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Structure-activity relationships of new agonists and antagonists of different metabotropic glutamate receptor subtypes

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1 We investigated the agonist and antagonist activities of 22 new phenylglycine and phenylalanine derivatives for metabotropic glutamate receptors (mGluRs) by examining their effects on the signal transduction of $mGluR_1$, $mGluR_2$ and $mGluR_6$ subtypes expressed in Chinese hamster ovary cells. This analysis revealed several structural characteristics that govern receptor subtype specificity of the agonist and antagonist activities of phenylglycine derivatives.

2 Hydroxyphenylglycine derivatives possessed either an agonist activity on $mGluR_1$/$mGluR_6$ or an antagonist activity on $mGluR_1$.

3 Carboxyphenylglycine derivatives showed an agonist activity on $mGluR_2$ but an antagonist activity on $mGluR_1$.

4 α-Methylation or α-ethylation of the carboxyphenylglycine derivatives converts the agonist property for $mGluR_2$ to an antagonist property, thus producing antagonists at both $mGluR_1$ and $mGluR_2$.

5 Structurally-corresponding phenylalanine derivatives showed little or no agonist or antagonist activity on any subtypes of the receptors.

6 This investigation demonstrates that the nature and positions of side chains and ring substituents incorporated into the phenylglycine structure are critical in determining the agonist and antagonist activities of members of this group of compounds on different subtypes of the mGluR family.

7 We also tested two α-methyl derivatives of mGluR agonists. (2S, 1'S, 2'S)-2-(2-Carboxycyclopropyl)glycine (L-CCG-I) is a potent agonist for $mGluR_2$ but α-methylation of this compound changes its activity to that of an $mGluR_2$-selective antagonist. In contrast, α-methylation of L-2-amino-4-phosphonobutyrate (L-AP4) results in retention of an agonist activity on $mGluR_6$. Thus, α-methylation produces different effects, depending on the chemical structures of lead compounds and/or on the subtype of mGluR tested.

Keywords: Metabotropic glutamate receptor; phenylglycine derivative; agonist and antagonist; receptor-expressing cell; receptor subtype

Introduction

Glutamate is a major excitatory neurotransmitter that plays a critical role in integrative brain function, the development of the nervous system, and neuronal cell survival and cell death (Watkins *et al.*, 1990; Choi & Rothman, 1990; Nakanishi, 1992; 1994; Bliss & Collingridge, 1993). Glutamate receptors in mammals are classified into two distinct groups (Nakanishi & Masu, 1994; Hollmann & Heinemann, 1994; Nakanishi, 1994). Ionotropic glutamate receptors are glutamate-gated cation channels and subdivided into the receptors for N-methyl-D-aspartate (NMDA) and the non-NMDA receptors for kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (Nakanishi & Masu, 1994; Hollmann & Heinemann, 1994). Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors that modulate the production of intracellular second messengers (Nakanishi, 1994; Pin & Duvoisin, 1995; Knöpfel *et al.*, 1995).

mGluRs consist of at least eight different subtypes and are classified into three groups on the basis of their sequence similarities, signal transduction mechanisms and agonist selectivities (Nakanishi, 1994; Pin & Duvoisin, 1995; Knöpfel *et al.*, 1995). $mGluR_1$ and $mGluR_5$ are coupled to the stimulation of the phosphatidylinositol (PI) hydrolysis/Ca^{2+} signal transduction and show a strong agonist selectivity to quisqualate (Nakanishi, 1994). All other six subtypes are linked to the inhibition of the adenosine 3':5'-cyclic monophosphate (cyclic AMP) cascade, but $mGluR_2$ and $mGluR_3$ potently react with *trans*-1-aminocyclopentane-1,3-dicarboxylate (ACPD) and (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I), whereas the remaining four subtypes effectively respond to L-2-amino-4-phosphonobutyrate (L-AP4). (Nakanishi, 1994; Duvoisin *et al.*, 1995). The different subtypes of the mGluR family are widely and distinctly expressed in various neuronal and glial cells and have been implicated in the specialized roles in synaptic transmission, modulation and plasticity (Schoepp & Conn, 1993; Nakanishi, 1994). However, the functions of individual mGluR subtypes in different brain regions still largely remain to be clarified mostly due to the difficulty in specifying the functions of different mGluR subtypes in glutamate transmission. Recently, synthetic glutamate analogues derived from different lead compounds have been developed as new agonists and antagonists of different mGluR subtypes. These include a series of phenylglycine derivatives (Birse *et al.*, 1993; Eaton *et al.*, 1993a,b; Jane *et al.*, 1993a; Kemp *et al.*, 1994c; Hayashi *et al.*, 1994; Watkins & Collingridge, 1994) and 2-(carboxycyclopropyl)glycine derivatives (Ishida *et al.*, 1990; 1993; Hayashi *et al.*, 1992; 1993).

In our previous study (Hayashi *et al.*, 1994), we reported the agonist and antagonist potencies and specificity of five pairs of stereoisomers of phenylglycine derivatives as assessed by clonal Chinese hamster ovary (CHO) cell lines expressing $mGluR_1$, $mGluR_2$ and $mGluR_4$ subtypes. These clonal cell lines can avoid any ambiguity derived from cross-reactivity with different mGluR subtypes and are very useful in determining the precise subtype specificity and potencies of test compounds. In

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this investigation, we examined the agonist and antagonist activities of 22 new phenylglycine and phenylalanine derivatives in CHO cells expressing individually $mGluR_1$, $mGluR_2$ and $mGluR_6$ to extend our analysis of the structure-activity relationships of these compounds. In addition, we examined two α-methyl derivatives of mGluR agonists, (S)-2-amino-2-methyl-4-phosphonobutyrate (MAP4) and (2S, 1'S, 2'S)-2-(2-carboxycyclopropyl)alanine (MCCG), which have been reported to antagonize L-AP4-sensitive and ACPD-sensitive mGluR subtypes, respectively (Jane *et al.*, 1994; Cao *et al.*, 1995; Salt & Eaton, 1995; Bushell *et al.*, 1995). On the basis of the characterization of the above new compounds, together with our previous analysis of the phenylglycine derivatives, we discuss the structure-activity relationships of agonists and antagonists for different subtypes of the mGluR family.

Methods

Materials

The compounds used in this study are abbreviated as indicated in parentheses (see also Table 1) and were chemically synthesized as follows: (**RS**)-3-carboxy-5-hydroxyphenylglycine (3C5HPG), (**RS**)-3,4-dicarboxyphenylglycine (3,4-DCPG), (**RS**)-5-carboxy-2-hydroxyphenylglycine (5C2HPG), (**RS**)-4-carboxy-2-iodophenylglycine (4C2IPG), (**RS**)-2-chloro-3-hydroxyphenylglycine (2Cl3HPG), (**RS**)-4-chloro-3-hydroxyphenylglycine (4Cl3HPG), (**RS**)-2,6-dichloro-3-hydroxyphenylglycine (2,6-DCl3HPG), (**RS**)-4,6-dichloro-3-hydroxyphenylglycine (4,6-DCl3HPG), (**RS**)-4-chloro-3,5-dihy-droxyphenylglycine (4Cl-3,5-DHPG), (**RS**)-3-carboxymethyl-phenylglycine (3CMPG) and (**RS**)-3,4,5-trihydroxyphenylglycine (3,4,5-THPG) were synthesized from the corresponding substituted benzaldehydes by the Strecker reaction (a variation of the method of Steiger, 1955). The intermediate aminonitriles were hydrolyzed in either 6N HCl or concentrated HBr, purified by ion-exchange chromatography and crystallized from an appropriate solvent. The α-alkyl phenylglycines, (**RS**)-α-methyl-3-carboxy-4-hydroxyphenyl glycine (M3C4HPG), (**RS**)-α-ethyl-4-carboxyphenylglycine (ECPG), (**RS**)-α-methyl-3-carboxymethylphenylglycine (M3CMPG), (**RS**)-α-methyl-4-carboxy-3-chlorophenylglycine (M4C3ClPG), (**RS**)-α-methyl-2-bromo-4-carboxyphenyl glycine (M2Br4CPG) and (**RS**)-α-methyl-4-carboxy-3-hydroxyphenylglycine (M4C3HPG), were synthesized from the corresponding acetophenones by the Bucherer-Berg reaction (a variation of the method of Henze & Long, 1941) followed by hydrolysis of the intermediate hydantoins in 6N HCl, purification by ion-exchange chromatography and crystallization from an appropriate solvent. (**S**)-MAP4, MCCG, (**S**)-1α-methyl-3-carboxyphenylalanine (M3CPA) and (**S**)-3-carboxy-2-hydroxyphenylalanine hydrochloride (3C2HPA) were prepared according to the procedures reported previously (Jane *et al.*, 1993b; Jane & Watkins, 1993). (**RS**)-3,4-Dicarboxyphenylalanine (3,4-DCPA) and (**RS**)-3-carboxy-4-chlorophenylalanine (3C4ClPA) were obtained by reaction of the sodium salt of diethyl acetamidomalonate with the appropriately substituted benzyl halides. (**S**)-6-Fluoro-3-hydroxyphenylglycine (6F3-HPG) and (**S**)-3,5-dihydroxyphenylglycine (3,5-DHPG) were prepared from the appropriate phenylacetic acid derivatives by enantioselective synthesis using the method of Evans *et al.* (1987). (**S**)-3,5-DHPG, (**RS**)-4Cl-3,5-DHPG and (**RS**)-3,4,5-THPG were dissolved in phosphate-buffered saline solution at desired concentrations in each experiment to avoid their decomposition. (**S**)-MAP4, MCCG, (**RS**)-M3C4HPG, (**RS**)-M3CMPG, (**S**)-M3CPA, (**RS**)-3C5HPG and (**RS**)-4,6-DCl3HPG were dissolved in an equivalent of NaOH solution. (**S**)-3C2HPA hydrochloride and (**RS**)-3,4-DCPG were dissolved in 2 equivalents of NaOH solution. All the rest were dissolved in 1.1 equivalents of NaOH solution. The stock solutions were made at 50 or 100 mM and stored at −20°C. All compounds were used after pH adjustment.

Measurements of PI hydrolysis and cyclic AMP formation

Clonal CHO cell lines expressing individually $mGluR_1$, $mGluR_2$, $mGluR_6$ and endothelin receptor ET_A were prepared as described previously (Aramori & Nakanishi, 1992a, b; Tanabe *et al.*, 1992; Nakajima *et al.*, 1993). The agonist and antagonist activities of test compounds on $mGluR_1$ were determined by measuring total inositol phosphate (IP) formation as described previously (Aramori & Nakanishi, 1992b; Hayashi *et al.*, 1992; 1994). The agonist and antagonist activities of test compounds on $mGluR_2$ and $mGluR_6$ were determined by measuring changes in level of the forskolin-stimulated cyclic AMP formation in $mGluR_2$-expressing and $mGluR_6$-expressing cells, respectively, as described previously (Hayashi *et al.*, 1992; 1994; Tanabe *et al.*, 1992; Nakajima *et al.*, 1993). Measurements of second messengers in endothelin receptor ET_A-expressing cells and cells transfected with vector DNA alone were performed with the same procedures as described above. All measurements of PI hydrolysis and cyclic AMP formation were carried out at least twice in triplicate determinations.

Statistical analysis

Statistical differences were checked by Student's *t* test for unpaired data and by analysis of variance with Williams multiple-range test.

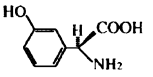
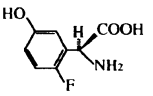
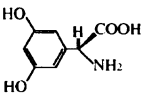
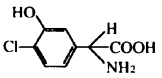
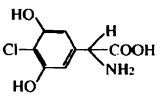
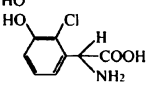
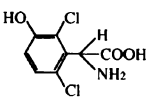
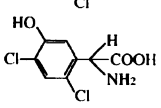
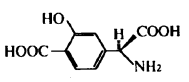
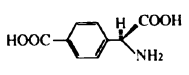
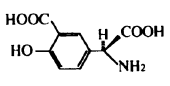
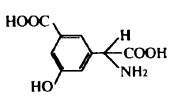
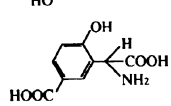
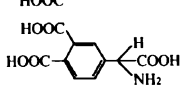
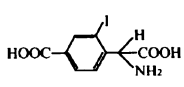
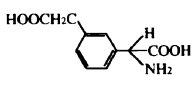
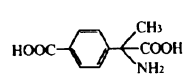
Results

Agonist activities of various test compounds on $mGluR_1$, $mGluR_2$ and $mGluR_6$

The chemical compounds we tested in this investigation consisted of 18 phenylglycine and 4 phenylalanine derivatives, together with the α-methyl derivatives of L-AP4 and L-CCG-I, (S)-MAP4 and MCCG, respectively. The new phenylglycine and phenylalanine derivatives were designed and synthesized on the basis of the chemical structures of 5 phenylglycine derivatives which have been shown to exhibit distinct agonist or/and antagonist activities on different mGluR subtypes (Hayashi *et al.*, 1994). These compounds were modified by variation of the substituents on the phenyl ring and the position, length and α-alkyl substitution of the amino acid side chains. They can be classified into several groups according to the chemical structures of the lead compounds used: seven non-carboxyl containing 3-hydroxyphenylglycine derivatives, five carboxyphenylglycine derivatives with or without hydroxyl groups, six α-methyl or α-ethylphenylglycine derivatives and four phenylalanine derivatives (Table 1). To test the agonist and antagonist activities of different compounds, we chose $mGluR_1$, $mGluR_2$ and $mGluR_6$ as the representative receptor subtypes of the mGluR family and examined the effects of the test compounds on the signal transduction characteristic of the respective receptor subtypes. These three subtypes were stably expressed in CHO cells and provided reproducible responses to test compounds in IP formation ($mGluR_1$) or cyclic AMP formation ($mGluR_2$ and $mGluR_6$).

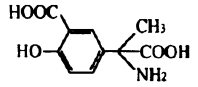
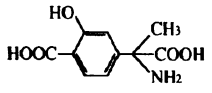
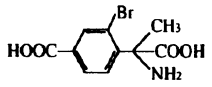
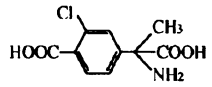
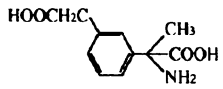
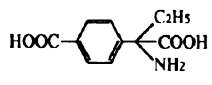
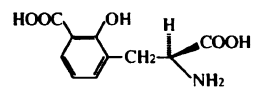
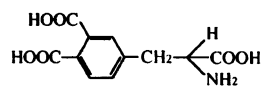
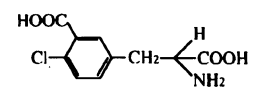
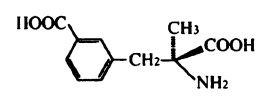
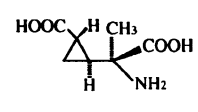
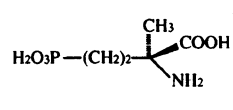
We first tested the agonist activities of the 24 compounds for each of the three mGluR subtypes by adding a constant concentration (1 mM) of the test compounds to receptor-expressing cells. The data obtained in this analysis are shown in Figure 1 and summarized in Table 1. In $mGluR_1$-expressing cells, L-glutamate (1 mM) induced about 6 fold increase in total IP formation above control levels (Figure 1a). Among the 24 compounds tested, (S)-6F3HPG, (S)-3,5-DHPG, (RS)-4C13HPG and (RS)-4Cl-3,5-DHPG showed agonist activities on $mGluR_1$, but none of these compounds elicited a full agonist activity comparable to that of L-glutamate. No other compounds showed any agonist activity on $mGluR_1$ (Figure 1a). In $mGluR_2$-expressing cells, L-glutamate (1 mM) inhibited

Table 1 Summary of agonist and antagonist potencies of the test compounds on $mGluR_1$, $mGluR_2$ and $mGluR_6$ expressed individually in CHO cells

Name	*Structure*	*$mGluR_1$*		*$mGluR_2$*		*$mGluR_6$ (or $mGluR_4$)*	
Hydroxyphenylglycine derivatives		*Agonist*	*Antagonist*	*Agonist*	*Antagonist*	*Agonist*	*Antagonist*
(S)-3-Hydroxyphenylglycine* (3HPG)		+	ND	–	–	–	–
(S)-6-Fluoro-3-hydroxyphenylglycine (6F3HPG)		+	ND	–	–	–	–
(S)-3,5-Dihydroxyphenylglycine (3,5-DHPG)		++	ND	–	–	+	ND
(RS)-4-Chloro-3-hydroxyphenylglycine (4Cl3HPG)		+	ND	–	–	+	ND
(RS)-4-Chloro-3,5-dihydroxyphenylglycine (4Cl-3,5-DHPG)		+	ND	–	–	++	ND
(RS)-2-Chloro-3-hydroxyphenylglycine (2Cl3HPG)		–	+	–	–	–	–
(RS)-2,6-Dichloro-3-hydroxyphenylglycine (2,6-DCl3HPG)		–	+	–	–	–	–
(RS)-4,6-Dichloro-3-hydroxyphenylglycine (4,6-DCl3HPG)		–	++	+	ND	–	–
Carboxyphenylglycine derivatives (S)-4-Carboxy-3-hydroxyphenylglycine* (4C3HPG)		–	+++ (3×10^{-5} M)	+++	ND	–	–
(S)-4-Carboxyphenylglycine* (4CPG)		–	+++ (4×10^{-5} M)	++	ND	–	–
(S)-3-Carboxy-4-hydroxyphenylglycine* (3C4HPG)		–	++ (4×10^{-4} M)	+++	ND	–	–
(RS)-3-Carboxy-5-hydroxyphenylglycine (3C5HPG)		–	+++	+++	ND	++	ND
(RS)-5-Carboxy-2-hydroxyphenylglycine (5C2HPG)		–	++	–	–	–	–
(RS)-3,4-Dicarboxyphenylglycine (3,4-DCPG)		–	+++	+	ND	++	ND
(RS)-4-Carboxy-2-iodophenylglycine (4C2IPG)		–	+++ (9×10^{-5} M)	++	ND	–	–
(RS)-3-Carboxymethylphenylglycine (3CMPG)		–	–	–	–	–	–
α-Methyl and α-ethylphenylglycine derivatives (+)-α-Methyl-4-carboxyphenylglycine* (MCPG)		–	+++ (7×10^{-5} M)	–	+++ (4×10^{-4} M)	–	–

(continued)

Table 1 (continued)

Name	*Structure*	*mGluR$_1$*		*mGluR$_2$*		*mGluR$_6$ (or mGluR$_4$)*	
		Agonist	*Antagonist*	*Agonist*	*Antagonist*	*Agonist*	*Antagonist*
(RS)-α-Methyl-3-carboxy-4-hydroxyphenylglycine (M3C4HPG)		–	+	–	+	–	–
(RS)-α-Methyl-4-carboxy-3-hydroxyphenylglycine (M4C3HPG)		–	++ (4×10^{-4} M)	–	++ (6×10^{-4} M)	–	–
(RS)-α-Methyl-2-bromo-4-carboxyphenylglycine (M2Br4CPG)		–	+	–	–	–	–
(RS)-α-Methyl-4-carboxy-3-chlorophenylglycine (M4C3ClPG)		–	+	–	–	–	–
(RS)-α-Methyl-3-carboxymethylphenylglycine (M3CMPG)		–	+	–	+	–	–
(RS)-α-Ethyl-4-carboxyphenylglycine (ECPG)		–	++	–	++	–	–
Phenylalanine derivatives (S)-3-Carboxy-2-hydroxyphenylalanine (3C2HPA)		–	+	–	–	–	–
(RS)-3,4-Dicarboxyphenylalanine (3,4-DCPA)		–	+	–	–	–	–
(RS)-3-Carboxy-4-chlorophenylalanine (3C4ClPA)		–	–	–	–	–	–
(S)-α-Methyl-3-carboxyphenylalanine (M3CPA)		–	–	–	–	–	–
MCCG & MAP4 (2S,1'S,2'S)-2-(2-Carboxycyclopropyl)alanine (MCCG)		–	–	–	++	–	–
(S)-2-Amino-2-methyl-4-phosphonobutyrate (MAP4)		–	–	+	ND	++	ND

The potencies of the test compounds are arbitrarily classified by taking the maximal stimulation (agonists) and inhibition (antagonists) of glutamate responses as 100% as follows: for the stimulatory effects of agonists, –, <15%; +, 15–50%; ++, 50–90%; +++, >90%; for the inhibitory effects of antagonists, –, <25%; +, 25–50%; ++, 50–90%; +++, >90%. ND, not determined. The values indicated in parentheses are IC_{50} values of the individual antagonists; the IC_{50} value of (+)-MCPG for mGluR$_2$ was calculated at the concentration of 30 μM L-glutamate. The agonist and antagonist potencies of the 5 phenylglycine derivatives (marked by asterisks) reported in the previous study (Hayashi *et al.*, 1994) are indicated for reference in this summary; in the previous study, mGluR$_4$ instead of mGluR$_6$ was used as a representative L-AP4-sensitive subtype and 10 μM L-glutamate was applied to mGluR$_4$-expressing cells in antagonist experiments.

the forskolin-stimulated cyclic AMP accumulation to about 10–20% of control levels (Figure 1b). Four of the 24 compounds tested, (RS)-4,6-DCl3HPG, (RS)-3,4-DCPG, (RS)-4C2IPG and (S)-MAP4, showed partial inhibition of the forskolin-stimulated cyclic AMP formation, while (RS)-3C5HPG had full agonist activity on mGluR$_2$. No other compounds were effective as agonists on mGluR$_2$ (Figure 1b). In mGluR$_6$-expressing cells, L-glutamate (1 mM) inhibited the cyclic AMP level to 30–40% of control levels (Figure 1c). Among the 24 compounds tested, (S)-3,5-DHPG, (RS)-4Cl3HPG, (RS)-4Cl-3,5-DHPG, (RS)-3C5HPG, (RS)-3,4-DCPG and (S)-MAP4 showed agonist activities on mGluR$_6$, but these six compounds were not as fully active as L-glutamate. No other compounds showed any agonist activity in mGluR$_6$-expressing cells.

Antagonist activities of various test compounds on mGluR$_1$, mGluR$_2$ and mGluR$_6$

We next examined the antagonist activities of the test compounds on each of the three mGluR subtypes by applying 1 mM of test compounds in the presence of an appropriate concentration of L-glutamate. In these experiments, the com-

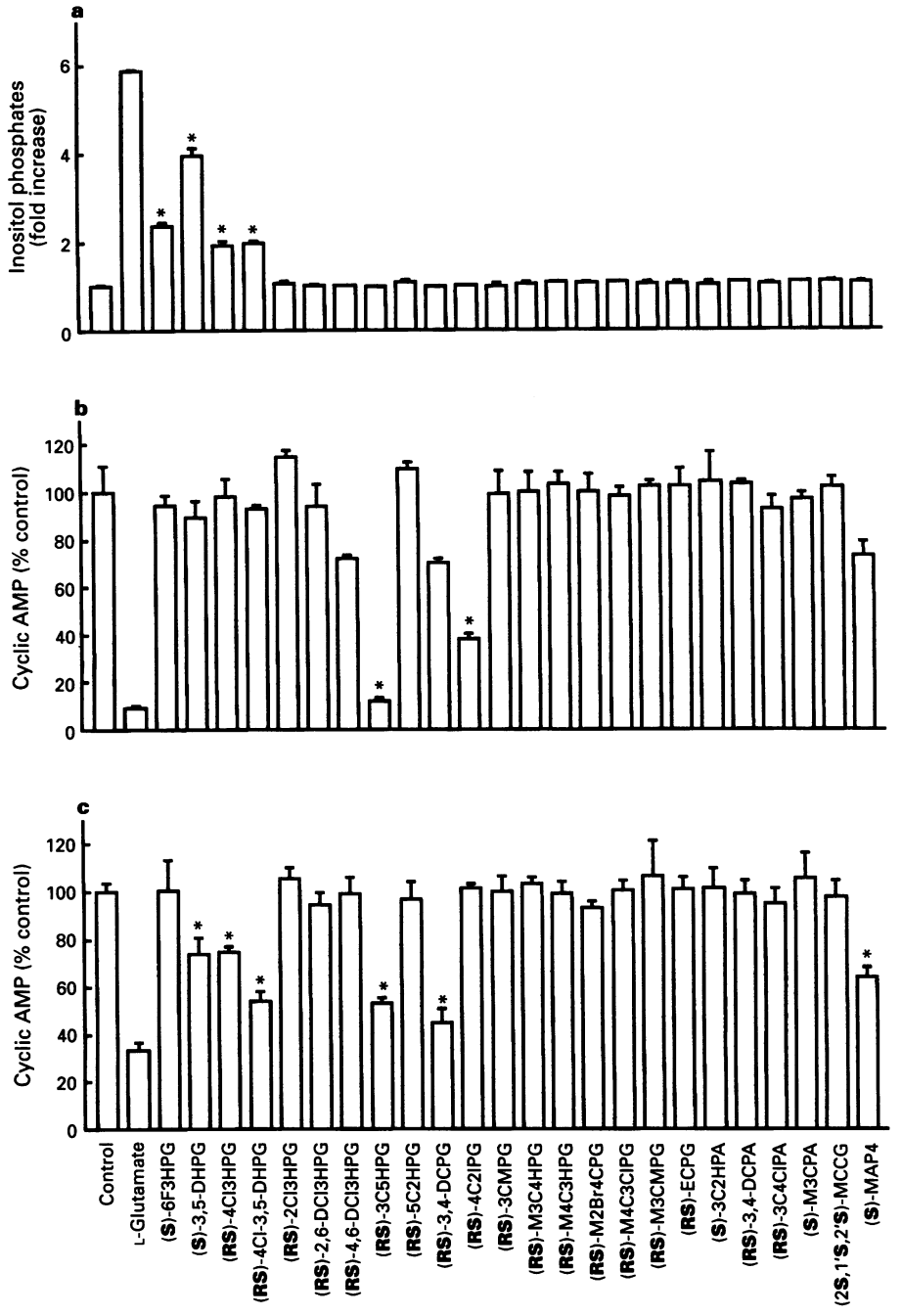


Figure 1 Agonist activities of 24 test compounds for $mGluR_1$, $mGluR_2$ and $mGluR_6$. In (a), $mGluR_1$-expressing cells were incubated with L-glutamate (1 mM) or test compounds (1 mM each) for 20 min, and total IP formation was determined. The IP formation is expressed as multiples of IP levels in agonist-untreated cells (Control). Basal levels (Control) of total inositol phosphates were 1227 ± 36 c.p.m. In (b), $mGluR_2$-expressing and in (c) $mGluR_6$-expressing cells were incubated with L-glutamate (1 mM) or test compounds (1 mM each) for 10 min in the presence of 10 μM forskolin, respectively, and intracellular cyclic AMP levels were determined. Cyclic AMP levels in cells treated and untreated with forskolin were 132.8 ± 14.8 and 4.3 ± 0.1 pmol per well ($mGluR_2$-expressing cells) and 51.2 ± 1.9 and 2.3 ± 0.4 pmol per well ($mGluR_6$-expressing cells), respectively. The cyclic AMP levels in forskolin-stimulated, L-glutamate-untreated cells (Control) are taken as 100%. The data indicated were taken from representative experiments. The values are means $\pm$ s.d. (error bars) of triplicate determinations. $^*P<0.001$ for (a) and $^*P<0.005$ for (b) and (c) compared with L-glutamate-untreated control cells.

pounds exhibiting an agonist activity in the above analysis were omitted, and the data obtained in this analysis are shown in Figure 2 and summarized in Table 1.

In the analysis of $mGluR_1$, 10 μM L-glutamate, the concentration corresponding to an approximate half-maximal effective concentration of L-glutamate for $mGluR_1$ was added to $mGluR_1$-expressing cells and was found to increase IP levels about three fold. Among the compounds tested, (RS)-2Cl3HPG, (RS)-2,6-DCl3HPG, (RS)-4,6-DCl3HPG, (RS)-3C5HPG, (RS)-5C2HPG, (RS)-3,4-DCPG, (RS)-4C2IPG, (RS)-M3C4HPG, (RS)-M4C3HPG, (RS)-M2Br4CPG, (RS)-M4C3ClPG, (RS)-M3CMPG, (RS)-ECPG, (S)-3C2HPA and (RS)-3,4-DCPA inhibited L-glutamate-induced IP increase in $mGluR_1$-expressing cells. Among them, (RS)-3C5HPG, (RS)-3,4-DCPG and (RS)-4C2IPG reduced the IP formation to control levels (Figure 2a).

The antagonist effects of the test compounds on $mGluR_2$ and $mGluR_6$ were analyzed by examining whether the L-

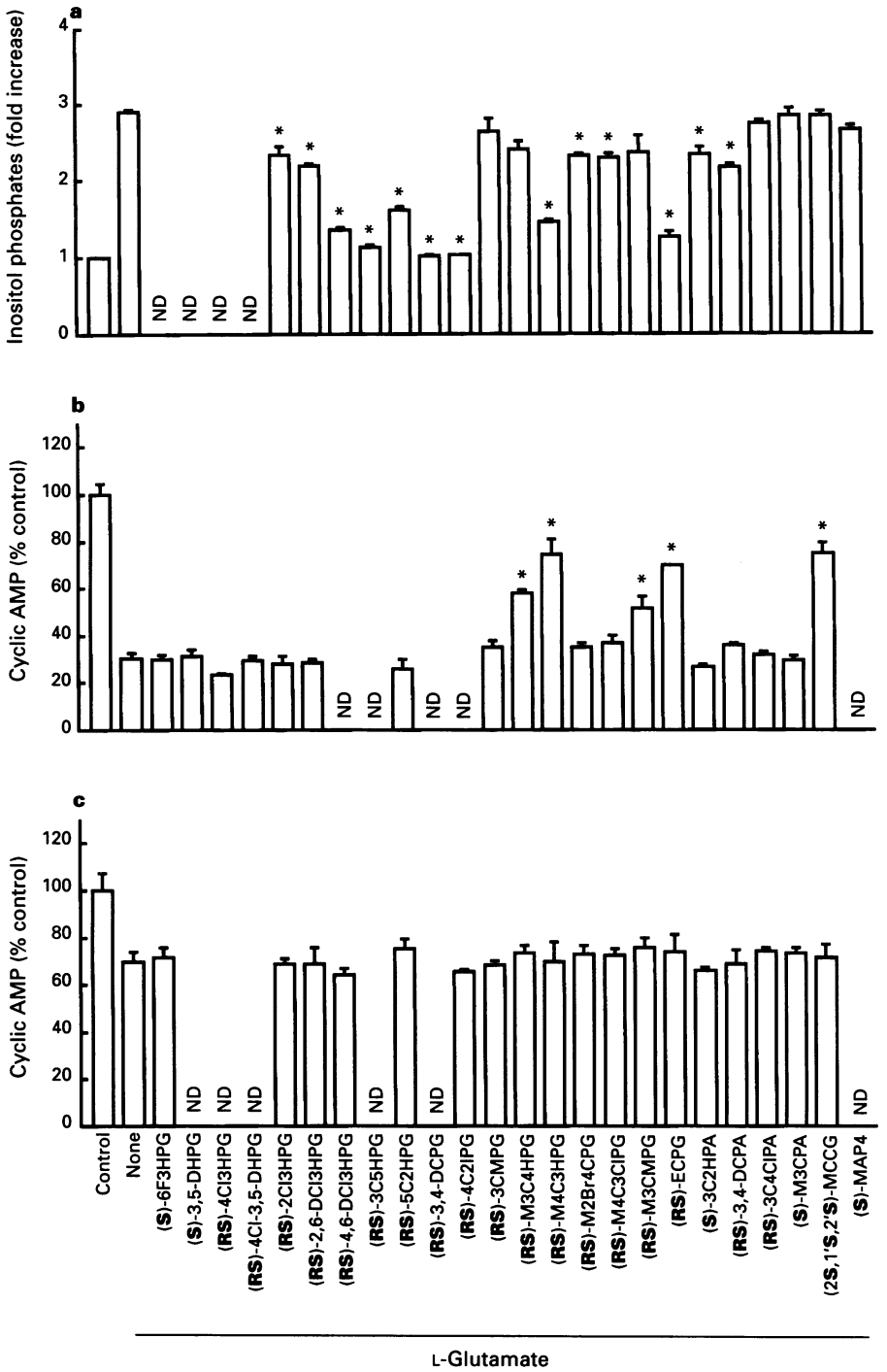


Figure 2 Antagonist activities of various test compounds for $mGluR_1$, $mGluR_2$ and $mGluR_6$. In (a), $mGluR_1$-expressing cells were preincubated with the indicated compounds (1 mM each) for 20 min and then incubated with 10 μM L-glutamate in the presence of the same compounds (1 mM each) for 20 min. Basal levels (Control) of total inositol phosphates were 1227 ± 15 c.p.m. In (b), $mGluR_2$-expressing and in (c) $mGluR_6$-expressing cells were preincubated with the indicated compounds (1 mM each) for 20 min and then incubated with 20 μM L-glutamate for $mGluR_2$ or 30 μM L-glutamate for $mGluR_6$, together with 10 μM forskolin, in the presence of the same test compounds (1 mM each) for 10 min. Intracellular cyclic AMP levels were then determined. Cyclic AMP levels in cells treated and untreated with forskolin were 192.3 ± 9.1 and 5.8 ± 1.0 pmol per well ($mGluR_2$-expressing cells) and 97.9 ± 7.0 and 3.7 ± 0.5 pmol per well ($mGluR_6$-expressing cells), respectively. The antagonist activity of some derivatives was not determined (indicated with ND) because they showed agonist activities. $^*P < 0.001$ for (a) and $^*P < 0.005$ for (b) compared with cells treated with L-glutamate alone. For other details, see Figure 1.

glutamate-mediated inhibition of the forskolin-induced cyclic AMP levels was antagonized by the addition of 1 mM of different test compounds to $mGluR_2$-expressing and $mGluR_6$-expressing cells, respectively. In the analysis of $mGluR_2$, 20 μM L-glutamate was added to $mGluR_2$-expressing cells, and this concentration of L-glutamate reduced cyclic AMP levels to about 30–40% of forskolin-stimulated cyclic AMP levels. Among the 19 compounds analyzed, (RS)-M3C4HPG, (RS)-M4C3HPG, (RS)-M3CMPG, (RS)-ECPG and MCCG partially antagonized the effect of L-glutamate (Figure 2b). We then examined the antagonist activities of 18 test compounds on $mGluR_6$. In this analysis, 30 μM or 100 μM L-glutamate was

added to $mGluR_6$-expressing cells, and the former and the latter concentrations of L-glutamate reduced cyclic AMP levels to about 60–70% and 30–40% of control levels, respectively. With both concentrations of L-glutamate, none of the 18 compounds tested showed any antagonist activity on $mGluR_6$ (Figure 2c).

The inhibitory potencies of (RS)-4C2IPG, (RS)-3,4-DCPG and (RS)-3C5HPG that exhibited a full antagonist activity on $mGluR_1$ at a concentration of 1 mM were also determined at 100 μM each. (RS)-4C2IPG, (RS)-3,4-DCPG and (RS)-3C5HPG showed 57.3±0%, 35.0±12.3% and 28.4±5.2% inhibition of L-glutamate (10 μM)-induced IP formation, respectively (data not shown). Concentration-response analysis of the most potent compound, (RS)-4C2IPG, was then carried out, and the half-maximal inhibitory concentration (IC_{50}) of (RS)-4C2IPG was calculated to be 9×10^{-5} M (Figure 3a).

Our previous study indicated that (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) acts as an effective antagonist on both $mGluR_1$ and $mGluR_2$ with IC_{50} values of 7×10^{-5} M and 4×10^{-4} M, respectively (Hayashi *et al.*, 1994). Similarly, (RS)-M4C3HPG showed an antagonist activity on both $mGluR_1$ and $mGluR_2$ (Figure 2a and b). The inhibitory potencies of (RS)-M4C3HPG on $mGluR_1$ and $mGluR_2$ were then analyzed by determining concentration-response relationships of this compound in $mGluR_1$-expressing and $mGluR_2$-expressing cells, respectively (Figure 3b and c). The IC_{50} values of (RS)-M4C3HPG for $mGluR_1$ and $mGluR_2$ were calculated to be 4×10^{-4} M and 6×10^{-4} M, respectively, indicating that α-methylation of 4C3HPG is less effective than α-methylation of 4CPG for producing antagonist activity at both $mGluR_1$ and $mGluR_2$ (Table 1).

In the above system, the agonist and antagonist properties were characterized by measuring their effects on intracellular second messengers characteristic of the respective mGluR subtypes. However, the same stimulatory or inhibitory effect could be evoked if a test compound acts directly on intracellular signalling machinery. This possibility for the potent agonists and antagonists identified in this study was ruled out by the following experiments. We adopted cell lines expressing the cloned endothelin receptor subtype ET_A for the IP formation and cells transfected with vector DNA alone for the cyclic AMP cascade. We confirmed that (S)-3,5-DHPG had no effect on IP formation in ET_A-expressing cells, nor did any potent $mGluR_1$ antagonists ((RS)-4,6-DCl3HPG, (RS)-3C5HPG, (RS)-5C2HPG, (RS)-3,4-DCPG, (RS)-4C2IPG, (RS)-M4C3HPG and (RS)-ECPG) inhibit the endothelin-induced IP formation in these cells (data not shown). We also ascertained that none of the agonists for $mGluR_2$ and $mGluR_6$ inhibited the forskolin-stimulated cyclic AMP accumulation in the vector-transfected cell. In addition, we observed that the $mGluR_2$ antagonists identified in this study are specific to $mGluR_2$, since they did not antagonize the L-glutamate-mediated inhibition of the cyclic AMP cascade in $mGluR_6$-expressing cells (Figure 2c; Table 1), indicating that these antagonists interact specifically with the receptor protein *per se*. Thus, these control experiments validated our identification of the mGluR agonist and antagonist activities described in this study.

We did, however, find an exceptional case, in which (RS)-3,4,5-trihydroxyphenylglycine (3,4,5-THPG) seemed to inhibit an intracellular signalling machinery directly rather than via an interaction with the receptor protein. In $mGluR_1$-expressing cells, this compound inhibited L-glutamate-stimulated IP formation in a concentration-dependent manner with an IC_{50} value of 5×10^{-5} M (Figure 4a). Furthermore, (RS)-3,4,5-THPG showed a dual action on the cyclic AMP cascade in both $mGluR_2$-expressing and $mGluR_6$-expressing cells, depending on the concentrations added (Figure 4c and d); it apparently antagonized the L-glutamate-mediated inhibition of the forskolin-stimulated cyclic AMP formation at a concentration of 100 μM and then reduced the cyclic AMP formation at 1 mM. When the effect of (RS)-3,4,5-THPG on endothelin-stimulated IP formation was examined in endothelin receptor ET_A-expressing cells, similar inhibition was observed, with an IC_{50} value of 3×10^{-5} M (Figure 4b). Furthermore, a similar pattern of increase and decrease in the forskolin-stimulated cyclic AMP formation was seen by the addition of 100 μM and 1 mM (RS)-3,4,5-THPG in the vector-transfected cells (Figure 4e). Thus, although the mechanism of this peculiar action of (RS)-3,4,5-THPG remains to be determined, (RS)-3,4,5-THPG seems to exert its action at a site other than the receptor protein, and the identification of agonist and antagonist activities warrants caution when these properties are characterized by measuring intracellular second messengers solely in a single receptor-expressing cell line or a nerve preparation.

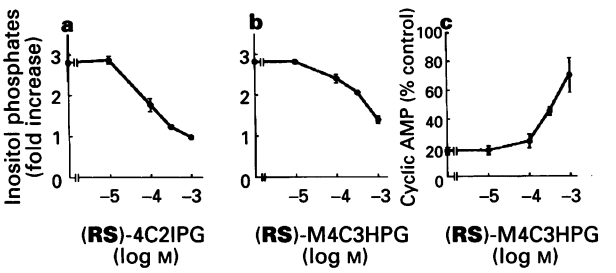


Figure 3 Concentration-response analysis of the antagonist effect of (RS)-4C2IPG on $mGluR_1$ (a) and antagonist effects of (RS)-M4C3HPG on $mGluR_1$ (b) and $mGluR_2$ (c). $mGluR_1$-expressing cells were preincubated with the indicated concentrations of (RS)-4C2IPG (a) and (RS)-M4C3HPG (b) for 20 min and further incubated with the same concentrations of these compounds for 20 min in the presence of 10 μM L-glutamate. The basal level of IP formation was 1552±47 c.p.m. (a and b). In (c), $mGluR_2$-expressing cells were preincubated with the indicated concentrations of (RS)-M4C3HPG for 20 min and further incubated with the same concentrations of this compound for 10 min in the presence of 20 μM L-glutamate together with 10 μM forskolin. Cyclic AMP levels in cells treated and untreated with forskolin were 117.9±5.1 and 3.3±0.7 pmol per well, respectively. Values significantly different ($P<0.01$) from values of cells treated with L-glutamate alone were obtained from 10^{-4} M (RS)-4C2IPG (a), 10^{-4} M (RS)-M4C3HPG (b) and 3×10^{-4} M (RS)-M4C3HPG (c). For other details, see Figure 1.

Discussion

The analysis of the structure-activity relationships of the mGluR ligands is important not only for understanding the interaction between the ligand and mGluR proteins but also for developing new agonists and antagonists for the mGluR family. The phenylglycine and phenylalanine derivatives investigated share the fundamental structure with L-glutamate in that they have an α-amino acid moiety and an ω-anionic group and act as conformationally restricted analogues of L-glutamate. In this investigation, we determined the agonist and antagonist properties of 22 new phenylglycine and phenylalanine derivatives and extended our previous observation that phenylglycine derivatives possess different agonist and antagonist activities, dependent on the nature and relative position of substituents in the phenyl ring and amino acid side chain (Hayashi *et al.*, 1994). Some characteristic features of the structure-activity relationships can be pointed out according to the chemical structures of phenylglycine derivatives, including those of the 5 phenylglycine derivatives described in our previous study (Hayashi *et al.*, 1994) (Table 1). We also discuss our data in relation to the properties of phenylglycine derivatives characterized in neuronal cells and slice preparations.

(S)-3-hydroxyphenylglycine (3HPG), as reported previously (Hayashi *et al.*, 1994), showed agonist activity on $mGluR_1$. Further hydroxyl and/or halogen substitution of the 3HPG molecule led to either agonist or antagonist activity at $mGluR_1$. Agonist activity was retained with hydroxyl and/or monohalogan substitution in the 4, 5 and 6 position. Other

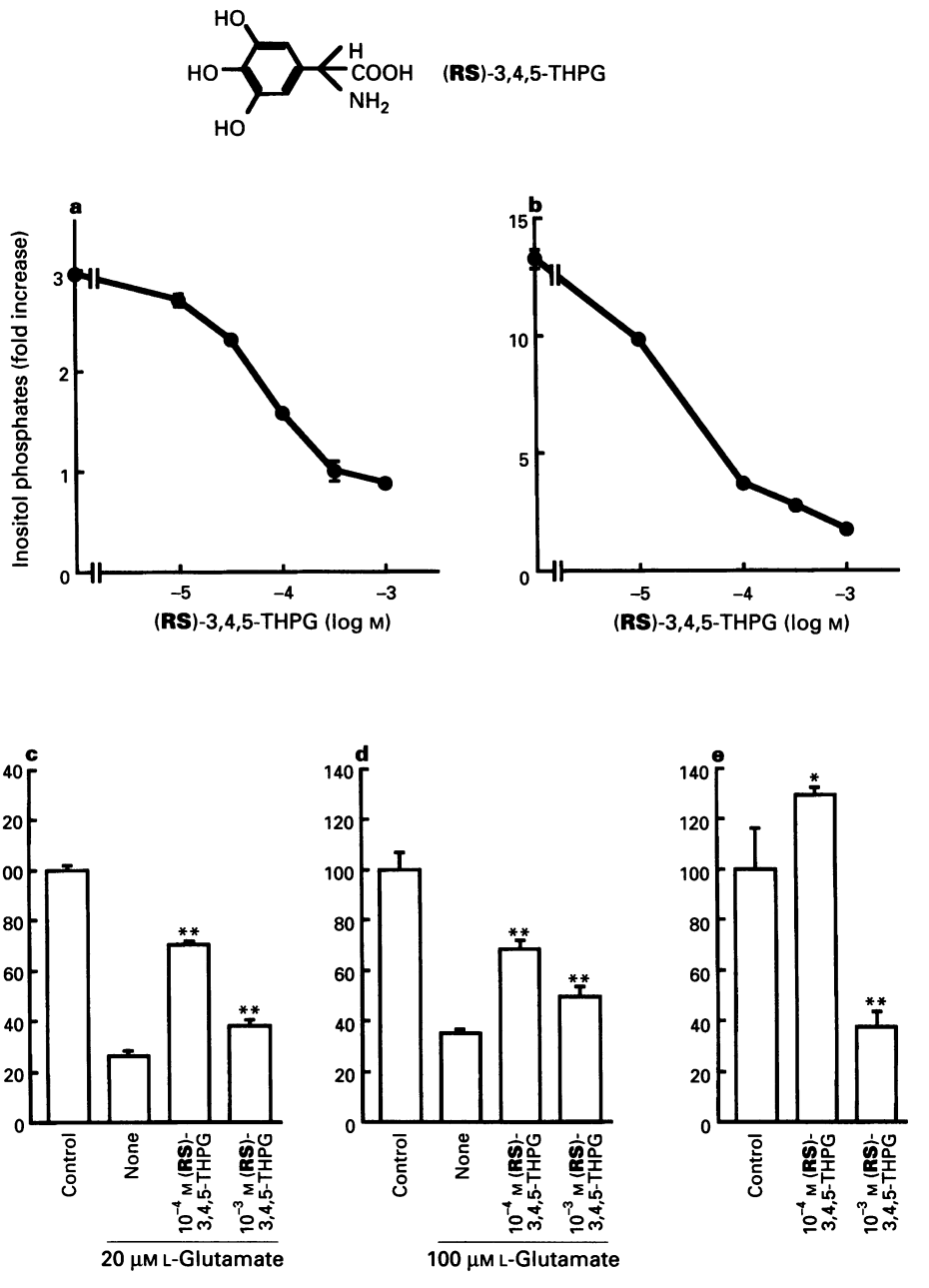


Figure 4 Effects of (RS)-3,4,5-THPG on signal transduction in cells expressing various types of receptors and cells transfected with vector DNA alone. In (a), $mGluR_1$-expressing cells and in (b) ET_A receptor-expressing cells were preincubated with the indicated concentrations of (RS)-3,4,5-THPG for 20 min, respectively, and further incubated with the same concentrations of this compound for 20 min in the presence of 10 μM L-glutamate (a) or 1 nM endothelin-1 (ET-1) (b). The basal levels of IP formation were 1135 ± 26 c.p.m. (a) and 298 ± 21 c.p.m. (b). Values significantly different ($P < 0.01$) from values of cells treated with L-glutamate alone (a) and ET-1 alone (b) were obtained from 10^{-5} M (RS)-3,4,5-THPG in both (a) and (b). In (c), $mGluR_2$-expressing cells, in (d) $mGluR_6$-expressing cells and in (e) those transfected with vector DNA alone were preincubated with the indicated concentrations of (RS)-3,4,5-THPG for 20 min and further incubated with the same concentrations of this compound for 10 min in the presence of 10 μM forskolin together with 20 μM L-glutamate (c), 100 μM L-glutamate (d) or without L-glutamate addition (e). Cyclic AMP levels in cells treated and untreated with forskolin in (c), (d) and (e) were 135.7 ± 2.5 and 4.7 ± 0.1, 81.1 ± 5.5 and 3.3 ± 0.3, and 52.8 ± 8.6 and 2.4 ± 0.1 pmol per well, respectively. In (c) and (d), $^{**}P < 0.01$ compared with cells treated with L-glutamate alone. In (e), $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control cells. For other details, see Figure 1.

modifications of 3HPG abolished agonist activity and, in several cases, resulted in antagonist activity (see (RS)-2Cl3HPG, (RS)-2,6-DCl3HPG, and (RS)-4,6-DCl3HPG).

Additional to their agonist activity at $mGluR_1$, (S)-3,5-DHPG, (RS)-4Cl3HPG and (RS)-4Cl-3,5-DHPG also showed an agonist activity on $mGluR_6$, and these compounds, together with (RS)-3C5HPG and (RS)-3,4-DCPG (see below), are the first phenylglycine derivatives reported to possess an agonist activity on L-AP4-sensitive mGluR subtypes.

We previously reported that three species of phenylglycine derivatives possessing a carboxyl substituent in the benzene ring, (S)-4-carboxy-3-hydroxyphenylglycine (4C3HPG), (S)-4-carboxyphenylglycine (4CPG) and (S)-3-carboxy-4-hydroxyphenylglycine (3C4HPG), have an agonist activity on $mGluR_2$ and an antagonist activity on $mGluR_1$ (Hayashi *et al.*, 1994). This profile of agonist and antagonist properties was found to be retained in three additional carboxyphenylglycine derivatives analyzed in this study (see (RS)-3C5HPG, (RS)-

3,4-DCPG and (**RS**)-4C2IPG). Useful $mGluR_1$ antagonist activity was observed for (**RS**)-4C2IPG ($IC_{50} = 9 \times 10^{-5}$ M for inhibition of L-glutamate-induced IP formation). This compares favourably with (**S**)-4CPG ($IC_{50} = 4 \times 10^{-5}$ M) and a further increase in potency is likely when the individual enantiomers of 4C2IPG have been synthesized. It is of interest to note that substitution of either a carboxyl or hydroxyl group at the 3-position or an iodo group at the 2-position of 4CPG results in either a slight increase or no significant loss of potency. This observation is important since it establishes the position of allowed volume in the $mGluR_1$ receptor.

Interestingly, (**RS**)-3C5HPG and (**RS**)-3,4-DCPG also showed an agonist activity on $mGluR_6$, while (**RS**)-5C2HPG was a selective antagonist on $mGluR_1$. The observed difference in selectivity between (**RS**)-3C5HPG and (**RS**)-5C2HPG indicates the importance of relative positions of the carboxyl group and the hydroxyl group attached to the benzene ring, reflecting the fact that molecules of different chain length and conformation are produced when the relative positions of the carboxyl and hydroxyl groups are altered. These effects could be important for further modification to produce a more potent $mGluR_1$-specific antagonist. (**RS**)-3CMPG which has a carboxymethyl group as an ω-anionic group showed neither agonist nor antagonist activity on all three mGluR subtypes, again suggesting the importance of conformation in interaction between a ligand and its receptor molecule.

Our previous investigation indicated that methylation of the α-carbon of (**S**)-4CPG (namely, MCPG) converts an $mGluR_2$ agonist to an $mGluR_2$ antagonist without losing its antagonist activity on $mGluR_1$. We extended analysis of the effects of methylation and ethylation at the α-carbon of phenylglycine derivatives by synthesizing 6 additional compounds. (**S**)-3C4HPG and (**S**)-4C3HPG possess agonist activity on $mGluR_2$, together with antagonist activity on $mGluR_1$ (Hayashi *et al.*, 1994). α-Methylation of these compounds ((**RS**)-M3C4HPG and (**RS**)-M4C3HPG) converts the $mGluR_2$ agonist actions of the parent compounds to antagonist activity, thus producing antagonists on both $mGluR_1$ and $mGluR_2$. Furthermore, whereas (**RS**)-3CMPG showed no activity on any mGluRs (this investigation), the α-methyl analogue of this compound was a weak antagonist on both $mGluR_1$ and $mGluR_2$ subtypes. In addition, ethylation of the α-carbon moiety of 4CPG is also effective in converting its agonist activity for $mGluR_2$ to antagonist activity at the same receptor. This analysis clearly indicates that the methyl or ethyl group at the α-carbon of phenylglycine derivatives plays a role in preventing activation of the receptor while still allowing the molecules to interact with the L-glutamate-binding site.

Substitution of either chloro or hydroxyl at the 3-position or bromo at the 2-position of the phenyl ring of MCPG is detrimental to antagonist activity at $mGluR_1$. It is likely that in the case of (**RS**)-M2Br4CPG there is a change in preferred conformation due to steric effects brought about by interaction of the bulky bromo substituent and the amino acid side chain. The reasons for the reduction of activity observed with (**RS**)-M4C3HPG and (**RS**)-M4C3ClPG are less clear (particularly since (**S**)-4C3HPG is the most potent $mGluR_1$ antagonist yet reported) but this again may be due to changes in preferred conformation causing the substituent at the 3-position of the phenyl ring to interact with excluded volume in the receptor.

We also tested the properties of several phenylalanine derivatives and found that these compounds have no activities on all three mGluR subtypes or only a weak antagonist activity on $mGluR_1$. This finding again supports the view that both conformational effects and the inter-acidic group chain length of the phenylglycine derivatives are critical factors in the interaction between the ligand and the receptor molecule.

With some exceptions discussed below, the above characterization of the phenylglycine derivatives is consistent with the reports of these compounds studied in neuronal cells, and slice preparations as well as mGluR-expressing *Xenopus laevis* oocytes. (**RS**)-3,5-DHPG stimulates PI hydrolysis in rat hippocampal slices (Schoepp *et al.*, 1994) and in $mGluR_1$-expressing *Xenopus laevis* oocytes (Ito *et al.*, 1992). (**S**)-3,5-DHPG, (**S**)-6F3HPG and (**RS**)-4Cl-3,5-DHPG also increase IP formation in cerebral cortical slices (Bedingfield *et al.*, 1994). (**RS**)-ECPG, (**RS**)-4C2IPG, (**RS**)-M4C3HPG and (**RS**)-3,4-DCPG all antagonize the (1**S**, 3**R**)-ACPD-stimulated IP formation in cerebral cortical slices (Bedingfield *et al.*, 1994). All these findings agree with the actions of the compounds determined in this work.

In this study, $mGluR_6$ was chosen as a representative L-AP4-sensitive receptor, because this subtype expressed in CHO cells is capable of producing reproducible responses to test compounds. However, $mGluR_6$ is localized selectively in the retina (Nakajima *et al.*, 1993) and therefore results in other tissues may not correlate with effects on $mGluR_6$. We detected no antagonist effects of (**RS**)-M3C4HPG, (**RS**)-M3CMPG and (**S**)-M3CPA on $mGluR_6$-mediated inhibition of forskolin-stimulated cyclic AMP formation. However, these compounds were found to antagonize potently the inhibitory effects of L-AP4 on the forskolin-stimulated cyclic AMP formation in rat forebrain slices (Kemp *et al.*, 1994a). Although it has been shown that the members of the same group of the mGluR family share a common sensitivity to various agonists and antagonists (Nakanishi, 1994), $mGluR_6$ and the L-AP4-sensitive mGluR subtype in the forebrain may differ in their responses to some phenylglycine compounds.

Finally, two α-methyl derivatives of mGluR agonists, (**S**)-MAP4 and MCCG, have recently been developed and their properties have been characterized in detail in neuronal cells and slice preparations. In this investigation, we defined the properties of MCCG and (**S**)-MAP4 in our assay system and showed that MCCG is a selective antagonist on $mGluR_2$ without any activities on the other subtypes. This property of MCCG is consistent with the property of this compound characterized in neonatal rat motoneurones (Jane *et al.*, 1994) and thalamic neurones (Salt & Eaton, 1995).

However, in contrast to several reports of the antagonist effects of (**S**)-MAP4 on the L-AP4-sensitive mGluR in neuronal preparations, our study indicated that (**S**)-MAP4 possesses a moderate agonist activity on $mGluR_6$ and a weak agonist activity on $mGluR_2$. In agreement with our finding, Gottesman *et al.* (1995) reported that (**S**)-MAP4 acts as an L-AP4-type agonist in retinal ON bipolar cells. Kemp *et al.* (1994b) have also reported that (**S**)-MAP4 acts as an agonist, when this activity was measured by the inhibitory effect of the forskolin-stimulated cyclic AMP formation in rat forebrain slices. These findings are, however, inconsistent with the reports of the (**S**)-MAP4 action studied electrophysiologically in several brain and spinal cord preparations. It was reported that (**S**)-MAP4 antagonizes the synaptic depressant actions of L-AP4 in neonatal motoneurones (Jane *et al.*, 1994) and lateral perforant path-dentate granule cell synapses in rat hippocampus (Bushell *et al.*, 1995) as well as the inhibitory action of L-AP4 on γ-aminobutyrate transmission in rat thalamic neurones (Salt & Eaton, 1995). Such L-AP4 effects are considered likely to be mediated by presynaptic L-AP4-sensitive mGluRs negatively coupled to the cyclic AMP cascade. Thus, it is concluded that (**S**)-MAP4 acts as a potent antagonist on the presynaptic L-AP4-sensitive mGluR (Jane *et al.*, 1994; Bushell *et al.*, 1995; Salt & Eaton, 1995). The apparently opposite effects of (**S**)-MAP4 on the L-AP4-sensitive mGluRs may result from the different tissues used in these various studies which may contain a different proportion of L-AP4-sensitive mGluR subtypes. Further examination of the compounds used in this study on other mGluR subtypes may help to resolve this problem.

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