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Pharmacological characterization of type 1α metabotropic glutamate receptor-stimulated [^{35}S]-GTPγS binding

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1 The activation of G proteins by type 1α metabotropic glutamate receptors (mGluRs) in membranes from recombinant baby hamster kidney cells expressing the cloned rat mGluR1α receptor has been studied by use of a [^{35}S]-guanosine 5′-[γ-thio]triphosphate ([^{35}S]-GTPγS) binding assay.

2 L-Glutamate increased the rate of [^{35}S]-GTPγS binding in a concentration-dependent manner ($-\log EC_{50}$ (M) 5.25 ± 0.07), with an optimal ($62.4 \pm 1.6\%$) increase over basal binding being observed following 60 min incubation at 30°C with 70 pM [^{35}S]-GTPγS, 1 μM GDP, 10 mM $MgCl_2$, 100 mM NaCl and 100 μg membrane protein ml^{-1}. The L-glutamate (100 μM)-stimulated increase in [^{35}S]-GTPγS binding was totally prevented in the presence of the group I mGluR antagonist (**S**)-4-carboxy-3-hydroxyphenylglycine (300 μM).

3 Quantitative analysis of the affinity and number of G proteins activated by a maximally effective concentration of L-glutamate revealed an equilibrium dissociation constant (K_D) for [^{35}S]-GTPγS binding of 0.76 ± 0.20 nM and a maximal number of GTPγS-liganded G proteins (B_{max}) of 361 ± 30 fmol mg^{-1} protein.

4 Metabotropic glutamate receptor agonists, quisqualate ($-\log EC_{50}$ (M) 6.74 ± 0.06), 1**S**,3**R**-ACPD (4.64 ± 0.08) and (**S**)-3,5-dihydroxyphenylglycine (5.16 ± 0.23) also increased [^{35}S]-GTPγS binding in a concentration-dependent manner, with the latter two agents behaving as partial agonists.

5 (+)-α-Methylcarboxyphenylglycine (300 μM) caused a parallel rightward shift of the L-glutamate concentration-effect curve for [^{35}S]-GTPγS binding, allowing an antagonist equilibrium dissociation constant (K_D) of 34.0 ± 7.8 μM to be calculated for this mGluR antagonist.

6 Pretreatment of BHK-mGluR1α cells with a concentration of pertussis toxin (PTX) shown to be maximally effective (100 ng ml^{-1}, 24 h) before membrane preparation resulted in a marked decrease in agonist-stimulated [^{35}S]-GTPγS binding (by $66.0 \pm 0.9\%$), and an altered concentration-effect relationship for agonist-stimulated [^{35}S]-GTPγS binding by the residual PTX-insensitive G-protein population.

7 The modulation of [^{35}S]-GTPγS binding by agonists and antagonists in membranes from recombinant cells provides an excellent system in which to study mGluR interactions with PTX-sensitive and -insensitive G proteins.

Keywords: Metabotropic glutamate receptors (mGluR1α); G proteins; [^{35}S]-GTPγS binding; pertussis toxin

Introduction

Metabotropic glutamate receptors (mGluRs) are divided into three groups (Group I–III) based on their sequence homologies, the selectivity/potency of mGluR agonists and the signal transduction pathways to which each receptor couples preferentially (Nakanishi, 1994; Pin & Duvosin, 1995). Group I receptors (which includes the splice variants of mGluR1 and 5) stimulate phospholipase C-β (PLC-β) to generate inositol 1,4,5-trisphosphate and diacylglycerol (Abe *et al*., 1992; Aramori & Nakanishi, 1992; Thomsen *et al*., 1993; Joly *et al*., 1995), whilst Group II (mGluR2 and 3) and III (mGluR4, 6, 7 and 8) receptors are negatively coupled to adenylyl cyclase (Nakanishi, 1994; Pin & Duvosin, 1995). Increasing evidence suggests a diverse array of roles for mGluRs in the central nervous system (Schoepp & Conn, 1993; Nakanishi, 1994; Pin & Duvosin, 1995; Riedel, 1996), making this receptor family potentially important therapeutic targets in a variety of disease states, including acute and chronic neurodegenerative disorders (Knöpfel & Gasparini, 1996; Nicoletti *et al*., 1996).

Although mGluRs are believed to possess a seven-transmembrane topology and to exert their actions via linkage to G proteins, they share little or no sequence homology with other members of the G protein-coupled receptor (GPCR) superfamily (Pin & Duvosin, 1995). Thus, structural studies have demonstrated that the ligand binding site is located in the large N-terminal extracellular domain of mGluRs (O'Hara *et al*., 1993; Takahashi *et al*., 1993), contrasting with the transmembrane domain agonist-binding pocket identified for many other GPCRs which bind small hydrophilic ligands (Kobilka *et al*., 1988). Furthermore, recent evidence suggests that the critical element in determining information exchange between receptor and G protein is the second (rather than the third) intracellular loop of mGluRs (Pin *et al*., 1994; Gomeza *et al*., 1996). Such studies suggest that mechanisms involved in mGluR coupling to cellular effectors may differ from those so far delineated for other GPCRs.

Agonist stimulation of a GPCR results in interaction between the receptor and G protein facilitating α-subunit guanosine 5′-triphosphate (GTP) for guanosine 5′-diphosphate (GDP) exchange (Gilman, 1987) and allowing Gα-GTP to dissociate from the βγ subunit (Conklin & Bourne, 1993). This phenomenon can be used as a functional measure of receptor-mediated G protein activation, by utilizing the hydrolysis-resistant and slowly dissociating GTP analogue [^{35}S]-guanosine 5′-[γ-thio]triphosphate ([^{35}S]-GTPγS) in a membrane system. [^{35}S]-GTPγS binding has been widely employed to investigate the action of other GPCRs including muscarinic acetylcholine (Lazareno *et al*., 1993; Offermanns *et al*., 1994), A_1-adenosine (Lorenzen *et al*., 1993), μ-opioid (Traynor & Nahorski, 1995; Sim *et al*., 1995) and D_2-dopamine (Gardner *et al*., 1996). In the present study we have utilized this method to investigate mGluR1α-G protein activation in membranes prepared from

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baby hamster kidney (BHK) cells stably expressing recombinant mGluR1α (Pickering *et al*., 1993; Thomsen *et al*., 1993). A preliminary account of this work has been presented to the British Pharmacological Society (Akam *et al*., 1996).

Methods

Cell culture techniques

BHK cells stably expressing the rat type 1α mGluR (T45A clone; Pickering *et al*., 1993; Thomsen *et al*., 1993) were kindly provided by Dr C. Thomson (Novo Nordisk A/S, Måløv, Denmark) and were cultured in Dulbecco's modified Eagle's (Glutamax-1) medium supplemented with 5% dialysed foetal calf serum, G418 (0.5 mg ml^{-1}), gentamicin (50 μg ml^{-1}) and methotrexate (1 μM). Vector control cells (BHK-570; Pickering *et al*., 1993) were cultured without G418 or methotrexate, but in the presence of neomycin (0.1 mg ml^{-1}). Cells were maintained at 37°C in a humidified atmosphere (95% air, 5% CO_2) and were passaged every 4–5 days. Pertussis toxin pretreatment of the cells involved the addition of the toxin to the cell culture medium at a final concentration of 30–300 ng ml^{-1} and the cells were left for a further 24 h before membrane preparation.

Preparation of cell membranes

BHK-mGluR1α or BHK-570 cells were grown to confluence in 175 cm^3 flasks and then washed with 10 ml HBS (10 mM HEPES, 9% NaCl, 0.2% EDTA, pH 7.4). The HBS was removed and then a further 5 ml of HBS was placed in the flasks and left for approximately 15 min or until the cells had lifted. The cells were then removed from the flasks and centrifuged at 200 x*g* for 4 min to allow a loose pellet to form. The supernatant was discarded and 1 ml of wash buffer A (10 mM HEPES, 10 mM EDTA, pH 7.4) was added to the cells pelleted from one 175 cm^3 flask. The pellet was homogenized on ice by a polytron homogenizer (speed 5, 4×5 s bursts separated by approximately 30 s). The homogenate was centrifuged at 40,000 x*g* for 15 min at 4°C and the resulting supernatant was discarded. The pelleted membranes were homogenized and recentrifuged as described above in wash buffer B (10 mM HEPES, 0.1 mM EDTA, pH 7.4). The final pellet of cell membranes were resuspended in wash buffer B at a final concentration of 1 mg ml^{-1} and snap-frozen in liquid nitrogen for storage until required.

[^{35}S]-GTPγS radioligand binding assay

The binding assay was performed according to the method of Lazareno & Birdsall (1993), with slight modifications. The assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) contained 70 pM [^{35}S]-GTPγS (approx. 2×10^5 d.p.m. per assay tube), 1 μM GDP and appropriate additions of agonists/antagonists in a total volume of 900 μl. Incubations were started by addition of 100 μl of the BHK-mGluR1α membrane preparation (as above) and continued, unless otherwise stated for 60 min at 30°C. Assay 'blanks' were identical, except that 100 μl buffer rather than membranes was added. Assays were terminated to separate free and bound radioactivity by rapid vacuum filtration through pre-wetted GF/B filters by use of a Brandel Cell Harvester. Filters were washed with 2×5 ml of ice-cold assay buffer and semi-dry filters were then transferred to vials and 5 ml of scintilliant added. Samples were left to extract for > 12 h before radioactivity was determined by liquid scintillation counting. Total membrane binding was always < 20% of [^{35}S]-GTPγS added.

Data analysis

All data are presented as means ± s.e.mean for the indicated number of experiments. Agonist concentration-response curves for increases in [^{35}S]-GTPγS binding were analysed by non-linear regression by a commercially available programme (Prism, GraphPad Software, San Diego, CA, U.S.A.) and used to generate EC_{50} values. Antagonist equilibrium dissociation constants (K_D) were estimated in the absence and presence of a fixed concentration of antagonist assuming parallel log concentration-response curves by use of the equation:

$$K_D = A/(DR - 1)$$

where A is the antagonist concentration and DR represents the ration of the concentrations required to evoke 50% of maximal responses in the presence and absence of the antagonist. Statistical significance was assessed by Student's *t* test for unpaired observations.

Materials

[^{35}S]-GTPγS (specific activity 1000–1400 Ci $mmol^{-1}$) was purchased from NEN, DuPont. L-Glutamate, L-aspartate (all h.p.l.c. grade) were purchased from Fisons Fine Chemicals (Loughborough, U.K.). Quisqualic acid, guanosine 5′-diphosphate (GDP) and pertussis toxin were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). The four diastereoisomers of 1-aminocyclopentane-1,3-dicarboxylic acid (1**S**, 3**R**-, 1**S**, 3**S**-, 1**R**, 3**R**- and 1**R**, 3S-ACPD), (**S**)-3,5-dihydroxyphenylglycine (3,5-DHPG), (**S**)-4-carboxy-3-hydroxyphenylglycine (4C3HPG), (+)-α-methylcarboxyphenylglycine ((+)-MCPG), (−)-α-methylcarboxyphenylglycine ((−)-MCPG), *N*-methyl-D-aspartate (NMDA) and (**S**)-α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) were purchased from Tocris Cookson Ltd. (Langford, U.K.).

Results

Optimizing conditions for [^{35}S]-GTPγS binding to mGluR1α

Initial experiments examined the ability of L-glutamate to increase [^{35}S]-GTPγS binding and whether agonist-stimulated [^{35}S]-GTPγS binding to membranes expressing mGluR1α could be inhibited by the group I mGluR antagonist 4C3HPG. Figure 1 shows the time-courses of [^{35}S]-GTPγS binding in the absence and presence of 100 μM L-glutamate and the ability of 4C3HPG (300 μM) to prevent completely the agonist-stimulated increase. The inset to Figure 1 shows that the increase in [^{35}S]-GTPγS binding (expressed as a percentage increase-over-basal), stimulated by a maximally effective concentration of L-glutamate (300 μM), increased to a peak (41.6 ± 6.2%) at 60 min. No stimulation of [^{35}S]-GTPγS binding above basal levels was observed in incubations of membranes from vector-transfected BHK cells for up to 120 min (data not shown).

The initial experiments shown in Figure 1 were carried out in the presence of 100 mM NaCl and 10 μM GDP. Studies in tissues (Hilf *et al*., 1989) and cell lines (Lazareno *et al*., 1993) have shown that optimal responses for receptor-mediated [^{35}S]-GTPγS binding in membrane systems are critically dependent upon both GDP and salt concentration. Thus, to optimize the mGluR1α-mediated increase in agonist-stimulated [^{35}S]-GTPγS binding we investigated the effects of altering both GDP and NaCl concentrations. L-Glutamate (300 μM)-stimulated [^{35}S]-GTPγS binding was assessed for a range of GDP concentrations (0.1–10 μM) at high (100 mM) and low NaCl (10 mM) concentrations (Figure 2a). Optimal agonist-stimulated [^{35}S]-GTPγS binding (62.4 ± 1.6% increase over basal ($n = 3$)) was observed in the presence of 1 μM GDP and 100 mM NaCl, whilst lowering the concentration of NaCl to 10 mM substantially reduced the response in the presence of 1 μM GDP (15.4 ± 0.4% increase over basal ($n = 3$)). Thus, the assay conditions adopted for all further studies in membranes prepared from BHK-mGluR1α cells were 60 min incubations

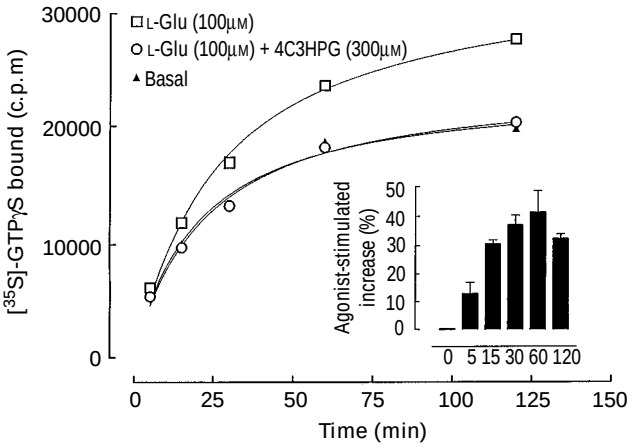

Figure 1 Time-courses of L-glutamate (L-Glu)-stimulated [^{35}S]-GTPγS binding to membrane preparations of BHK-mGluR1α cells. The main figure shows the time-courses of [^{35}S]-GTPγS binding following addition of glutamate; glutamate-plus-(**S**)-4-carboxy-3-hydroxyphenylglycine (4C3HPG) or vehicle (Basal). The inset shows % stimulation-over-basal obtained at the indicated times (in min) by comparison of [^{35}S]-GTPγS in the absence and presence of a maximally effective concentration of glutamate (300 μM). Data are shown as means±s.e.mean (vertical lines) for 3 (main Figure) or 5 (inset) separate experiments performed in duplicate.

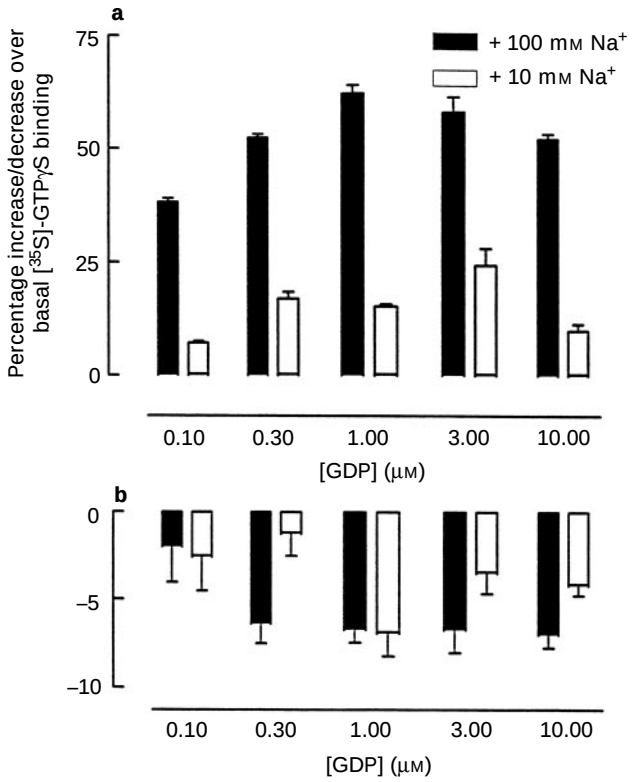

Figure 2 Effects of guanosine-5′-diphosphate (GDP) and Na^+ concentration on L-glutamate-stimulated, or 4C3HPG-inhibited [^{35}S]-GTPγS binding to membrane preparations of BHK-mGluR1α cells. Membranes were incubated with [^{35}S]-GTPγS in the presence and absence of (a) L-glutamate (300 μM), or (b) (**S**)-4-carboxy-3-hydroxyphenylglycine (4C3HPG, 300 μM) and the indicated concentrations of GDP in assay buffer containing either 10 mM or 100 mM NaCl for 60 min. Agonist/antagonist effects on [^{35}S]-GTPγS binding are expressed as % stimulation/inhibition-over-basal and are shown as means±s.e.mean for 4 separate experiments performed in duplicate.

at 30°C with 70 pM [^{35}S]-GTPγS, 1 μM GDP, 10 mM $MgCl_2$, 100 mM NaCl and 100 μg membrane protein ml^{-1}.

In parallel studies we investigated whether the group I mGluR antagonist 4C3HPG could suppress basal [^{35}S]-GTPγS binding. For a range of GDP and NaCl concentrations, 4C3HPG (300 μM) caused small decreases in basal [^{35}S]-GTPγS binding which never exceeded 8% (Figure 2b).

Pharmacological characterization of [^{35}S]-GTPγS binding

Although the increase in [^{35}S]-GTPγS binding stimulated by mGluR agonists in BHK-mGluR1α cell membranes is small, it is sufficient to investigate the actions of receptor agonists and antagonists at the level of G protein activation. The mGluR agonists quisqualate, L-glutamate, 3,5-DHPG and 1**S**,3**R**-ACPD all increased [^{35}S]-GTPγS binding in a concentration-dependent manner (Figure 3). At maximally effective concentrations both L-glutamate and quisqualate caused similar increases in [^{35}S]-GTPγS binding, whilst 3,5-DHPG and 1**S**,3**R**-ACPD appeared to behave as partial agonists with respect to this response. The apparent EC_{50} values for these agents are shown in Table 1, and are in approximate agreement with other studies where EC_{50} values were derived by use of either specific L-[^{3}H]-glutamate binding or total [^{3}H]-inositol phosphate measurements (Aramori & Nakanishi, 1992; Pickering *et al*., 1993; Thomsen *et al*., 1993; Desai *et al*., 1995). The lack of significant effects of a variety of other glutamate analogues, including 1**S**, 3**S**-, 1**R**, 3**R**-, and 1**R**, 3**S**-ACPD, are also shown in Table 1.

In addition to generating agonist potency ranking profiles, mGluR antagonist action was also investigated. In the presence of the mGluR antagonist (+)-MCPG (300 μM), a parallel rightward shift was observed in the concentration-response curve for L-glutamate-stimulated [^{35}S]-GTPγS binding (Figure 4). No shift of the concentration-response curve was observed in the presence of 300 μM (−)-MCPG (data not shown). The dose-ratio (11.7±2.1 (n=5)), for EC_{50} values obtained in the absence and presence of (+)-MCPG, was used

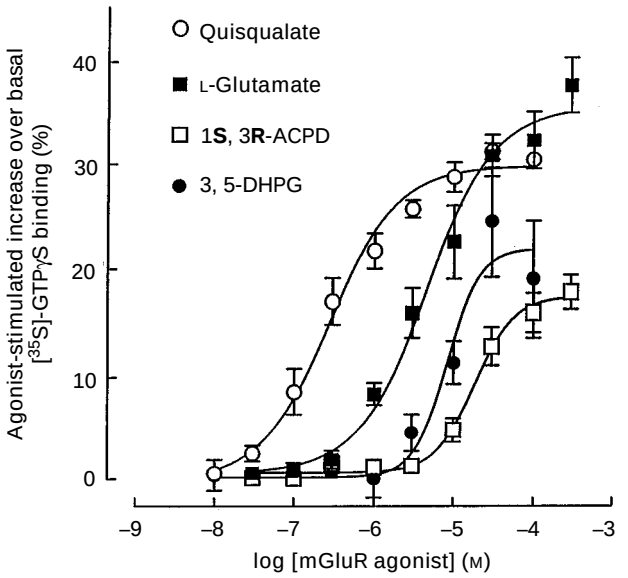

Figure 3 Pharmacological characterization of metabotropic glutamate receptor agonist-stimulated [^{35}S]-GTPγS binding to membrane preparations of BHK-mGluR1α cells. The indicated concentrations of quisqualate, L-glutamate, 1**S**, 3**R**-ACPD and (**S**)-3,5-DHPG were incubated with BHK-mGluR1α cell membranes and [^{35}S]-GTPγS under optimal assay conditions (see Methods). Data expressed as % stimulation-over-basal and presented as means±s.e.mean (vertical lines) for 3–6 independent experiments carried out in duplicate.

Table 1 Pharmacological characterization of [^{35}S]-GTPγS binding to membrane preparations of BHK-mGluR1α cells stimulated by a variety of agonists

Agonist	$-log\ EC_{50}$ (M)
L-Glutamate	5.25±0.07
Quisqualate	6.74±0.06
(**S**)-3,5-DHPG	5.16±0.23
1**S**,3**R**-ACPD	4.64±0.08
1**S**,3**S**-ACPD	N/A[1]
1**R**,3**R**-ACPD	N/A[1]
1**R**,3**S**-ACPD	N/A[1]
L-Aspartate	N/A[2]
L-Glutamine	N/A[2]
(**S**)-AMPA	N/A[2]
NMDA	N/A[2]

All incubations were carried out under optimal binding conditions (i.e. in the presence of 10 mM $MgCl_2$, 1 μM GDP and 100 mM NaCl) for 60 min at 30°C. Data are shown as −log EC_{50} values±s.e.mean for 3–5 separate experiments carried out in duplicate. N/A[1] <10% of L-glutamate activity at 300 μM; N/A[2] <10% of L-glutamate activity at 10 mM.

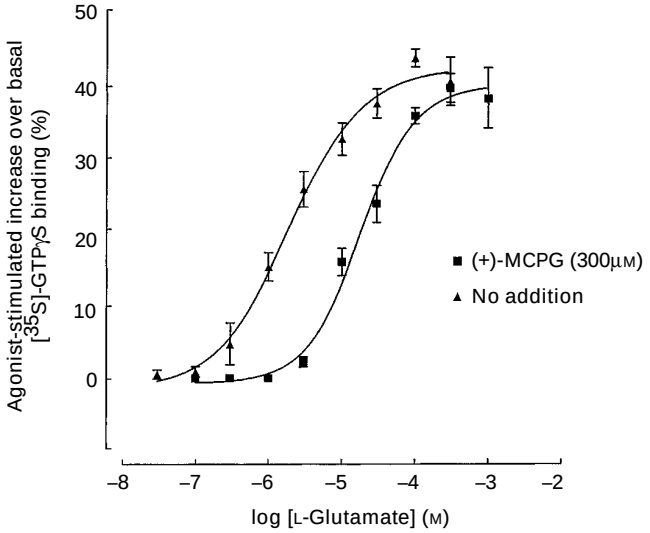

Figure 4 Antagonism of L-glutamate-stimulated [^{35}S]-GTPγS binding to membrane preparations of BHK-mGluR1α cells by (+)-α-MCPG. Concentration-response curves for glutamate-stimulated [^{35}S]-GTPγS binding was performed in the absence and presence of (+)-α-MCPG. Data expressed as % stimulation-over-basal and presented as means±s.e.mean (vertical lines) for 4 separate experiments performed in duplicate.

to calculate a K_D for (+)-MCPG (see Methods) of 34.0±7.8 μM.

Quantitation of G proteins activated by agonist stimulation

Quantitative estimates of the number of G proteins activated by agonist stimulation of mGluR were also made in BHK-mGluR1α membranes. Isotope-dilution experiments were performed where a fixed concentration of [^{35}S]-GTPγS (70 pM) and increasing concentrations of GTPγS were incubated with BHK-mGluR1α membranes in the absence and presence of 300 μM L-glutamate (Figure 5). The difference in [^{35}S]-GTPγS binding determined in the presence versus the absence of L-glutamate provides an estimate of the G protein receptor population activated by mGluR activation. Scatchard analysis of these data (Figure 5 inset) suggested that L-glutamate activates a population of G proteins with a K_D of 0.76±0.20 nM (n=3) and a B_{max} value for G protein activation of 361± 30 fmol mg^{-1} protein (n=3).

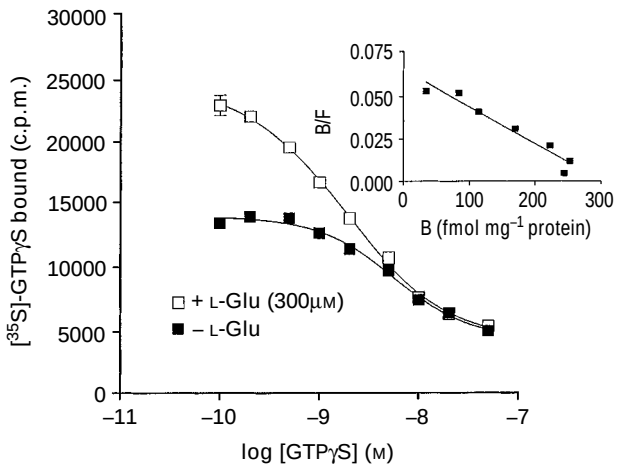

Figure 5 Isotope-dilution analysis of L-glutamate (L-Glu)-stimulated [^{35}S]-GTPγS binding to membrane preparations of BHK-mGluR1α cells. Membranes were incubated with a single concentration of [^{35}S]-GTPγS (70 pM) and increasing concentrations of unlabelled GTPγS (0.1–50 nM) in the absence and presence of glutamate. The inset shows a Scatchard plot of these data obtained from the difference between [^{35}S]-GTPγS binding in the absence and presence of glutamate. Data are shown as means±s.e.mean (vertical lines) for a representative experiment performed in triplicate. K_D and B_{max} values given in the text are derived from this and 2 further experiments.

Pertussis toxin effects on agonist-stimulated [^{35}S]-GTPγS binding

Pretreatment of BHK-mGluR1α cells with pertussis toxin (PTX; 30–300 ng ml^{-1}, 24 h), followed by subsequent harvesting of cells and membrane preparation, showed that maximal inhibition of agonist-stimulated [^{35}S]-GTPγS binding could be observed in the presence of 100 ng ml^{-1} (Figure 6a) and suggested that under control conditions mGluR1α interacts with both PTX-sensitive and -insensitive G proteins to elicit effector modulation. Thus, pretreatment with PTX *per se* reduced basal [^{35}S]-GTPγS binding by 60% (control, 173.6±4.2; +PTX, 69.6±0.4 fmol mg^{-1} protein; n=3). In addition, the maximal L-glutamate-stimulated increase in [^{35}S]-GTPγS binding was substantially attenuated (by 66.0±0.9%) in membranes from PTX-treated BHK-mGluR1α cells (Figure 6b), and the concentration-dependency of L-glutamate-stimulated [^{35}S]-GTPγS binding was significantly rightward shifted ($-\log EC_{50}$ values for the L-glutamate-stimulated response: control, 5.44±0.07; +PTX, 4.68±0.20 (n=4) (P<0.02).

Discussion

The present study is the first where the interactions of the group I metabotropic glutamate receptor mGluR1α with G proteins have been assessed directly by use of the [^{35}S]-GTPγS binding assay. This method allows agonist-stimulated guanine nucleotide exchange to be quantified with radiolabelled GTPγS, a hydrolysis-resistant GTP analogue (Kurose *et al.*, 1986; Hilf *et al.*, 1989), and therefore can provide information on the potency and efficacy of agonists to stimulate receptor-G protein coupling. Despite the radical differences, with respect to both ligand binding and receptor-G protein coupling (O'Hara *et al.*, 1993; Pin *et al.*, 1994; Gomeza *et al.*, 1996), between mGluRs and other members of the G protein-coupled receptor superfamily, preliminary experiments demonstrated that optimal conditions for L-glutamate-stimulated [^{35}S]-GTPγS binding (i.e. relatively long incubation times (30–60 min at 30°C), the presence of a low concentration of GDP

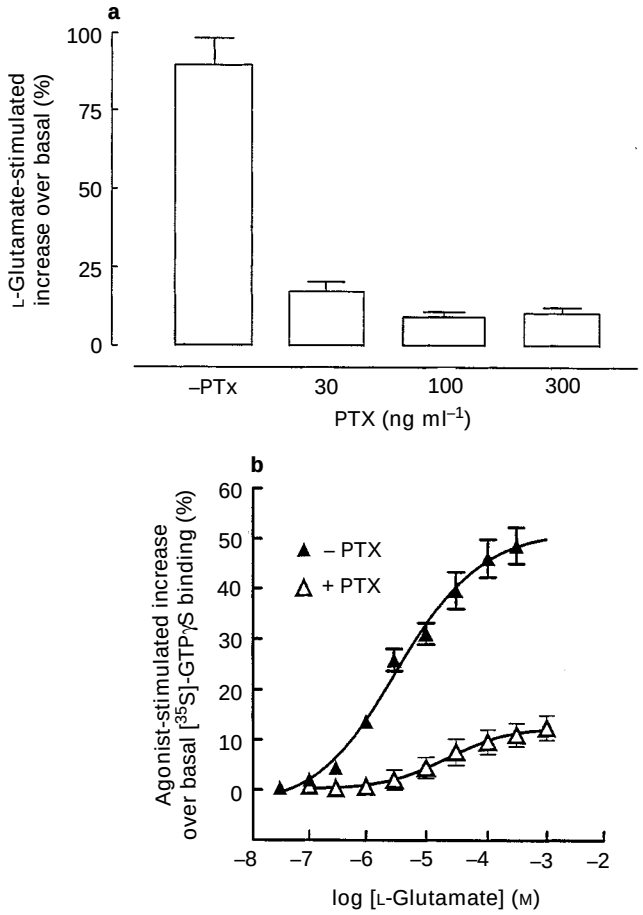

Figure 6 Effects of pertussis toxin (PTX) on L-glutamate-stimulated [^{35}S]-GTPγS binding to membrane preparations of BHK-mGluR1α cells. BHK-mGluR1α cells were treated with vehicle or PTX (0–300 ng ml^{-1} (a); or 100 ng ml^{-1} (b)) for 24 h before being harvested and cell membrane preparation. Data are expressed as % stimulation of [^{35}S]-GTPγS binding-over-basal and presented as means ± s.e.mean (vertical lines) for 3 (a) or 4 (b) separate experiments carried out in duplicate.

(1 μM) and a high salt concentration (100 mM NaCl)) were similar to those previously described for a variety of other G protein-coupled receptors (Hilf *et al*., 1989; Lazareno *et al*., 1993; Offermanns *et al*., 1994; Traynor & Nahorski, 1995; Gardner *et al*., 1996). The agonist-stimulated increase in [^{35}S]-GTPγS binding could be completely prevented by addition of the mGluR antagonist 4C3HPG. Although this antagonist completely annulled the effect of L-glutamate, it had little effect on basal [^{35}S]-GTPγS binding, suggesting either that mGluR1α expressed in BHK-cell membranes exhibits no constitutive activity or that 4C3HPG possesses no inverse agonist activity. A recent study supports the latter conclusion. Prézeau and colleagues have demonstrated that transient expression of mGluR1α (but not mGluR1β or mGluR1c splice variants) in LLC-PK1 cells increases basal, agonist-independent phosphoinositide turnover, but this constitutive activity of the type 1α receptor is not inhibited by 4C3HPG or two other mGluR antagonists (Prézeau *et al*., 1996).

Analysis of basal and L-glutamate-stimulated [^{35}S]-GTPγS binding in the presence of increasing concentrations of unlabelled GTPγS allowed estimation of the affinity of mGluR1α-activated G proteins for GTPγS ($K_D = 0.76 \pm 0.20$ nM) and the total number of G proteins activated by a maximally effective concentration of L-glutamate ($B_{max} = 361 \pm 30$ fmol mg^{-1} protein). For a number of other G protein-coupled receptor systems it has been possible to compare the agonist-stimulated increase in [^{35}S]-GTPγS binding with the receptor density derived from saturation analysis and, hence, obtain an estimate of the average number of G proteins activated by an agonist-liganded receptor (Hilf *et al*., 1989; Giershik *et al*., 1991; Traynor & Nahorski, 1995). Unfortunately, we were unable to obtain a reliable estimate of the receptor density of mGluR1α in BHK cells with L-[^{3}H]-glutamate, due to high non-specific binding and the presence of a non-receptor; L-[^{3}H]-glutamate binding site which may be the L-glutamate transporter present in these cells (Scott & Pateman, 1978; Thomsen *et al*., 1994b). Thomsen *et al*. (1993) have obtained a B_{max} value for mGluR1α of 840 fmol mg^{-1} protein with L-[^{3}H]-glutamate binding to a membrane preparation from this cell-line. However, a true quantitative comparison between mGluR1α density and G protein activation would require an estimate of what proportion of the cellular mGluR1α population is expressed at the plasma membrane, as immunofluorescence studies have clearly demonstrated that a substantial proportion of mGluR1α expressed in BHK T45A clone cells is localized to intracellular organelles (Pickering *et al*., 1993) and is, therefore, unlikely to be in an appropriate cellular sub-compartment for agonist activation and productive interaction with G proteins. Thus, the present study provides, for the first time, an accurate quantitative assessment of the magnitude of the immediate downstream signalling event following maximal agonist activation of receptor in BHK-mGluR1α cells, but estimation of the number of G proteins stimulated to undergo GTP for GDP exchange by agonist-activated mGluR1α will have to await superior methods for quantitation of cell-surface mGluR1α expression.

Although the maximal increase in [^{35}S]-GTPγS binding stimulated by L-glutamate was modest (~60% above basal), it did provide a satisfactory window in which pharmacological analysis of the functional effects of mGluR agonists and antagonists on G protein activation could be studied. The potencies of a number of mGluR agonists for stimulation of [^{35}S]-GTPγS binding to membranes prepared from BHK-mGluR1α cells were in agreement, both qualitatively and quantitatively, with those from previous studies in BHK-mGluR1α cells (Pickering *et al*., 1993; Thomsen *et al*., 1993; 1994b) and AV12 cells co-expressing human mGluR1α and a glutamate transporter (Desai *et al*., 1996), where stimulation of inositol phosphate accumulation was employed as an index of receptor-G protein-effector activation. However, a number of significant discrepancies between the present and previous studies do emerge. Thus, although there is a general consensus on the partial agonist activity of 1**S**, 3**R**-ACPD at mGluR1α compared to both L-glutamate and quisqualate, our data suggesting that 3,5-DHPG is also a partial agonist contrast with those of Desai *et al*. (1996). Also, our finding that the other three diastereoisomers of ACPD are essentially inactive contrasts with the finding of weak agonist activity of 1**S**, 3**S**-ACPD at mGluR1α by Thomsen *et al*. (1994b). However, it should be emphasized that a direct comparison of these studies may not be appropriate. The present study utilized [^{35}S]-GTPγS binding to membrane preparations, whereas Desai *et al*. (1996) and Thomsen *et al*. (1994b) measured phosphoinositide hydrolysis in whole cells. Thus, it is uncertain whether the same receptor-G protein complex(es) are involved in these studies (see below).

Antagonist activity could also be investigated with the [^{35}S]-GTPγS binding assay. The mGluR antagonist (+)-MCPG (300 μM) caused a significant parallel rightward-shift of the L-glutamate-stimulated [^{35}S]-GTPγS binding concentration-effect curve, whilst at a similar concentration the (−)-enantiomer of MCPG was without effect. The calculated K_D (34.0 ± 7.8 μM) for (+)-MCPG agrees with previous estimates of the activity of this antagonist at mGluR1α made by analysis of [^{3}H]-inositol phosphate data (Ferraguti *et al*., 1994; Hayashi *et al*., 1994; Thomsen *et al*., 1994a).

Another unanswered question regarding mGluR1α signalling relates to the association of the receptor with particular G proteins. The present study has revealed that in membranes prepared from BHK cells expressing mGluR1α, [^{35}S]-GTPγS binding is predominantly associated with PTX-sensitive G

proteins. These observations are in agreement with previous studies where [^{35}S]-GTPγS binding has been used to investigate coupling to PLC-β. Thus, human M_3- (and to a lesser extent M_1-) muscarinic cholinoceptors in membrane preparations from recombinant Chinese hamster ovary cells (Lazareno *et al*., 1993; Burford *et al*., 1995) and human embryonic kidney cells (Offermanns *et al*., 1994) stimulate [^{35}S]-GTPγS binding in a PTX-sensitive manner. We have attempted to investigate functional mGluR1α-$G_{i/o}$-coupling by measuring agonist-mediated effects on adenylyl cyclase activity in the presence of forskolin. Although unsuccessful in BHK-mGluR1α membranes, we have observed small, but significant inhibitions of forskolin-stimulated adenylyl cyclase activity in intact BHK-mGluR1α cells (e.g. quisqualate (0.1 μM) inhibits forskolin (10 mM)-stimulated cyclic AMP accumulation by 16±3% ($n=4$; $P<0.02$), whilst this inhibitory effect is lost in cells pretreated with PTX (100 ng ml^{-1};24 h): R.A.J. Challiss, R. Mistry & S.R. Nahorski, unpublished results). True quantitative assessment of the magnitude of the $G_{i/o}$-mediated inhibitory effect in these cells is not possible as at higher mGluR1α agonist concentrations any further inhibitory effects are obscured by a profound stimulation of adenylyl cyclase activity, consistent with previous findings (Aramori & Nakanishi, 1992; Thomsen, 1996). The ability of mGluR1α to associate with both PTX-sensitive and -insensitive G proteins could also be consistent with our recent findings that phosphoinositide hydrolysis mediated by this receptor may be under both positive (via $G_{q/11}$) and negative (via $G_{i/o}$) regulatory control (Carruthers *et al*., 1996; 1997). Although the apparent promiscuity of mGluR1α-G protein interactions may reflect a relative overexpression of receptors in these model cells, the quantitatively modest increase in [^{35}S]-GTPγS binding promoted by L-glutamate argues against this (see above). These issues may be addressed when suitable radioligand assays for mGluR1α (and mGluRs in general) have been developed.

In summary, the present studies provide the first characterization of mGluR1α-mediated guanine nucleotide exchange by use of the [^{35}S]-GTPγS binding assay. This method has provided new information on the activity of agonist and antagonist ligands at this level of transmembrane signalling and has indicated the possible association of this receptor with more than one G protein. It will now be important to integrate this information with other assessments of transduction coupling that have been made in intact cells.

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