhibit potently forskolin-stimulated cyclic AMP formation in adult rat cerebrocortical slices (Kemp *et al*., 1996). These 'additional' actions of MCCG and MAP4 might be the cause of the discrepancies between potencies observed in the two present studies. Gomeza *et al*. (1996) have also performed a functional analysis of mGlu$_2$ receptor antagonists and when the IC_{50} values obtained are converted into K_i values, by use of the Cheng and Prusoff equation, it is possible to compare the results with the data of the present study. Although many of the K_i values are similar between the studies, MAP4 appears to be approximately 10 fold more potent in the GTPγ^{35}S binding test in comparison with the potency derived from the study by Gomeza *et al*. (1996). Such a difference in potency might be explained by the uptake of MAP4 into the HEK 293 cells used in the latter study.

In addition to different potencies found in [^{3}H]-DCG IV binding and GTPγ^{35}S experiments, L-AP4 was without effect on the inhibition of forskolin-stimulated cyclic AMP formation by 1**S**,3**R**-ACPD in mGlu$_2$ receptor-transfected CHO cells. As L-AP4 inhibited both [^{3}H]-DCG IV and GTPγ^{35}S binding in membranes, we checked if the lack of effect of L-AP4 might be due to the compound being taken up into the CHO cells. However, even in the presence of Na^+-dependent uptake blocker, L-THA (10 μM), L-AP4 was ineffective up to a concentration of 3 mM. It is possible that L-AP4 is taken up into CHO cells by a Cl^--dependent uptake system, but this was not tested. It is odd that of the compounds active in the binding assays and subsequently tested on the cyclic AMP functional assay, L-AP4 was the only one without activity.

In contrast, both L-AP3 and L-AP5 were able to reverse 1**S**,3**R**-ACPD-elicited inhibition of the forskolin cyclic AMP response in mGlu$_2$-expressing CHO cells. To our knowledge, this is the first study of the activity of L-AP3 on the mGlu$_2$ receptor, and might help to explain previously published anomalies regarding the effects of the compound. At a concentration of 1 mM, AP3 has been shown to elicit only 20% inhibition of PI hydrolysis mediated via group I mGlu receptors expressed in CHO cells (Aramori & Nakanishi, 1992). However, Schoepp & Johnson (1989) showed that, in neonatal rat hippocampal slices, 1 mM AP3 produced over 80% inhibition of PI hydrolysis stimulated by the non-selective mGlu receptor agonist, ibotenate. Recently, it has been demonstrated that, in rat brain slices, co-stimulation of group II mGlu receptors can result in a potentiation of group I mGlu receptor-stimulated PI hydrolysis (Schoepp *et al*., 1996). Therefore, it is possible that in rat brain, L-AP3 directly reduces the PI response mediated via group I receptors, but also inhibits group II receptors and thus abolishes the synergistic cross-talk between the two subtypes of mGlu receptors. This would result in a far greater reduction of the apparent PI response in brain slices, in comparison with the limited inhibitory effects of L-AP3 observed in cells expressing the individual group I mGlu receptor clones.

This study is the first to describe the binding of the new radioligand, [^{3}H]-DCG IV, and furthermore, this is the first time that mGlu receptor ligand binding has been compared with functional studies. The data presented here show corresponding potencies for a large number of compounds tested in [^{3}H]-DCG IV and GTPγ^{35}S binding, and in addition, we have identified compounds previously unknown as mGlu$_2$ receptor ligands.

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