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The effects of (RS)-α-cyclopropyl-4-phosphonophenylglycine ((RS)-CPPG), a potent and selective metabotropic glutamate receptor antagonist

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1 In this study we describe the potent antagonist activity of a novel metabotropic glutamate (mGlu) receptor antagonist (**RS**)-α-cyclopropyl-4-phosphonophenylglycine ((**RS**)-CPPG) which exhibits selectivity for mGlu receptors (group II and III) negatively coupled to adenylyl cyclase in the adult rat cortex.

2 Both the L-2-amino-4-phosphonobutyrate (L-AP4) and (2S, 1'S, 2'S)-2-(carboxycyclopropyl)glycine (L-CCG-1) inhibition of forskolin-stimulated cyclic AMP accumulation were potently reversed by (**RS**)-CPPG (IC_{50} values: 2.2 ± 0.6 nM and 46.2 ± 18.2 nM, respectively).

3 In contrast, (**RS**)-CPPG acted as a weak antagonist against group I mGlu receptors. In neonatal rat cortical slices, (**RS**)-CPPG antagonized ($K_B = 0.65 \pm 0.07$ mM) (1**S**,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD)-stimulated phosphoinositide hydrolysis. (**RS**)-CPPG (100 μM) failed to influence L-quisqualate-stimulated phosphoinositide hydrolysis in cultured cerebellar granule cells.

4 In the rat cerebral cortex, (**RS**)-CPPG is the most potent antagonist of group II/III mGlu receptors yet described (with 20 fold selectivity for group III mGlu receptors), having negligible activity at group I mGlu receptors.

Keywords: (**RS**)-α-cyclopropyl-4-phosphonophenylglycine ((**RS**)-CPPG); metabotropic glutamate receptor antagonist; cyclic AMP; phosphoinositide hydrolysis

Introduction

L-Glutamate is the principal excitatory amino acid neurotransmitter in the mammalian central nervous system (Monaghan *et al.*, 1989). Glutamate receptors are divided into two groups termed ionotropic (iGlu) and metabotropic glutamate (mGlu) receptors. iGlu receptors are ligand-gated ion channels consisting of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. mGlu receptors are G-protein coupled to several signal transduction pathways and have been divided into three groups according to their amino acid sequence, signal transduction pathway and agonist preference (Pin & Duvoisin, 1995).

Group I mGlu receptors ($mGlu_1$ and $mGlu_5$) are coupled to phosphoinositide hydrolysis and are potently activated by L-quisqualate (Aramori & Nakanishi, 1992; Abe *et al.*, 1992). Group II ($mGlu_2$ and $mGlu_3$) and group III ($mGlu_4$ and $mGlu_{6-8}$) receptors are negatively coupled to adenylyl cyclase and are activated by (2S, 1'S, 2'S)-2-(carboxycyclopropyl)glycine (L-CCG-1) and L-2-amino-4-phosphonobutyrate (L-AP4), respectively (Tanabe *et al.*, 1992; 1993; Nakajima *et al.*, 1993; Okamoto *et al.*, 1994; Saugstad *et al.*, 1994; Duvoisin *et al.*, 1995).

Previously we have synthesized and characterized an extensive series of substituted α-methyl-phenylglycine derivatives which demonstrate varying degrees of activity at group I and group II/III mGlu receptors (Watkins & Collingridge, 1994; Roberts, 1995). Our initial investigations led to the discovery of (**RS**)-α-methyl-4-carboxyphenylglycine (MCPG) which proved to be a non-selective mGlu receptor antagonist acting at group I mGlu receptors in neonatal rat cortical slices and group II and III mGlu receptors in adult rat cortical slices (Eaton *et al.*, 1993; Kemp *et al.*, 1994). Recently, we have found a large increase in antagonist potency and selectivity for group II and III mGlu receptors with the substitution of the 4-carboxy group of MCPG by a 4-phosphono group to form (**RS**)-α-methyl-4-phosphonophenylglycine (MPPG) (Kemp *et al.*, 1994; 1995).

In the present study we have investigated the activity of the cyclopropyl derivative of MPPG, (**RS**)-α-cyclopropyl-4-phosphonophenylglycine ((**RS**)-CPPG, for structure see Figure 1), against group I mGlu receptors (in both neonatal rat cortical slices and cultured cerebellar granule cells) and groups II and III mGlu receptors (in adult rat cortical slices). We describe here, the potent and selective antagonism of (**RS**)-CPPG against groups II and III mGlu receptors (showing moderate selectivity for group III) and weak activity against group I mGlu receptors. Part of this work has already been published (Kemp *et al.*, 1996).

Methods

Cyclic AMP determination in adult rat cortical slices

The effect of (**RS**)-CPPG on 10 μM L-AP4 and 300 nM L-CCG-1-mediated inhibition of forskolin-stimulated cyclic AMP levels was investigated by use of a modified method of that previously described by Brown *et al.* (1972) and Voss & Wallner (1992). After pre-incubation for 60 min at 37°C in Krebs buffer (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 5.5, $CaCl_2$ 2.5), 25 μl aliquots of gravity-packed cerebrocortical slices (200 μm × 200 μm) were incubated for 20 min at 37°C with (**RS**)-CPPG, plus either L-AP4 or L-CCG-1. Adenosine deaminase (0.4 u) and the selective phosphodiesterase IV inhibitor 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Ro 20-1724) (100 μM) were also added. The assay was started with the addition of forskolin (30 μM) and incubation continued for a further 10 min. The reaction was terminated by the addition of ice-cold chloroform/methanol (1:1 v/v) and the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of the sample determined by measuring the displacement of [^{3}H]-

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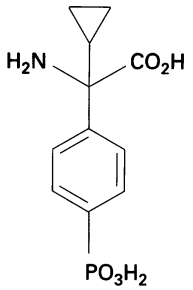

Figure 1 Structure of (**RS**)-α-cyclopropyl-4-phosphonophenylglycine ((**RS**)-CPPG).

cyclic AMP binding to bovine adrenal cortex cyclic AMP-binding protein and comparison with unlabelled cyclic AMP standards.

Phosphoinositide hydrolysis in cerebrocortical slices

Phosphoinositide hydrolysis was measured by monitoring the accumulation of [^{3}H]-inositol monophosphate ([^{3}H]-IP$_1$), in the presence of LiCl (Berridge *et al.*, 1982; Porter *et al.*, 1992). Briefly, cerebrocortical slices (300 μm × 300 μm), prepared from 6–8 day old Wistar rats, were pre-labelled with D-*myo*-[^{3}H]-inositol (75 μCi 15 ml^{-1} Krebs) for 120 min at 20°C. After extensive washing, 50 μl aliquots of the slices were exposed to (**RS**)-CPPG (1 mM or 3 mM) for 20 min at 37°C followed by the addition of (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3**R**)-ACPD) for 45 min at 37°C. LiCl (10 mM) was present throughout the assay. The reaction was terminated by the addition of chloroform/methanol (1:2 v/v) and [^{3}H]-IP$_1$ isolated by ion-exchange chromatography on Dowex-1X8-200 (formate form) columns.

Results

(RS)-CPPG inhibition of group II and group III mGlu receptors

Forskolin-stimulated cyclic AMP accumulation in adult rat cortical slices was inhibited by L-CCG-1 (EC_{50} = 300 nM) and L-AP4 (EC_{50} = 10 μM), selective group II and group III mGlu receptor agonists, respectively (data not shown). The effect of the novel phenylglycine derivative, (**RS**)-CPPG was tested against EC_{50} concentrations of both L-CCG-1 and L-AP4 (Figure 2). (**RS**)-CPPG potently reversed both L-AP4 (IC_{50} = 2.2 ± 0.6 nM)- and L-CCG-I (IC_{50} = 46.2 ± 18.2 nM) -mediated inhibition of forskolin-stimulated cyclic AMP accumulation in adult rat cortical slices. Although (**RS**)-CPPG reversed both group II and group III mGlu receptor activity, an approximate 20 fold selectivity for group III mGlu receptors was observed.

(RS)-CPPG action at group I mGlu receptors

The effects of (**RS**)-CPPG (1 mM and 3 mM) against (1S,3**R**)-ACPD-stimulated phosphoinositide hydrolysis in neonatal rat cortical slices was tested (Figure 3). (**RS**)-CPPG produced parallel dextral shifts in the (1S,3**R**)-ACPD concentration-response curve, consistent with the action of a weak competitive antagonist (K_B = 0.65 ± 0.07 mM). Previously, we have described the action of several phenylglycine derivatives against group I mGlu receptors in cultured cerebellar granule cells which have a different pharmacology from that of group I mGlu receptors in the neonatal rat cortex (Birse *et al.*, 1993; Toms *et al.*, 1995). In the present study, 100 μM (**RS**)-CPPG was inactive against the EC_{50} (2 μM) of L-quisqualate in stimulating phosphoinositide hydrolysis in cultured cerebellar granule cells (data not shown).

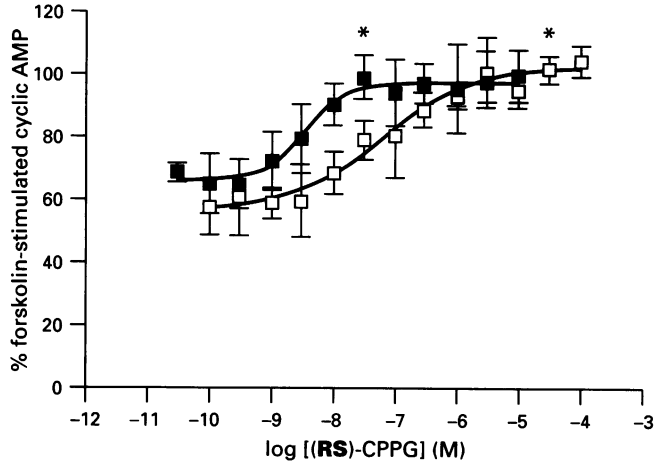

Figure 2 Reversal of 10 μM L-2-amino-4-phosphonobutyrate (L-AP4) (■) and 300 nM (2S, 1'S, 2'S)-2-(carboxycyclopropyl)glycine (L-CCG-1) (□)-inhibition of forskolin-stimulated cyclic AMP accumulation in adult rat cortical slices by (**RS**)-CPPG (IC_{50} values ± s.e.mean: 2.2 ± 0.6 nM and 46.2 ± 18.2 nM, respectively). Control values (% inhibition of forskolin-stimulated cyclic AMP accumulation) were 10 μM L-AP4 (31.1 ± 2.9%) and 300 nM L-CCG-1 (42.5 ± 8.5%). *P < 0.05 control values versus both 30 nM (**RS**)-CPPG (L-AP4 curve) and 30 μM (**RS**)-CPPG (L-CCG-1 curve) as determined by Mann-Whitney U test. Data are represented as the means of four experiments performed in quadruplicate; vertical lines show s.e.mean.

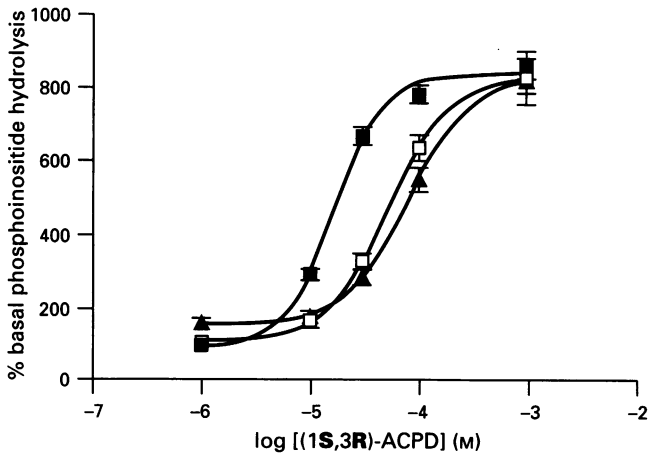

Figure 3 Stimulation of phosphoinositide hydrolysis in neonatal rat cerebrocortical slices by (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3**R**)-ACPD) alone (■) and in the presence of 1 mM (□) and 3 mM (▲) (**RS**)-CPPG. Data are represented by the means ± s.e.mean of three experiments performed in triplicate.

Discussion

The determination of the physiological roles of mGlu receptors in the CNS has been impeded principally by the lack of availability of suitable discriminatory pharmacological tools. In the present study we describe an important, recently synthesized, addition to mGlu receptor pharmacology, namely, the compound (**RS**)-CPPG.

The data from the current study, together with electrophysiological data of Jane *et al.* (1996) permit us to conclude that (**RS**)-CPPG is the most potent group II/III mGlu receptor antagonist yet described and exhibits some selectivity (approximately 20 fold) for group III (IC_{50} = 2.2 ± 0.6 nM) over group II (IC_{50} = 46.2 ± 18.2 nM) mGlu receptors. As with the prototypic, but non-selective mGlu receptor antagonist, (**RS**)-MCPG, (**RS**)-CPPG was a low potency antagonist

($K_B = 0.65 \pm 0.07$ mM) at group I mGlu receptors in neonatal rat cortical slices. In primary cultures of cerebellar granule cells, the active (S)-enantiomer of MCPG again showed a weak antagonism of L-quisqualate-stimulated phosphoinositide hydrolysis ($IC_{50} = 243 \pm 62$ μM) (Toms *et al.*, 1995). (**RS**)-CPPG, at a concentration of 100 μM, was devoid of antagonist activity in these cells. It is noteworthy that (**RS**)-α-methyl-4-phosphonophenylglycine ((**RS**)-MPPG) shows some selectivity for group II ($IC_{50} = 69.5 \pm 0.5$ nM) over group III ($IC_{50} = 156 \pm 29$ nM) mGlu receptors in the adult rat cortex (Bedingfield *et al.*, 1996). Clearly the substitution of the α-methyl group by a cyclopropyl moiety has a profound effect on both group III mGlu receptor antagonist potency and selectivity.

Because of the heterogeneity of mGlu receptors that are present in the cortical slice, it is not possible to attribute the action of (**RS**)-CPPG to any individual mGlu receptor subtype involved in second messenger generation in this preparation. However, it is noteworthy that strong expression of $mGlu_3$ and $mGlu_7$ (in adult rat cortex) and $mGlu_5$ receptors (in neonatal rat cortex) have been demonstrated (Ohishi *et al.*, 1993; Shigemoto *et al.*, 1993; Okamoto *et al.*, 1994; Saugstad *et al.*, 1994; Catania *et al.*, 1994). Cultured cerebellar granule cells demonstrate a pharmacology comparable to that of cloned $mGlu_1$ receptors; a finding that is in accord with the high levels of $mGlu_1$ receptor mRNA in these cells (Santi *et al.*, 1994; Brabet *et al.*, 1995; Toms *et al.*, 1995). However, caution is required, since it is feasible that other, as yet undescribed, mGlu receptor subtypes may also be present. Clearly, determining the selectivity of (**RS**)-CPPG for individual mGlu receptors is imperative and further work using clonal cell lines will be performed in the near future.

A corresponding antagonist action is found with (**RS**)-CPPG in the neonatal rat spinal cord, where (**RS**)-CPPG reverses both L-AP4- and (1S,3S)-ACPD-induced depression of the monosynaptic component of the dorsal root-evoked ventral root potential (DR-VRP). A similar degree of selectivity for group III ($K_D = 1.7$ μM) over group II ($K_D = 53$ μM) mGlu receptors (activated by L-AP4 and (1S,3S)-ACPD, respectively) was observed. Additionally, experiments to examine mGlu *versus* possible iGlu receptor activity, showed that 1 mM (**RS**)-CPPG failed to antagonize either postsynaptic AMPA- or NMDA-induced motoneurone depolarizations, thus confirming mGlu receptor selectivity (Jane *et al.*, 1996).

In summary, (**RS**)-CPPG was found to be a potent antagonist against group II/III mGlu receptors in the adult rat cortex and shows moderate selectivity for group III mGlu receptors. Conversely, (**RS**)-CPPG has weak effects at group I mGlu receptors in both the neonatal rat cortex and cultured cerebellar granule cells. With the rapid advances in our knowledge of mGlu receptor molecular biology over the past few years, there is a considerable demand for potent and selective mGlu receptor ligands. (**RS**)-CPPG should therefore be a useful tool for the delineation of mGlu receptor pharmacology and the further investigation of the physiological roles of mGlu receptors.

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