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Characterization of metabotropic glutamate receptor-stimulated phosphoinositide hydrolysis in rat cultured cerebellar granule cells

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1 The pharmacology of excitatory amino acid (EAA)-stimulated phosphoinositide (PI) hydrolysis, monitored via [^{3}H]-inositol monophosphate accumulation, was investigated in primary cultures of rat cerebellar granule cells.

2 EAA-stimulated PI hydrolysis peaked after 4-5 days *in vitro* and subsequently declined.

3 The agonist order of potency was found to be (EC_{50}): L-quisqualic acid (Quis) (2 μM)$\gg$L-glutamate (50 μM)>(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD) (102 μM). L-Glutamate (E_{max}=873% of basal activity) elicited the largest stimulation of PI hydrolysis, whereas Quis (E_{max}=603%) and (1S,3R)-ACPD (E_{max}=306%) produced somewhat lower stimulations.

4 Several phenylglycine derivatives were found to be active in inhibiting 2 μM Quis-stimulated PI hydrolysis, in order of potency (IC_{50}): (S)-4-carboxy-3-hydroxyphenylglycine (41 μM)$\geq$(S)-4-carboxyphenylglycine (51 μM)$\gg$(+)-α-methyl-4-carboxyphenylglycine (243 μM).

5 Cultured cerebellar granule cells of the rat appear to have Group I mGluR pharmacology similar to that reported for cloned mGluR1 and provide an ideal system for investigating novel mGluR1 ligands in a native environment.

Keywords: Cultured cerebellar granule cells of rat; metabotropic glutamate receptor; (1S,3R)-ACPD; L-quisqualic acid; phosphoinositide hydrolysis; phenylglycine derivatives

Introduction

L-Glutamate is widely accepted as being the principal excitatory neurotransmitter in the mammalian brain (Monaghan *et al.*, 1989). Its receptors can be divided into two groups termed ionotropic and metabotropic. Ionotropic glutamate receptors are ligand-gated ion channels comprising N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate subtypes. Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors linked to multiple signal transduction pathways.

To date, eight mGluRs have been cloned in the rat and have been subdivided into three groups based on their second messenger coupling, amino acid sequences and agonist selectivities observed in transfected mammalian cell lines. Group I mGluRs (mGluR1 and 5) are coupled to phospholipase C and are potently activated by quisqualic acid (Quis). Both Group II (mGluR2 and 3) and Group III (mGluR 4, 6, 7 and 8) mGluRs are negatively coupled to adenylyl cyclase, with potent agonists being (2S, 1'S, 2'R)-α-(carboxycyclopropyl)-glycine (L-CCG-I) and L-2-amino-4-phosphonobutanoic acid (L-AP4), respectively (Nakanishi, 1992; Pin & Duvoisin, 1995). This classification is complicated further by the existence of mGluR splice variants for mGluR1 (a,b,c and e), mGluR4 (a and b) and mGluR5 (a and b) (Pin *et al.*, 1992; Tanabe *et al.*, 1992; Minakami *et al.*, 1993; Simoncini *et al.*, 1993; Pin & Duvoisin, 1995). Several diverse roles for mGluRs have been suggested including, hippocampal long-term potentiation (LTP) (Bashir *et al.*, 1993; Bortolotto *et al.*, 1994), cerebellar long-term depression (LTD) (Daniel *et al.*, 1992; Shigemoto *et al.*, 1994), neurotoxicity (McDonald *et al.*, 1993) and neuroprotection (Koh *et al.*, 1991; Bruno *et al.*, 1994; Copani *et al.*, 1995).

Recently, several phenylglycine derivatives have been reported to show discriminatory mGluR antagonist activity (Watkins & Collingridge, 1994; Roberts, 1995). In particular, the 4-carboxyphenylglycine congeners: (S)-4-carboxyphenylglycine ((S)-4CPG), (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4C3HPG) and (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) have proved to be active against glutamate-stimulated phosphoinositide (PI) hydrolysis in mGluR1a transfected cells (Hayashi *et al.*, 1994; Thomsen *et al.*, 1994). However, whereas both (+)-MCPG and (S)-4CPG were shown to be antagonists of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD)-stimulated PI hydrolysis in neonatal rat cerebrocortical slices, (S)-4C3HPG was found to be a weak agonist (Birse *et al.*, 1993; Eaton *et al.*, 1993).

Several excitatory amino acids (EAA's) have been shown to stimulate PI hydrolysis in cultured cerebellar granule cells (Nicoletti *et al.*, 1986; Suzdak *et al.*, 1993). These neurones are reported to contain high levels of mGluR1 mRNA, with negligible amounts of mGluR5 mRNA (Prézeau *et al.*, 1994; Santi *et al.*, 1994). Therefore, the aim of the present study was to investigate the pharmacology of native mGluR1 responses coupled to PI hydrolysis in primary granule cell cultures.

Methods

Cell culture

Cerebellar granule cells for culture were prepared from neonatal rat cerebellum according to methods previously described by Thangnipon *et al.* (1983) and Van Vliet *et al.* (1989). Cerebella were removed from 8-day-old Wistar rat pups, placed in phosphate-buffered saline (containing 33 mM glucose) and the meninges dissected free. Cerebella were then teased apart, incubated in versene (10 ml, 5 min) and then dissociated by use of a series of flame-narrowed Pasteur pipettes (diameter 1 to 0.2 mm). Dissociated cells were collected by centrifugation (500 *g*, 5 min) and re-suspended in basal Eagle's medium supplemented with foetal calf serum (10% v/v), KCl (25 mM), L-glutamine (4 mM), penicillin (50 u ml^{-1}) and streptomycin (50 μg ml^{-1}). The cell sus-

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pension was filtered (70 μm mesh) and plated at a density of 1.1×10^6 cells/well onto 12-well culture dishes which were previously coated with 5 μg ml^{-1} poly-L-lysine (mol. wt. $70-150 \times 10^3$). Cells were incubated at 37°C in a humidified atmosphere of 6% CO_2-94% air. Cytosine β-D-arabino-furanoside (10 μM) was added 20 h after plating to prevent glial cell proliferation.

Measurement of phosphoinositide hydrolysis

Phosphoinositide hydrolysis was determined by monitoring the accumulation of inositol monophosphate in the presence of LiCl (10 mM) in a modified protocol as described by Aronica *et al.* (1993a). Cultures were pre-labelled with 2 μCi ml^{-1} [^{3}H]-*myo*-inositol for 24 h. The culture medium was then removed and the cells washed extensively with buffer (composition, mM: NaCl 154, KCl 5.6, $MgSO_4$1.0, $NaHCO_3$ 3.6, glucose 5.6, $CaCl_2$ 1.3, HEPES 5.0, LiCl 10, pH 7.4). To each well, 500 μl of buffer, supplemented with 50 μM D-2-amino-5-phosphonopentanoic acid (D-AP5), 30 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 1 μM tetrodotoxin (TTX), was added. If required the antagonist was then added, immediately followed by the agonist, and incubated at 37°C for 30 min. The assay was terminated by aspiration of the buffer and the addition of ice-cold 500 μl $HClO_4$ (7.5% v/v) and left on ice for 15 min. The cell extract was then neutralized with Na_2CO_3 and [^{3}H]-inositol monophosphate ([^{3}H]-IP$_1$) was isolated via ion exchange chromatography and quantified by liquid scintillation spectrometry (Porter *et al.*, 1992).

Materials

Phenylglycine derivatives were either synthesized in house or, as with EAA analogues, obtained from Tocris-Cookson Ltd., Bristol. [^{3}H]-*myo*-inositol (15 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. Foetal calf serum, basal Eagle's medium, penicillin (10,000 u ml^{-1})/streptomycin (10,000 μg ml^{-1}) solution and versene were purchased from Gibco. Twelve well culture clusters were obtained from Costar. All other chemicals were obtained from Sigma.

Results

Agonist-stimulated PI hydrolysis

In order to eliminate involvement of ionotropic glutamate receptors and effects due to any released glutamate, all experiments were performed in the presence of 30 μM CNQX, 50 μM D-AP5 and 1 μM TTX, all of which have been reported not to have any direct effects, at these concentrations on PI hydrolysis (Nicoletti *et al.*, 1987; Van Vliet *et al.*, 1989; Gallo *et al.*, 1990; Suzdak *et al.*, 1993).

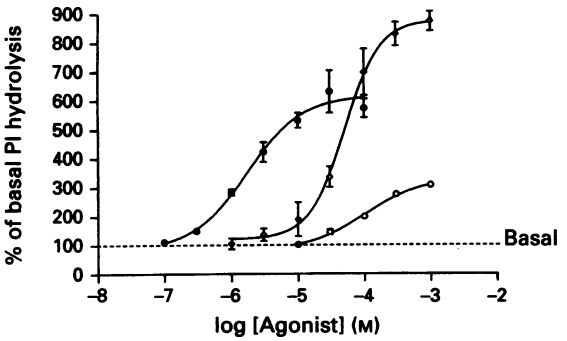

Figure 2 Concentration-dependent accumulation of [^{3}H]-inositol monophosphate in response to Quis (●), L-glutamate (◇) and (1S,3R)-ACPD (○) in rat cultured cerebellar granule cells (4–6 days *in vitro*). The data points represent the means (± s.e.mean) of 3 separate experiments performed in triplicate. For abbreviations, see text.

Figure 1 depicts the effects of culture time upon agonist-induced PI hydrolysis. In agreement with Aronica *et al.* (1993a), maximal responses were observed with each of the agonists (1S,3R)-ACPD, Quis and glutamate at approximately 4 days *in vitro* (DIV) with a decline thereafter. Subsequently, in all further experiments described, cells were used between 4–6 DIV.

The same agonists were tested for their ability to influence PI hydrolysis (Figure 2). Quis was found to be the most potent ($EC_{50} = 2 \pm 1.5$ μM), followed by L-glutamate ($EC_{50} = 50 \pm 3.8$ μM) and (1S,3R)-ACPD ($EC_{50} = 102 \pm 11.5$ μM). However, L-glutamate exhibited a much greater efficacy ($E_{max} = 873\%$ of basal; $n_H = 1.61$), than either Quis ($E_{max} = 603\%$; $n_H = 1.05$) or (1S,3R)-ACPD ($E_{max} = 306\%$; $n_H = 0.99$).

Effects of phenylglycine derivatives on Quis-stimulated PI hydrolysis

Since (1S,3R)-ACPD and L-glutamate are non-selective agonists for Group I mGluRs, Quis (in the presence of the AMPA/kainate antagonist, CNQX) was the agonist of choice for the following antagonist studies. Several phenylglycine compounds were tested for their ability to influence Quis (2 μM)-stimulated PI hydrolysis, with the 4-carboxy phenylglycine derivatives producing parallel inhibition curves (Figures 3 and

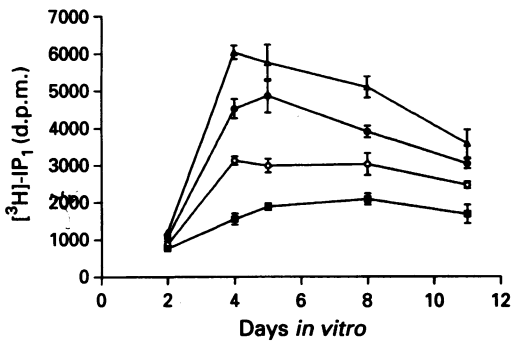

Figure 1 Developmental profile of basal (■), 100 μM (1S,3R)-ACPD-(○), 2 μM L-quisqualate-(●) and 50 μM L-glutamate- (△) stimulated [^{3}H]-inositol monophosphate accumulation in rat cultured cerebellar granule cells. The data points represent the means (± s.e.mean) of 3 separate experiments performed in triplicate. For abbreviations, see text.

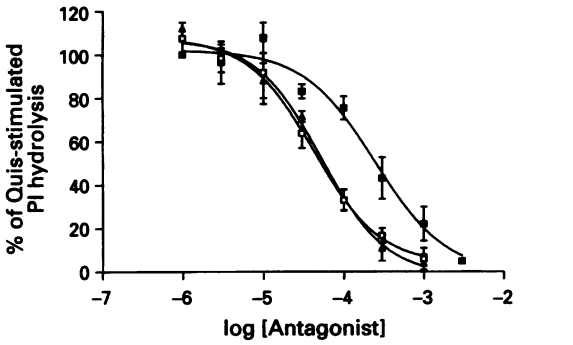

Figure 3 Concentration-dependent inhibition of 2 μM Quis-stimulated [^{3}H]-inositol monophosphate production by (S)-4C3HPG (□), (S)-4CPG (▲) and (+)-MCPG (■) in rat cultured cerebellar granule cells (4–6 days *in vitro*). The data points represent the means (± s.e.mean) of 3 separate experiments performed in triplicate. For abbreviations, see text.

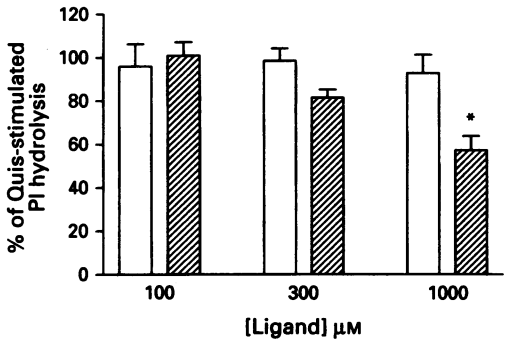

Figure 4 Effects of (RS)-4SPG (open column) and (RS)-4PPG (hatched columns) on 2 μM Quis-stimulated [^{3}H]-inositol monophosphate production in rat cultured cerebellar granule cells (4–6 days *in vitro*). The data points represent the means (± s.e.mean) of 4 separate experiments performed in triplicate. *$P<0.05$ 1 mM vs. 100 μM (RS)-4PPG as determined with Mann-Whitney U Test. For abbreviations, see text.

4). Of the compounds tested, the most effective were (in order of potency (IC_{50})): (S)-4C3HPG (41 ± 7.8 μM) ≥ (S)-4CPG (51 ± 2.9 μM) > (+)-MCPG (243 ± 62 μM). None of the 4-carboxyphenylglycine congeners stimulated PI hydrolysis (tested at 1 mM) (data not shown).

Recently, the inhibition of mGluR-mediated depression of dorsal root-evoked monosynaptic excitation by α-methyl-4-sulphonophenylglycine (MSPG) and α-methyl-4-phosphonophenylglycine (MPPG) has been described in neonatal rat motoneurones (Thomas *et al.*, 1995). This finding, coupled with data from Figure 3 (α-methyl introduction to 4CPG decreases antagonist potency), led us to investigate the effects of 4-sulphonophenylglycine ((RS)-4SPG) and 4-phosphonophenylglycine ((RS)-4PPG) on Quis-stimulated PI hydrolysis (Figure 4). However, both compounds proved to be less active than (+)-MCPG; (RS)-4SPG produced no significant difference up to 1 mM, whereas 1 mM (RS)-4PPG inhibited Quis-stimulated PI hydrolysis (57 ± 6% control, $P<0.05$).

Discussion

At present, our knowledge of mGluR molecular biology exceeds that of mGluR pharmacology. However, recent developments in the search for novel mGluR ligands (such as the discovery of phenylglycine derivatives) are redressing this imbalance. The present study is part of our ongoing investigations into mGluR pharmacology and was undertaken to evaluate the effects of several phenylglycine derivatives upon Quis-stimulated PI hydrolysis in a mammalian neuronal preparation.

The initial experiments demonstrated that the development of mGluR-mediated EAA-stimulated PI hydrolysis peaked at 4 DIV and then declined after 5 DIV. Aronica *et al.* (1993a, b) and others (Santi *et al.*, 1994) have attributed this decline to decreases in mGluR1 mRNA levels, possibly mediated via mGluR1-activated protein kinase C activity (Aronica *et al.*, 1993b; Bessho *et al.*, 1993).

Upon exposing the cells (4–6 DIV) to various EAA agonists and measuring the subsequent increases in PI hydrolysis, the following rank order of agonist potency was established: (Quis) ≫ L-glutamate > (1S,3R)-ACPD), with Quis and (1S,3R)-ACPD demonstrating lower efficacy than L-glutamate. The reason(s) why the L-glutamate concentration-effect curve yields a Hill coefficient significantly different from unity ($n_H = 1.61$) is unclear from this study. Although positive cooperativity cannot be excluded, other mGluR participation can be discounted, since a similar agonist profile has been reported for cloned mGluR1 (Aramori & Nakanishi, 1992). Conversely, the agonist profile seen in this study is markedly different from that described for cloned mGluR5 where Quis and *trans*-ACPD are reported to stimulate PI hydrolysis to similar extents (Abe *et al.*, 1992). However, our data differ from those of previous studies carried out using rat cerebellar granule cells where glutamate was found to be more potent than Quis (Nicoletti *et al.*, 1986).

It should be noted that the latter study was carried out on older cells (7–9 DIV), in the absence of ionotropic glutamate receptor antagonists and TTX. It is noteable that ionotropic glutamate receptor activation has been shown to influence PI hydrolysis (Nicoletti *et al.*, 1986; 1987). Indeed, a more recent study using mouse cultured cerebellar granule cells (with antagonists present) provided a similar agonist profile to that observed here, thereby indicating little species difference in mGluR1 pharmacology between rat and mouse cerebellar granule cells *in vitro* (Chavis *et al.*, 1994).

The rank order of potency of the 4-carboxyphenylglycine derivatives closely corresponded to data from mammalian cell lines transfected with mGluR1 (in order of potency: (S)-4C3HPG ≥ (S)-4CPG ≫ (+)-MCPG) (Hayashi *et al.*, 1994; Thomsen *et al.*, 1994). Clearly, the introduction of an α-methyl moiety into 4CPG to yield MCPG results in a decrease in antagonist potency in this system. Furthermore, substitution of the 4-carboxy moiety of 4CPG, to yield either 4-sulphono- or 4-phosphono-phenylglycine resulted in a virtual abolition of antagonist activity. This is in contrast to the inhibition of mGluR-mediated depression of dorsal root-evoked monosynaptic excitation of neonatal rat motoneurones where α-methyl phenylglycine derivatives α-methyl-4-sulphonophenylglycine (MSPG) and α-methyl-4-phosphonophenylglycine (MPPG) are found to be more potent antagonists than MCPG (Thomas *et al.*, 1995).

In summary, EAA-stimulated PI hydrolysis in rat cultured cerebellar granule cells closely resembles that for mouse cultured cerebellar granule cells and is consistent with mGluR1 being present in these neurones. Quis-stimulated PI hydrolysis can be antagonized by several 4-carboxyphenylglycine derivatives with (S)-4C3HPG being the most potent. However, alterations in either the 4-position acidic group (SPG and PPG) or the introduction of an α-methyl group in 4CPG, to yield MCPG, results in a marked decrease in antagonist potency.

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