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ACCELERATED COMMUNICATION

(2S,1′S,2′S,3′R)-2-(2′-Carboxy-3′-phenylcyclopropyl)glycine, a Potent and Selective Antagonist of Type 2 Metabotropic Glutamate Receptors

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SUMMARY

The pharmacological profile of (2S,1′S,2′S, 3′R)-2-(2′-carboxy-3′-phenylcyclopropyl)glycine (PCCG-IV) at metabotropic glutamate receptor (mGluR) subtypes mGluR1a, mGluR2, mGluR4a, and mGluR5 was examined. PCCG-IV potently antagonized glutamate-induced inhibition of forskolin-stimulated cAMP formation in baby hamster kidney cells expressing mGluR2 in a competitive manner (K_B = 8.2 ± 0.4 μM). PCCG-IV was a weak agonist at mGluR4a but inactive at the cloned phosphoinositide-coupled mGluRs (mGluR1a and mGluR5a). PCCG-IV was significantly more potent and selective as an antagonist at mGluR2 compared with previously described mGluR2 antagonists, including α-methyl-4-carboxyphenylglycine. In mice cortical neurons, PCCG-IV antagonized the neuroprotective effects of a selective mGluR2 agonist, (2S,1′R,2′R,3′R)-2-(2,3-dicarboxycyclopropyl)glycine, at low doses (0.2–20 μM), whereas a higher dose of PCCG-IV (80 μM) was similarly neuroprotective to L-2-amino-4-phosphonobutanoate. The neuroprotective effect of PCCG-IV was blocked by an antagonist of mGluR4a, α-methyl-4-phosphonophenylglycine. Thus, PCCG-IV is a novel and useful tool for delineating the physiological roles of group II mGluRs in the central nervous system.

A selective agonist of the G protein-coupled mGluRs, 1S,3R-ACPD has been shown to produce different and often opposing effects on second messenger formation, synaptic transmission, and neuron survival in a variety of preparations derived from the rodent brain (1, 2). Eight subtypes of mGluRs, termed mGluR1–8, provide the molecular basis for the numerous effects of (1S,3R)-ACPD in the central nervous system (1). According to their amino acid sequence homology and coupling to signal transduction pathways in transfected mammalian cell lines, these subtypes have been divided into three distinct groups (3). Group I mGluRs (mGluR1 and mGluR5) are coupled to the phosphoinositide/Ca^{2+} cascade, and group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) mGluRs are both negatively coupled to adenylate cyclase (1, 3). To some extent, these groups can be discriminated pharmacologically with the use of subtype-selective agonists and antagonists. Accordingly, group I, II, and III mGluRs are selectively activated by 3,5-dihydroxyphenylglycine, DCG-IV, and L-AP4, respectively (3–6). At mGluR2, only MCPG has been demonstrated as an effective antagonist, which, however, is equally potent at the cloned subtypes mGluR1a and mGluR2 (7, 8). With subgroup-selective agonists, it has been demonstrated that the original opposing effects of (1S,3R)-ACPD on neuron survival (9, 10) are likely to be related to excarbation of the neurotoxic effects through activation of group I mGluRs (11, 12) as well as to neuroprotective effects resulting from activation of group II and III mGluRs (12–16).

In the present study, PCCG-IV is demonstrated as a potent and selective antagonist of mGluR2 that blocked DCG-IV-induced neuroprotection in cultured cortical neurons. These

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ABBREVIATIONS: (1S,3R)-ACPD, (1S,3R)-1-aminocyclopentane dicarboxylic acid; L-AP4, L-2-amino-4-phosphonobutanoate; BHK, baby hamster kidney; PCCG-IV, (2S,1′S,2′S,3′R)-2-(2′-carboxy-3′-phenylcyclopropyl)glycine; DCG-IV, (2S,1′R,2′R,3′R)-2-(2,3-dicarboxycyclopropyl)-glycine; mGluR, metabotropic glutamate receptor; MCPG, α-methyl-4-carboxyphenylglycine; MPPG, α-methyl-4-phosphonophenylglycine; MTPG, α-methyl-4-tetrazolylphenylglycine; NMDA, *N*-methyl-D-aspartate; PI, phosphoinositide.

data indicate that PCCG-IV is a novel and promising tool for investigating the physiological roles of group II mGluRs.

Experimental Procedures

Materials. PCCG-IV (Fig. 1) is one of the 16 carboxyphenylcyclopropylglycine diastereoisomers synthesized in the laboratory of Dr. R. Pellicciari (University of Perugia, Italy). DCG-IV was kindly provided from Dr. Shinozaki (Tokyo Metropolitan Institute for Medical Sciences, Japan). (1*S*,3*R*)-ACPD, L-AP4, MCPG, MPPG, and MTPG were obtained from Tocris Cookson (Essex, UK). *myo*-[^{3}H]Inositol (specific activity, 17 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Forskolin was obtained from Calbiochem Corp. (La Jolla, CA).

Cell cultures. BHK cells stably expressing mGluR1a, mGluR2, mGluR4, or mGluR5a were cultured in Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal calf serum, 2 mM glutamine, 0.05 mg/ml gentamycin, and 0.1 mg/ml neomycin in a humidified atmosphere (95% air/5% CO_2) at 37°. The medium was supplemented with 0.5 mg/ml G-418 (geneticin) and 1 μM methotrexate (mGluR1a), 0.5 mg/ml G418 (mGluR2), 10 μM methotrexate (mGluR4a), or 2 μM methotrexate (mGluR5a).

Cultures of cortical neurons containing both neurons and glia cells were prepared from fetal mice at 14–16 days of gestation. The cultures were prepared as detailed previously (11) and cultured at 37° in a humidified atmosphere (5% CO_2/95% air) with Eagle's minimal essential medium supplemented with 5% horse serum (heat inactivated), 5% fetal calf serum, 2 mM glutamine, and 21 mM glucose. After 3–5 days *in vitro*, the cells were exposed to cytosine arabinoside (10 μM) for 3 days to inhibit non-neuronal cell division.

Measurements of PI hydrolysis and cAMP formation in transfected BHK cells. Measurements of PI hydrolysis in BHK cells expressing mGluR1a or mGluR5a and accumulation of cAMP in BHK cells expressing mGluR2 or mGluR4a were performed as described previously (8).

Measurements of viability of cultured mice cortical neurons. Measurements of neuron injury were performed with mature cultures of mice cortical neurons/glia cells (13–14 days *in vitro*) through examination of the cultures with the use of phase-contrast microscopy at 24 hr after the insult. The neuroprotective effects of mGluR ligands were examined by exposing the cultures for 10 min to 100 μM NMDA in the presence of various concentrations of test compounds as described previously (16). The method used for assessment of neuron damage on Trypan blue-stained cultures was described previously (16).

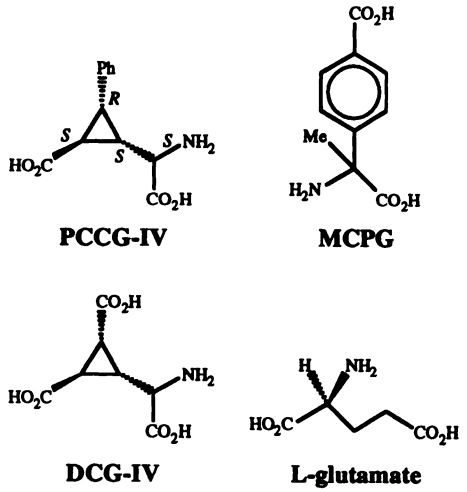

Fig. 1. Chemical structures of antagonists (PCCG-IV and MCPG) and agonists (glutamate and DCG-IV) for mGluR2.

Results and Discussion

In the current study, the pharmacological profile of PCCG-IV, which is a novel derivative of the potent mGluR2 agonist, DCG-IV (Fig. 1), was characterized at subtypes of mGluRs. To determine the activity of PCCG-IV for mGluRs, measurements of PI hydrolysis (mGluR1a and mGluR5a) or cAMP formation (mGluR2 and mGluR4) were performed. PCCG-IV did not increase basal PI hydrolysis or inhibit glutamate-induced PI hydrolysis in BHK cells expressing mGluR1a or mGluR5a (Fig. 2), demonstrating that PCCG-IV lacks affinity for mGluRs linked to PI hydrolysis. In parallel experiments, MCPG, MTPG, and MPPG blocked responses mediated by mGluR1a, whereas only MCPG was a weak antagonist at mGluR5a (Fig. 2). The antagonist potency (IC_{50}) of MTPG and MPPG for mGluR1a was 350 and 1100 μM, respectively (data not shown). When measuring forskolin-induced cAMP formation in BHK cells expressing mGluR2, the effect of glutamate in mGluR2-expressing cells was reversed with the use of PCCG-IV in a dose-dependent manner (Fig. 3A). The mGluR1a/mGluR2 antagonist MCPG (7, 8) completely reversed the effects of glutamate (Fig. 3A). In these experiments, MPPG was also an antagonist at mGluR2 (IC_{50} = 320 ± 40 μM), whereas MTPG was inactive (Fig. 3A). To determine the mode of action of PCCG-IV at mGluR2, dose-response curves for inhibition of cAMP formation by glutamate were performed in the absence or presence of PCCG-IV (Fig. 3B). A rightward shift in the dose-response curves for glutamate was observed in the presence of PCCG-IV, suggesting that PCCG-IV is a competitive antagonist at mGluR2. The potency of PCCG-IV for mGluR2 (K_B = 8.2 ± 0.4 μM) was calculated through linear regression analysis of Schild plots (graph not shown; slope = 0.9 ± 0.1, four experiments). In BHK cells expressing mGluR4a, PCCG-IV did not

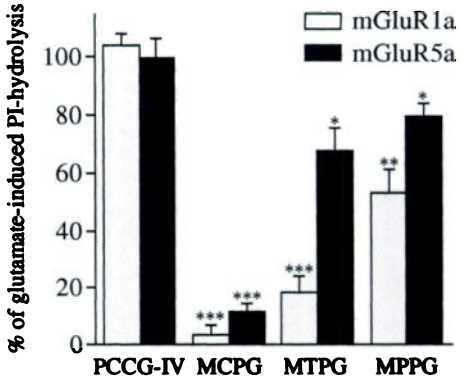

Fig. 2. The effects of PCCG-IV (0.3 mM), MCPG (1 mM), MTPG (1 mM), and MPPG (1 mM) on PI hydrolysis in BHK cells expressing mGluR1a or mGluR5a. The results are expressed as percentage of the response to glutamate with basal levels of PI hydrolysis subtracted and are mean ± standard error of three to five individual experiments performed in triplicate. Antagonists were applied 5 min before a submaximal concentration of glutamate for stimulation of PI hydrolysis (the concentration of glutamate was 10 μM for mGluR1a and 5 μM for mGluR5a). In BHK cells expressing mGluR1a and mGluR5a, the respective levels of basal PI hydrolysis were 4,900 ± 400 and 4,600 ± 500 dpm/mg protein and in the presence of glutamate the levels were 18,400 ± 1,000 and 43,300 ± 2,900 dpm/mg protein. [astc, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by a two-tailed *t* test, significant decrease in glutamate-induced PI hydrolysis in the presence of antagonists compared with control levels. At concentrations of ≤300 μM, PCCG-IV did not increase basal levels of PI hydrolysis in cells expressing mGluR1a or mGluR5a ($p > 0.5$, two-tailed *t* test, six experiments).

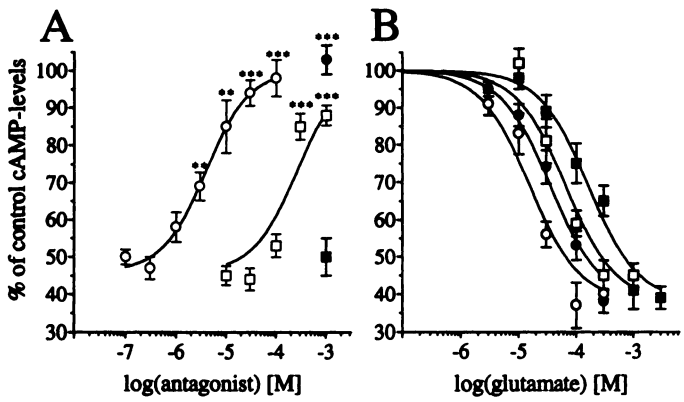

Fig. 3. A, Effects of PCCG-IV (○), MCPG (●), MPPG (□), and MTPG (■) on glutamate-induced inhibition of forskolin-stimulated cAMP-formation in BHK cells expressing mGluR2. B, Effects of glutamate on forskolin-stimulated cAMP formation in the absence (○) or presence of PCCG-IV in concentrations of 10 μM (●), 30 μM (□), or 100 μM (■). Antagonists were applied 2 min before 50 μM glutamate. Values are expressed as percentage of cAMP levels obtained with 10 μM forskolin alone (37 ± 4 pmol cAMP/mg protein) and are mean ± standard error of five (A) or four (B) experiments performed in duplicate. **, $p < 0.01$; ***, $p < 0.001$ by a two-tailed t test, significant reversal of the effects of glutamate.

reverse the inhibition of cAMP-formation induced by glutamate (Fig. 4), whereas MPPG was an effective antagonist at mGluR4a (IC_{50} = 110 ± 20 μM). In contrast, PCCG-IV mimicked the effects of glutamate on cAMP formation in BHK cells expressing mGluR4a (Fig. 4). Thus, PCCG-IV is a potent and competitive antagonist of mGluR2 that shows ≥40-fold selectivity over group I mGluRs and ~20-fold selectivity over mGluR4a in the respective functional assays. Furthermore, the affinities of PCCG-IV for ionotropic glutamate receptors (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate, NMDA, and kainate receptors) or Na^+- and Ca^{2+}/Cl^--dependent glutamate uptake sites are >300 μM.[1] suggesting that PCCG-IV is highly selective for mGluRs. However, although the results of this set of experiments demonstrate that PCCG-IV is a potent and selective antagonist of mGluR2, they do not rule out the possibility that PCCG-IV shows affinity for additional mGluRs (mGluR3, mGluR6, mGluR7, and mGluR8). In particular, it remains to be determined whether PCCG-IV discriminates between mGluR2 and mGluR3, because these subtypes are considered to have a similar agonist selectivity (1, 3, 5). Nevertheless, compared with the mGluR1a/mGluR2 antagonist (+)-MCPG (7), PCCG-IV is 6–7-fold more potent at mGluR2 and shows no activity at mGluR1a. Recently, a derivative of MCPG, MTPG, has been shown to antagonize (1*S*,3*S*)-ACPD-mediated responses in spinal cord neurons, suggesting that this compound is an antagonist of group II mGluRs (17). However, the lack of functional activity of MTPG for mGluR2 (Fig. 3A) suggests that these effects are mediated by other mGluRs, such as mGluR3. In contrast, the antagonist activity of MPPG at mGluR4a (Fig. 4) is good agreement with the ability of MPPG to block L-AP4-mediated responses in spinal cord neurons (17).

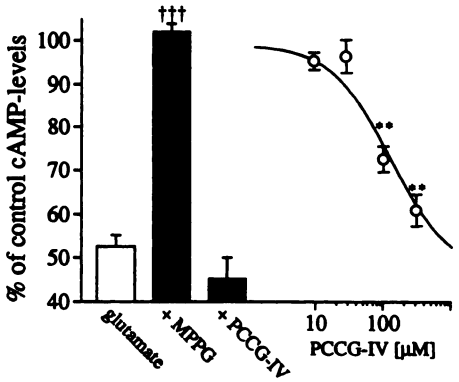

Fig. 4. The effects of PCCG-IV and MPPG at mGluR4a. For testing of antagonist activity, MPPG (1 mM) or PCCG-IV (0.3 mM) was applied 2 min before glutamate (50 μM) and forskolin (10 μM). For testing of agonist activity, PCCG-IV (○) was applied before forskolin. The values are expressed as percentage of cAMP levels in the presence of forskolin and are mean ± standard error of three or four individual experiments performed in duplicate. The basal levels of cAMP formation (2.6 ± 0.3 pmol/mg protein) were stimulated 9–15-fold by forskolin, and the maximal inhibition of forskolin-stimulated cAMP formation by 1 mM glutamate was 47 ± 3%. **, $p < 0.01$ by a two-tailed t test (four experiments, a significant decrease in cAMP levels compared with levels with forskolin alone. †††, $p < 0.001$ by a two-tailed t test (four experiments), reversal of the inhibitory effects of glutamate on cAMP formation by MPPG.

Neuron viability has been shown to be affected differentially by subtype-selective agonists of mGluRs (11–16). We used mixed cultures of cortical neurons and astrocytes to study the influence of PCCG-IV on neurodegeneration. As shown in Fig. 5A, a significant number of cortical neurons were rescued from NMDA-induced cell death with the use of DCG-IV, L-AP4, and PCCG-IV (at 80 μM). Thus, relatively high concentrations of PCCG-IV elicit neuroprotective effects similar to the reported effects of other agonists of group II and III mGluRs (12–16). The neuroprotective effects of PCCG-IV and L-AP4 were fully reversed with the mGluR4 antagonist MPPG (Fig. 5B), further suggesting that the neuroprotective effects of L-AP4 and PCCG-IV are mediated through a group III mGluR, possibly mGluR4a. It should, however, be kept in mind that the selectivity of MPPG over mGluR2 is only 3-fold (see Figs. 2, 3A, and 4). Although lower concentrations of PCCG-IV (0.2–20 μM) did not affect neuron survival, the neuroprotective effect of DCG-IV was dose-dependently reversed by PCCG-IV (ED_{50} = 2 μM) (Fig. 5C). The good agreement between the potency of PCCG-IV for reversing DCG-IV-induced protection of cultured cortical neurons and the observed mGluR2 antagonist activity of PCCG-IV further suggests that a group II mGluR plays an important role in rescuing neurons from excitotoxicity. The most likely neuroprotective mechanisms triggered by mGluR2 activation may include specific down-regulation of NMDA receptor activity (12, 15) and/or a decrease in voltage-gated calcium channel activities (18). Finally, because mGluR2 is highly enriched in the subthalamic nucleus (19), which has been implicated in the pathophysiology of Parkinson's disease (20), mGluR2 antagonists have been proposed as a novel approach for the treatment of this disease (21, 22). In that respect, a compound with a pharmacological profile such as PCCG-IV may have a reduced risk of producing neuron damage (13, 16) due to its mGluR4a agonist activity at higher doses.

[1] C. Thomsen, unpublished observations.

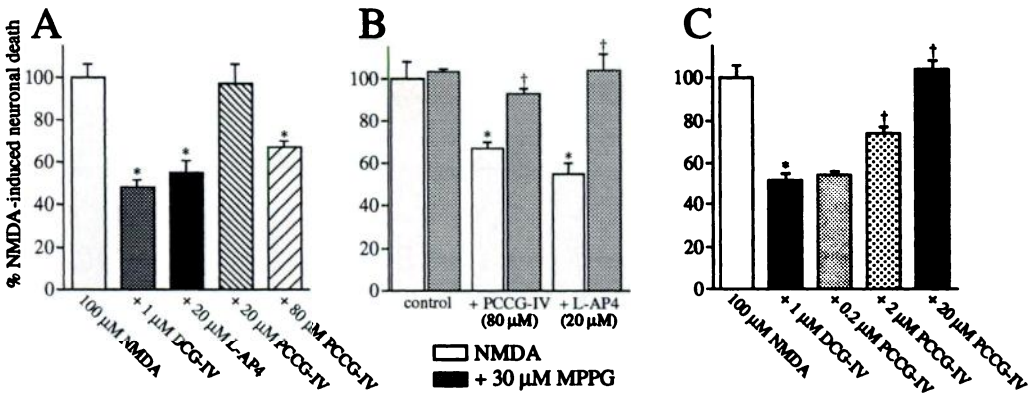

Fig. 5. A, Effects of PCCG-IV, L-AP4, and DCG-IV on NMDA-induced neuron death in cultured mice cortical neurons. B, Reversal of the neuroprotective effects of PCCG-IV and L-AP4 by the mGluR4 antagonist MPPG. C, Reversal of the neuroprotective effects of DCG-IV by PCCG-IV. Compounds were applied to the cells in the doses indicated together with 100 μM NMDA as described in Experimental Procedures. The results (mean ± standard error of three independent experiments performed with at least six determinations) are expressed as percentage of the number of cells stained with Trypan blue after exposure to NMDA and measured 24 hr after the insult (140 ± 8 cells/determination). *, $p < 0.05$ by a one-way analysis of variance and Fisher's PLSD test, significant reduction in the number of cells dying from exposure to NMDA in the presence compared with the absence of mGluR agonists. †, $p < 0.05$ by a one-way analysis of variance and Fisher's PLSD test, reversal of the neuroprotective effects of mGluR agonists by MPPG or PCCG-IV.

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