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Metabotropic glutamate receptor mGlu2 is resistant to homologous agonist-induced desensitization but undergoes protein kinase C-mediated heterologous desensitization

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ABSTRACT

To investigate the susceptibility of the group II metabotropic glutamate receptor mGlu2 to agonist-induced desensitization, the receptor was stably expressed in Chinese hamster ovary (CHO-mGlu2) or C6 glioma cells (C6-mGlu2). Exposure of CHO-mGlu2 cells to the group II mGlu receptor agonist (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine (LCCG-1; 10 μM) for up to 15 h did not affect the subsequent ability of LCCG-1 to inhibit forskolin-stimulated cAMP accumulation. Similarly, in C6-mGlu2 cells, prolonged exposure to LCCG-1 also did not affect the subsequent ability of LCCG-1 to inhibit cAMP formation. In contrast, exposure of CHO-mGlu2 cells to the protein kinase C activator phorbol myristate acetate (PMA) suppressed the ability of LCCG-1 to inhibit cAMP formation. Using an *in vitro* model of group II mGlu receptor activity, the hemisected neonatal rat spinal cord preparation, the ability of the selective group II agonist (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate ((2*R*,4*R*)-APDC) to depress the fast component of the dorsal root-evoked ventral root potential (fDR-VRP) did not desensitize when applied for up to 2 h. Together these results indicate that in contrast to most G protein-coupled receptors, the mGlu2 receptor is resistant to agonist-induced homologous desensitization, and that *in vitro* data suggests that resistance to desensitization is a physiologically relevant property of this mGlu receptor subtype.

1. Introduction

The metabotropic glutamate receptors (mGlu receptors) are G protein-coupled receptors implicated in the modulation of both glutamatergic and non-glutamatergic transmission (Pin and Duvoisin, 1995; Schoepp et al., 1999; Ferraguti et al., 2008). Eight such receptors have been identified to date, classified on the basis of amino acid sequence similarity, pharmacology and signal transduction mechanisms. Those identified as the group I mGlu receptors (mGlu1 and mGlu5) couple via $G_{q/11}$ to phospholipase C activation, whilst those of group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7 and mGlu8) couple via $G_{i/o}$ proteins to inhibition of adenylyl cyclase activity. A number of these receptors are also reported to modulate ion channel activity (Conn and Pin, 1997). In native tissue, group II mGlu receptors act as presynaptic autoreceptors, inhibiting the release of glutamate (Flavin et al., 2000; Macek et al., 1998), GABA (Hayashi et al., 1993; Gereau and Conn, 1995; Salt and Eaton, 1995) and dopamine (Verma and Moghaddam, 1998; Feenstra et al., 1998). These effects appear to be mediated by a number of intracellular mechanisms, including the G protein-mediated inhibition of N-type Ca^{2+} channels (McCool et al., 1996) implicated in the control of transmitter release (Dunlap et al., 1994), and the activation of inwardly rectifying potassium conductances (Sharon et al., 1997) which contribute to the stabilization of the membrane potential. An oral prodrug, LY2140023, of the potent and selective group II mGlu receptor agonist, LY404039, has been shown to have efficacy for treatment of schizophrenia in a randomized phase II clinical trial, demonstrating the therapeutic relevance of group II mGlu receptor agonists (Patil et al., 2007).

Most G protein-coupled receptors are known to undergo agonist-dependent homologous desensitization, resulting either from prolonged or repeated exposure to agonist (Krupnick and Benovic, 1998; Premont and Gainetdinov, 2007; Kelly et al., 2008). The mechanisms commonly include phosphorylation of the receptor by second messenger-dependent or G protein-coupled receptor kinases (GRKs). For example, group I mGlu receptors are rapidly desensitized by both protein kinase C-dependent and GRK/arrestin-dependent mechanisms (Iacovelli et al., 2004; Mundell et al., 2003; Dhami and Ferguson, 2006; Ferraguti et al., 2008). Less is known about the susceptibility of group II mGlu receptors to agonist-induced desensitization, although a very recent study reported that mGlu2 coupling to the inhibition of adenylyl cyclase activity does not undergo GRK-mediated desensitization (Iacovelli et al., 2009). In the present study we have examined the ability of mGlu2 receptors, stably expressed in

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two different mammalian cell lines, to undergo agonist-induced desensitization. We find that the rat mGlu2 receptor does not undergo agonist-induced homologous desensitization but is instead susceptible to heterologous desensitization via a protein kinase C-dependent pathway.

2. Methods

2.1. Materials

Chinese hamster ovary (CHO) cells stably expressing the rat mGlu2 or the mGlu5 receptor were kindly provided by Professor S. Nakanishi (University of Kyoto, Japan). The rat mGlu2 cDNA in $pcDNA_3$ was kindly supplied by Dr A. Doherty and Professor J. Henley (University of Bristol, UK). (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine (LCCG-1), (*S*)-α-ethylglutamate (EGLU), (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate ((2*R*,4*R*)-APDC), (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ((*S*)-AMPA), (*S*)-3,5-dihydroxyphenylglycine ((*S*)-3,5-DHPG), (2 *S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) and (*R*)-2-amino-5-phosphonopentanoate (D-AP5) were obtained from Tocris Cookson, Bristol, UK. [^{3}H]cAMP (925 GBq mmol^{-1}) was from Amersham Life Science, Little Chalfont, UK. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum and cell culture plasticware were obtained from Life Technologies, Paisley, UK, whilst Superfect was from Qiagen, Crawley, UK. All other materials were obtained from Sigma-Aldrich, Dorset, UK.

2.2. Cell culture

CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 25 mM glucose, 1% (w/v) proline, 100 units/ml penicillin, 1 mg/ml streptomycin, 0.25 μg/ml amphotericin B, and 2 mM glutamine. C6 glioma cells were maintained in DMEM with 10% fetal bovine serum, supplemented with 100 units/ml penicillin and 1 mg/ml streptomycin, 2 mM glutamine and 50 μg/ml gentamycin. All cells were maintained at 37 °C in a humidified atmosphere containing 6% CO_2. Cells were passaged when the cell layer reached confluence, using a solution of 5 mg/ml porcine trypsin, 2 mg/ml EDTA to remove the cells from the surface of the flask.

2.3. Stable expression of the mGlu2 receptor in C6 glioma cells

The cDNA encoding the rat mGlu2 receptor, contained in the mammalian expression vector $pcDNA_3$, was transfected into the C6 glioma cell line using the transfection reagent Superfect. Cells for transfection were plated onto a 6-well plate 48 h before transfection, such that on the day of transfection they were approximately 70% confluent. Fifteen microlitres of Superfect was added to a mixture of 3 μg DNA and 300 μl of culture medium containing no serum or antibiotics. This mixture was incubated at room temperature for 10 min. During this period, cells were washed twice with phosphate buffered saline (PBS). One millilitre of culture medium containing serum and antibiotics was then added to the Superfect–DNA mixture, and this was then pipetted onto the cells, and incubated for 2 h. The culture medium was then removed and the cells washed with PBS, before fresh culture medium was applied. After 2 days, geneticin (300 μg/ml) was added to the culture medium to select positive transformants. A number of days after the addition of geneticin, surviving clones were picked and seeded onto a 24-well plate, and were assessed for mGlu2 receptor activity by a functional cyclic AMP assay (see below), assessing the ability of LCCG-1 to inhibit forskolin-stimulated cAMP accumulation.

2.4. Whole cell cAMP accumulation studies

Experiments were performed 24–48 h after seeding cells onto a 24-well plate, during which time the cell layer grew to approximately 80% confluence. At 1 h before the assay, complete growth medium was replaced with DMEM (0.5 ml/well) lacking fetal bovine serum and glutamine. Following this time, mGlu receptor agonists were applied directly to the medium in the well in the presence of both forskolin (a direct activator of adenylyl cyclase) and 250 μM (4-(3-butoxy-4-methoxy-benzyl)imidazolidin-2-one (Ro201724) as a phosphodiesterase inhibitor. Cells were incubated at 37 °C for 10 min and the reaction was terminated by addition of 20 μl of an ice-cold solution of 100% (w/v) trichloroacetic acid.

For desensitization experiments, CHO-$mGlu_2$ or C6-$mGlu_2$ cells were incubated for 2, 15 or 24 h in the presence of LCCG-1, glutamate or vehicle. The plate was then placed on ice and the cells washed twice with fresh ice-cold DMEM (0.5 ml/well). This was removed and replaced with DMEM at 37 °C (0.5 ml/well), before addition of forskolin/agonists/Ro201724 as above.

In most cases 50 μl of the supernatant was transferred to a fresh tube containing 50 μl of NaOH (1 M) and 200 μl of Tris (50 mM, pH 7.4) and EDTA (4 mM) (TE buffer). A further 100 μl of this mixture was transferred to a fresh tube containing 50 μl of TE buffer, 100 μl of [^{3}H]cAMP in TE buffer (about 20,000 counts per minute) and 100 μl of cAMP binding protein (to give a final concentration of ~750 μg of protein/ml, prepared from bovine adrenal cortex). Tubes containing 50 μl of cAMP (0.125–40 pmol) were used to construct a standard curve. After at least 90 min incubation at 4 °C, 200 μl of TE buffer containing charcoal (Norit GSX: 50 mg/ml final concentration) and bovine serum albumin (2 mg/ml final concentration) was added to each tube. After 15 min bound and non-bound [^{3}H]cAMP were separated by centrifugation at 2900×*g* for 15 min at 4 °C. The resulting supernatant was transferred into vials for liquid scintillation counting. The cAMP accumulation was expressed as pmol cAMP/mg protein in a 10 min incubation.

2.5. Western blotting and immunodetection

For the immunodetection of mGlu2 in cell lysates, CHO and C6 cells stably transfected with mGlu2 were plated onto poly-L-lysine-coated 60 mm dishes and grown to 90% confluence. The cells were washed with ice-cold PBS and harvested into ice-cold lysis buffer: 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 120 mM NaCl, 40 mM glycerol-2-phosphate, 1% Triton X-100, 0.5 mM sodium orthovanadate and the following proteinase inhibitors: 0.1 μM microcystin (Alexis, Exeter, UK), 4 mg/ml leupeptin, 4 mg/ml pepstatin A, 4 mg/ml antipain (all from Roche Diagnostics GmbH, Mannheim, Germany) and 4 mg/ml benzamidine, (Sigma-Aldrich, Gillingham, Dorset, UK). The lysed cells were spun at 17,600×*g* for 15 min at 4 °C and the pellet was discarded. The supernatants containing the mGlu2 receptor were diluted in SDS sample buffer and boiled at 95 °C for 4 min.

Rat brain tissue was obtained by decapitating adult male Wistar rats (130–170 g) obtained from Bantin and Kingman (Aldborough, Hull, UK). Experimental procedures were conducted in strict accordance with the UK Animals (Scientific Procedures) Act 1986, the University of Bristol ethical review document and with the European Community Council Directive (86/609/EEC).

Immunodetection of mGlu2 in rat brain, CHO-mGlu2 and C6-mGlu2 cells was carried out in plasma membrane fractions which were prepared as follows: Tissue from rat brain frontal cortex, hippocampus and caudate putamen and from CHO-mGlu2 or C6-mGlu2 cells was homogenized at 4 °C in ice-cold 20 mM Tris–HCl, 292 mM sucrose, pH = 7.4 buffer. Homogenates were centrifuged at 1,500×g for 20 min at 4 °C and the resulting supernatant was centrifuged at 17,500×*g* for 10 min at 4 °C to obtain the P2 fraction corresponding to plasma membrane fraction. Pellets were