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# Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells

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**1** 2-(Carboxycyclopropyl)glycines (CCGs) are conformationally restricted glutamate analogues and consist of eight isomers including L- and D-forms. The agonist potencies and selectivities of these compounds for metabotropic glutamate receptors (mGluRs) were studied by examining their effects on the signal transduction of representative mGluR1, mGluR2 and mGluR4 subtypes in Chinese hamster ovary cells expressing the individual cloned receptors.

**2** Two extended isomers of L-CCG, L-CCG-I and L-CCG-II, effectively stimulated phosphatidylinositol hydrolysis in mGluR1-expressing cells. The rank order of potencies of these compounds was L-glutamate > L-CCG-I > L-CCG-II.

**3** L-CCG-I and L-CCG-II were effective in inhibiting the forskolin-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in mGluR2-expressing cells. Particularly, L-CCG-I was a potent agonist for mGluR2 with an $EC_{50}$ value of $3 \times 10^{-7}$ M, which was more than an order of potency greater than that of L-glutamate.

**4** L-CCG-I evoked an inhibition of the forskolin-stimulated cyclic AMP production characteristic of mGluR4 with a potency comparable to L-glutamate.

**5** In contrast to the above compounds, the other CCG isomers showed no appreciable effects on the signal transduction involved in the three mGluR subtypes.

**6** This investigation demonstrates not only the importance of a particular isomeric structure of CCGs in the interaction with the mGluRs but also a clear receptor subtype specificity for the CCG-receptor interaction, and indicates that the CCG isomers would serve as useful agonists for investigation of functions of the mGluR family.

**Keywords:** Excitatory amino acids; glutamate analogue; agonist potency; agonist selectivity; metabotropic glutamate receptor; receptor-expressing cell; signal transduction

## Introduction

L-Glutamate, a major excitatory neurotransmitter in the central nervous system (CNS), plays an important role in neuronal plasticity and neurotoxicity (Monaghan *et al.*, 1989; Collingridge & Singer, 1990; Meldrum & Garthwaite, 1990). Glutamate receptors are classified into two distinct groups termed ionotropic receptors and metabotropic receptors (mGluRs) (Monaghan *et al.*, 1989). The ionotropic receptors comprise integral cation-specific channel complexes and are further subdivided into the receptors for N-methyl-D-aspartate (NMDA) and the non-NMDA receptors for kainate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (Monaghan *et al.*, 1989). The mGluRs are coupled to intracellular signalling transduction via guanine nucleotide-binding proteins (G proteins) (Schoepp *et al.*, 1990; Récasens *et al.*, 1991). Recently, we isolated complementary DNAs (cDNAs) for five different subtypes of the rat mGluR family (mGluR1-mGluR5) (Masu *et al.*, 1991; Tanabe *et al.*, 1992; Abe *et al.*, 1992). The mGluR family shares no sequence similarity with the other members of G protein-coupled receptors and possesses a large extracellular domain preceding the seven putative transmembrane segments (Masu *et al.*, 1991; Houamed *et al.*, 1991; Tanabe *et al.*, 1992). The mGluR subtypes differ in the signal transduction and agonist selectivity and are expressed in specialized neuronal and glial cells of the CNS (Masu *et al.*, 1991; Tanabe *et al.*, 1992; Abe *et al.*, 1992).

Specific agonists and antagonists for glutamate receptors are indispensible for receptor research and have been extensively developed for the ionotropic receptors (Watkins *et al.*, 1990). However, there have been no selective or potent agonists and no antagonists for the mGluR subtypes (Schoepp *et al.*, 1990). Furthermore, the precise characterization of the agonist and antagonist selectivity has been limited in tissue and cell preparations, because of the ambiguity resulting from the presence of multiple subtypes of the glutamate receptors in these preparations. The functional assay of a single cloned receptor expressed in animal cells provides a useful system to determine accurately the selectivity and potencies of agonists and antagonists.

2-(Carboxycyclopropyl)glycine (CCG) is a conformationally restricted glutamate analogue in which the cyclopropyl group fixes the glutamate chain in either an extended or a folded form (Kurokawa & Ohfune, 1985; Yamanoi *et al.*, 1988; Shimamoto *et al.*, 1991). CCG has 8 stereoisomers including L- and D-forms (Figure 1) and serves as a valuable compound for investigating the interaction of glutamate molecules with glutamate receptor subtypes (Shinozaki *et al.*, 1989). Shinozaki and his colleagues reported that the two extended isomers of CCG with L-configuration (2**S**, 1'**S**,2'**S**) isomer (L-CCG-I) and (2**S**,1'**R**,2'**R**) isomer (L-CCG-II), are capable of selectively activating mGluR and thus cause the induction of an electrophysiological response in *Xenopus* oocytes injected with rat brain poly(A)$^+$ RNA (Ishida *et al.*, 1990) and the stimulation of phosphatidylinositol (PI) hydrolysis in rat hippocampal synaptoneurosome (Nakagawa *et al.*,

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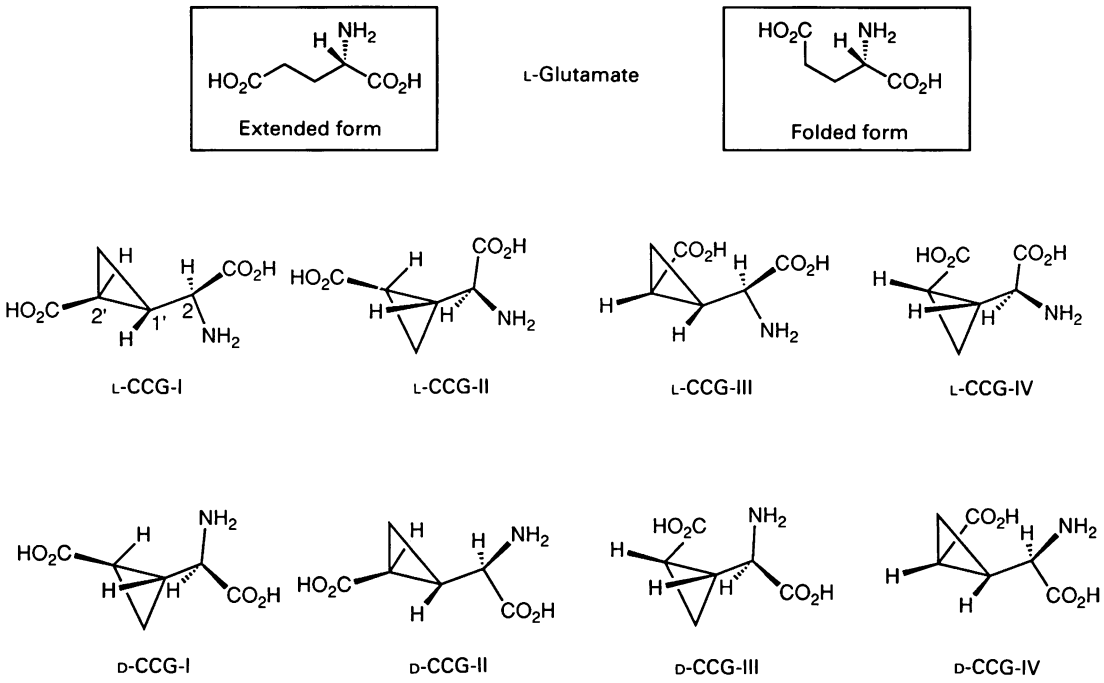


**Figure 1** Eight diastereomers of 2-(carboxycyclopropyl)glycine (CCG).

1990). In this investigation, we tested the agonist effects and selectivities of the 8 CCG isomers on the activities of different mGluR members by measuring intracellular second messengers in Chinese hamster ovary (CHO) cells expressing individual receptor subtypes. We confirmed that L-CCG-I and L-CCG-II specifically activate PI hydrolysis of mGluR1 among the 8 CCG isomers. Furthermore, we found that L-CCG-I acts as a more potent and selective agonist for mGluR2 than for the other members of mGluRs.

## Methods

### *Measurements of phosphatidylinositol hydrolysis*

CHO cell lines stably expressing mGluR1 and mGluR2 were described in our previous papers (Aramori & Nakanishi, 1992; Tanabe *et al.*, 1992). Those expressing mGluR4 were established according to the procedures used for the development of mGluR1-expressing cell lines (Aramori & Nakanishi, 1992). The agonist activity for mGluR1 was assessed by measuring agonist-induced stimulation of total inositol phosphate formation in mGluR1-expressing cells as described previously (Aramori & Nakanishi, 1992). Briefly, mGluR1-expressing cells were seeded in 6-well plates at a density of $3 \times 10^5$ cells per well and cultured for 24 h. The cells were labelled with fresh medium containing [$^3$H]-inositol (1 $\mu$Ci ml$^{-1}$) for 24 h. They were washed with phosphate buffered-saline solution (PBS) and incubated with the same solution for 20 min and then with PBS containing 10 mM LiCl (PBS-Li) for 20 min at 37°C. Agonist stimulation was started by replacing the medium with fresh PBS-Li containing various concentrations of test agents. After incubation for 20 min, the medium was removed, and the reaction was terminated with 5% (w/v) trichloroacetic acid (TCA). The TCA fraction was applied on a Bio-Rad AG1-X8 anion exchanger column, and a mixture of $^3$H-labelled inositol mono-, bis- and tris-phosphates was eluted from the column with 0.1 M formic acid/1.0 M ammonium formate. The radioactivity of eluate was determined by a liquid scintillation spectrometer.

### *Measurements of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation*

Agonist activities for mGluR2 and mGluR4 were evaluated by measuring an agonist-dependent inhibition of forskolin-induced cyclic AMP formation in mGluR2- and mGluR4-expressing cells as described (Tanabe *et al.*, 1992). Briefly, CHO cells expressing either mGluR2 or mGluR4 were seeded in 12-well plates at a density of $1.5 \times 10^5$ cells per well and grown for 2 to 3 days. The cells were washed with PBS containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) (PBS-IBMX) and incubated for 20 min in the same solution. The reaction was started by replacing the medium with fresh PBS-IBMX containing 10 $\mu$M forskolin and various concentrations of test agents. After incubation for 10 min, the medium was removed, and the reaction was terminated with 5% (w/v) TCA. Cyclic AMP levels were determined by the cyclic AMP radioimmunoassay kit.

### *Materials*

Eight stereoisomers of CCG [the (2S,1'S,2'S) isomer (L-CCG-I), (2S,1'R,2'R) isomer (L-CCG-II), (2S,1'S,2'R) isomer (L-CCG-III), (2S,1'R,2'S) isomer (L-CCG-IV), (2R, 1'R,2'R) isomer (D-CCG-I), (2R,1'S,2'S) isomer (D-CCG-II), (2R,1'R, 2'S) isomer (D-CCG-III) and (2R,1'S,2'R) isomer (D-CCG-IV)] (Figure 1) were synthesized according to previously described procedures (Yamanoi *et al.*, 1988; Shimamoto *et al.*, 1991); the carbons 1' and 2' were previously numbered as 3 and 4, respectively, and are renumbered according to the IUPAC nomenclature. Enantiomeric purity of these compounds (>99%) was ascertained by high performance liquid chromatography analysis using an optically active column (Daicel Chemical Industries Ltd., Crownpack CR(+), aqueous $HClO_4$ (pH 2.0) as an eluent). Other chemicals used were of reagent grade and were obtained as described previously (Tanabe *et al.*, 1992).

### *Statistical analysis*

All experiments for measurements of PI hydrolysis and cyclic

AMP formation were carried out in triplicate at least twice. Statistical significance was examined by analysis of variance with Williams multiple-range test.

## Results

### *Effects of CCG isomers on mGluR1*

The five mGluR subtypes can be subclassified into three groups on the basis of the sequence similarity of their primary structures and their signalling cascades: mGluR1/5, mGluR2/3, and mGluR4. They show about 60–70% amino acid homology within the same group of the receptors and about 40% sequence similarity between different groups of the receptors (Tanabe *et al.*, 1992; Abe *et al.*, 1992). mGluR1 and mGluR5 are coupled to PI hydrolysis with a similar profile of the agonist selectivity (Abe *et al.*, 1992). In contrast, mGluR2 and mGluR4 are linked to the inhibitory cyclic AMP cascade in response to the interaction with L-glutamate (Tanabe *et al.*, 1992; see also below). However, the agonist selectivity profiles of these two receptors are clearly different from each other. The rank order of potencies of agonists for mGluR2 was L-glutamate≈(1S*,3R*)-1-aminocyclopentane-1,3-dicarboxylate (tACPD)>ibotenate>quisqualate (Tanabe *et al.*, 1992), whereas the agonist activity of tACPD, quisqualate and ibotenate was very weak or almost negligible for mGluR4 (Tanabe & Nakanishi, unpublished data). This difference probably reflects a low homology between the primary structures of mGluR2 and mGluR4. We chose mGluR1, mGluR2 and mGluR4 as representatives of each group of the receptors and investigated the effects of the 8 CCG isomers on the signal transduction characteristic of the respective receptors. In addition, we included L-glutamate as a control compound for the subsequent analysis of the 8 isomers in receptor-expressing cells. We also confirmed that none of the 8 CCG isomers produced any effects on the signal transduction described below in untransfected cells or cells transfected with the vector DNA alone (data not shown).

The potencies of the 8 CCG isomers for mGluR1 were first examined by determining dose-response curves of these compounds for the stimulation of PI hydrolysis in mGluR1-expressing cells (Figure 2). PI hydrolysis was measured by incubating cells with an individual CCG isomer for 20 min to allow maximal stimulation of inositol phosphate formation, while blocking further dephosphorylation by LiCl. L-Glutamate added to mGluR1-expressing cells evoked stimulation of PI hydrolysis with an $EC_{50}$ value of $1 \times 10^{-5}$ M. The two extended CCG isomers with L-configuration, L-CCG-I and L-CCG-II, similarly showed the stimulation of PI hydrolysis in mGluR1-expressing cells (Figure 2a). The $EC_{50}$ value of L-CCG-I was about $5 \times 10^{-5}$ M, while the potency of L-CCG-II was much lower than that of L-CCG-I. In contrast, no response in the stimulation of PI hydrolysis was evoked by the two folded isomers of L-CCG (L-CCG-III and IV) or by the four D-CCG isomers (D-CCG-I to IV), regardless of whether the structure of D-CCG was extended or folded (Figure 2a and 2b). The result obtained thus demonstrates that the extended isomers of L-CCG specifically interact with mGluR1 but are less potent than L-glutamate in inducing mGluR1-mediated PI hydrolysis.

### *Effects of CCG isomers on mGluR2*

As shown previously (Tanabe *et al.*, 1992), mGluR2 mediates the inhibitory cyclic AMP cascade. In the experiments shown in Figure 3, incubation of mGluR2-expressing cells with 10 μM forskolin evoked cyclic AMP accumulation of approximately 30 times above the basal levels. This accumulation was reduced in a dose-dependent manner by increasing concentrations of L-glutamate and was lowered to about 10% of the maximally accumulated cyclic AMP levels at $1 \times 10^{-4}$ M L-glutamate; the $EC_{50}$ value of L-glutamate was about $2 \times 10^{-5}$ M. Similarly, L-CCG-I and L-CCG-II effectively inhibited the forskolin-stimulated cyclic AMP accumulation and maximally reduced the cyclic AMP formation (Figure 3a). Furthermore, L-CCG-I was highly effective for this receptor and was more potent than L-glutamate in inhibiting the cyclic AMP accumulation. The $EC_{50}$ values of L-CCG-I and L-CCG-II were $3 \times 10^{-7}$ M and $3 \times 10^{-4}$ M, respectively. Folded isomers of L-CCG, on the other hand, showed only a weak agonist activity at the concentration of $1 \times 10^{-3}$ M (Figure 3a). Among the 4 isomers with D-configuration, D-CCG-I exhibited a significant inhibitory activity at high concentrations (Figure 3b). The purity of all isomers of the CCG tested was estimated to be more than 99%. However, because D-CCG-I and its enantiomer, L-CCG-I, showed about 3 orders of magnitude difference in the effective concentrations, it could not be ascertained whether the effect of D-CCG-I reflects its intrinsic activity or arises from the possible presence of a trace amount of L-CCG-I in our D-CCG-I preparation.

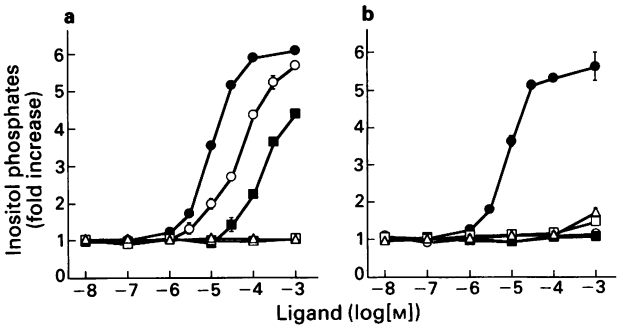


**Figure 2** Dose-response curves of 8 2-(carboxycyclopropyl)glycine (CCG) isomers for stimulation of phosphatidylinositol (PI) hydrolysis in mGluR1-expressing cells. Receptor-expressing cells were incubated with indicated concentrations of L-CCG isomers (a) and D-CCG isomers (b) for 20 min and inositol phosphate formation determined. In (a), L-glutamate (●), L-CCG-I (○), L-CCG-II (■), L-CCG-III (□) and L-CCG-IV (△) were added. In (b), L-glutamate (●), D-CCG-I (○), D-CCG-II (■), D-CCG-III (□) and D-CCG-IV (△) were examined. The inositol phosphate formation is expressed as multiples of inositol phosphate levels in agonist-untreated cells. Basal levels of total inositol phosphates were 1254 ± 142 c.p.m. The data indicated were taken from a representative experiment and the values are means with s.e. (vertical bars) of triplicate determinations.

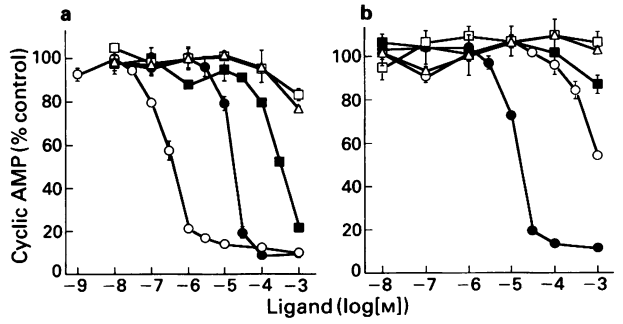


**Figure 3** Dose-response curves of 8 2-(carboxycyclopropyl)glycine (CCG) isomers for inhibition of the forskolin-stimulated cyclie AMP formation in mGluR2-expressing cells. Receptor-expressing cells were incubated with indicated concentrations of L-CCG isomers (a) and D-CCG isomers (b) in the presence of 10 μM forskolin and intracellular cyclic AMP levels determined. Cyclic AMP levels in cells treated and untreated with 10 μM forskolin were 169.1 ± 2.9 and 5.5 ± 0.1 nmol per well, respectively. The former levels were taken as 100%. For further explanation, see Figure 2.

### *Effects of CCG isomers on mGluR4*

As shown in Figure 4, L-glutamate inhibited the forskolin-stimulated cyclic AMP accumulation in mGluR4-expressing cells, but this inhibition did not extend beyond about 40% of the maximally stimulated levels even by the addition of higher concentrations of L-glutamate. Because the partial inhibition of the cyclic AMP formation was consistently observed in several independent mGluR4-expressing cell lines, this inhibition pattern probably represents a feature characteristic of the signal transduction mediated by mGluR4. In mGluR4-expressing cells, L-CCG-I elicited a partial inhibition of the forskolin-stimulated cyclic AMP accumulation (Figure 4a). The potency of this compound was comparable to L-glutamate; the $EC_{50}$ value of L-CCG-I was about $5 \times 10^{-5}$ M, when the maximal inhibition was assumed to occur at $1 \times 10^{-3}$ M L-CCG-I. In contrast to L-CCG-I, none of the other compounds including D-CCG isomers produced any remarkable effects on the mGluR4 activity, though some of these compounds showed a slight inhibition at the concentration of $1 \times 10^{-3}$ M.

## Discussion

The mGluR family consists of multiple receptor subtypes and the expressions of the individual receptors overlap in many neuronal cells (Tanabe *et al.*, 1992; Abe *et al.*, 1992). The determination of the subtype specificity of possible agonists thus requires an assay system in which a single receptor subtype can be characterized without any cross-reactivity of the agonists to other receptor subtypes. In this investigation, we adopted clonal cell lines expressing an individual cloned mGluR subtype to examine the agonist properties of the 8 CCG isomers for mGluRs and to clarify their subtype specificity. Our results unequivocally demonstrate that L-CCG-I and L-CCG-II specifically interact with mGluR1 and thus stimulate PI hydrolysis in mGluR1-expressing cells. This observation is in good agreement with previous studies on rat hippocampal synaptoneurosome and *Xenopus* oocytes injected with brain poly(A)$^+$ RNA (Nakagawa *et al.*, 1990; Ishida *et al.*, 1990). Furthermore, the extended isomers of L-CCG act not only on mGluR1 but also on other mGluR subtypes. Notably, L-CCG-I has a strong potency to interact with mGluR2 and thus effectively inhibits the forskolin-stimulated cyclic AMP formation in mGluR2-expressing cells. The $EC_{50}$ value of L-CCG-I for mGluR2 is $3 \times 10^{-7}$ M, and this potency is more than one order higher than those of L-glutamate and tACPD (Tanabe *et al.*, 1992). The latter compound is the only one that is currently available as an mGluR-specific agonist, but L-CCG-I is more potent than tACPD as an agonist for both mGluR1 and mGluR2; the $EC_{50}$ values of tACPD for mGluR1 and mGluR2 are about $1 \times 10^{-3}$ M and $5 \times 10^{-6}$ M, respectively (Aramori & Nakanishi, 1992; Tanabe *et al.*, 1992). With respect to the subtype specificity of L-CCG-I, the possible reactivity of this compound with mGluR3 must be taken into consideration. We have not yet succeeded in characterizing the agonist selectivity of mGluR3, because of the difficulty in developing cell lines expressing mGluR3 (the reasons for this are unknown). However, mGluR3 shows a high degree of sequence similarity (about 70%) with mGluR2 (Tanabe *et al.*, 1992). It is thus likely that mGluR3 shares similar properties with mGluR2 in signal transduction and the agonist selectivity. Remarkably, L-CCG-I is capable of producing an almost full activity of mGluR2 at low concentrations (e.g. $1 \times 10^{-6}$ M), where virtually no effects on mGluR1 and mGluR4 are produced. Furthermore, electrophysiological and binding studies have indicated that L-CCG-I is only weakly active for the NMDA receptor at high concentrations ($1 \times 10^{-4}$ M) and is totally inactive for the kainate/AMPA receptors (Ishida *et al.*, 1990; Kawai *et al.*, 1992). L-CCG-I may thus be useful for distinguishing functions not only between the ionotropic and metabotropic receptors but also within different subtypes of the mGluR family.

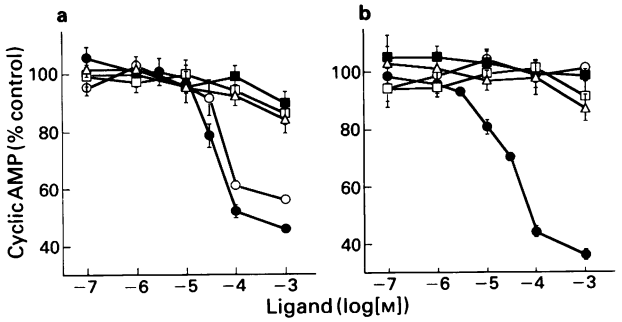


**Figure 4** Dose-response curves of L-2-(carboxycyclopropyl)glycine (L-CCG) isomers (a) and D-CCG isomers (b) for inhibition of the forskolin-stimulated cyclic AMP formation in mGluR4-expressing cells. Intracellular cyclic AMP levels in cells treated and untreated with 10 μM forskolin were 182.2 ± 14.1 and 5.3 ± 0.5 nmol per well, respectively. For further explanation, see Figure 3.

Recently accumulated evidence has indicated that mGluRs play an important role in mediating synaptic transmission in the CNS. Activation of mGluR has been reported to regulate neuronal excitability in the hippocampus through suppression of a $Ca^{2+}$-dependent $K^+$ current and a voltage-gated $K^+$ current (Charpak *et al.*, 1990; Baskys *et al.*, 1990; Desai & Conn, 1991). The involvement of mGluR has also been reported in the depression of synaptic transmission in the hippocampal CA1 synapses (Baskys & Malenka, 1991). mGluRs may also be involved in the neuronal functions of glutamate in other parts of the brain. For example, the functional cooperation of both the AMPA receptor and mGluR seems to be required for long-term depression in cerebellar Purkinje cells (Linden *et al.*, 1991). Glutamate-induced depression of spike discharge observed in cerebellar slice preparations could also result from activation of mGluR in Golgi cells (Yamamoto *et al.*, 1976), where mGluR2 mRNA is prominently expressed (Tanabe *et al.*, 1992). It has been thought that the major effects associated with mGluR activation result solely from the stimulation of PI hydrolysis/$Ca^{2+}$ signal transduction. However, it has now been established that there are several subtypes of mGluRs which are coupled to distinct signal transduction (Tanabe *et al.*, 1992). It has also recently been reported that the selective mGluR activation results in the inhibition of cyclic AMP formation in hippocampal slices (Schoepp *et al.*, 1992). L-CCG isomers selective to mGluR subtypes would thus provide a new tool to investigate which subtype of the mGluRs is responsible for the induction and depression of neuronal transmission as described above.

L-CCG isomers are not only valuable as specific mGluR agonists but also may serve as leading compounds for the synthesis of more specific ligands for mGluRs. Our study has revealed that the agonist activity of CCG isomers for mGluRs is confined to the extended form with the L-configuration at the α-carbon moiety. Conformationally restricted glutamate analogues have provided useful tools for the development of agonists and antagonists for glutamate receptors (Watkins *et al.*, 1990). Fixation of the main chain of glutamate or its longer chain analogues by incorporation of a cyclic structure or a double bond directs the ω-anionic group into a particular steric arrangement with respect to the α-carbon moiety. This strategy has been used successfully for the synthesis of NMDA receptor antagonists (Davies *et al.*, 1986; Murphy *et al.*, 1988; Sills *et al.*, 1991). Therefore, structural modification of L-CCG isomers may be designed to produce specific agonists and antagonists for a certain mGluR subtype. Noteworthy also here is that the only avail-

able radiolabelled compound for the mGluR family is [$^3$H]-glutamate. However, no significant binding of [$^3$H]-glutamate to membrane fractions prepared from mGluR-expressing cells was discernible by filter binding and centrifugation assays, due to high levels of endogenous [$^3$H]-glutamate binding (Aramori & Nakanishi, 1992; Abe *et al.*, 1992). In addition, rapid dissociation of [$^3$H]-glutamate binding is expected from the $EC_{50}$ value of this compound. Thus, incorporation of a tritium into L-CCG-I or its derivative may provide a high-affinity radioligand applicable for a simple binding assay of an mGluR subtype.

In conclusion, this investigation has explored the selectivity and potencies of the 8 CCG isomers for 3 representative subtypes of the mGluR family by examining the effects of these compounds on the signal transduction characteristic of the individual receptors. Our results demonstrate that the two extended isomers of L-CCG specifically interact with the mGluR family. It is particularly interesting that L-CCG-I acts as a potent agonist for mGluR2. These compounds may thus facilitate investigations of the function and role of mGluRs as well as development of specific agonists and antagonists for these receptors.

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