Potent NMDA-like actions and potentiation of glutamate responses by conformational variants of a glutamate analogue in the rat spinal cord

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1 Neuropharmacological actions of all possible-state isomers of α -(carboxycyclopropyl)glycine (CCG), conformationally restricted analogues of glutamate, were examined for electrophysiological effects in the isolated spinal cord of the newborn rat.

2 Eight CCG stereoisomers demonstrated a large variety of depolarizing activities. Among them, the (2R, 3S, 4S) isomers of CCG (D-CCG-II) showed the most potent depolarizing activity, followed by the (2S, 3R, 4S) isomer (L-CCG-IV).

3 The depolarization evoked by L-CCG-IV, D-CCG-II and other D-CCG isomers was effectively depressed by N-methyl-D-aspartate (NMDA) antagonists. D-CCG-II was about 5 times more potent than NMDA in causing a depolarization.

4 The (2S, 3S, 4S) isomer of CCG (L-CCG-I) was more potent than L-glutamate in causing a depolarization of spinal motoneurons. The depolarization was slightly depressed by NMDA antagonists, but residual amplitudes of responses to L-CCG-I in the presence of NMDA antagonists were almost insensitive to 6,7-dinitro-quinoxaline-2,3-dione (DNQX) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), suggesting that L-CCG-I might be a novel potent agonist.

5 After application of the (2S, 3S, 4R) isomer of CCG (L-CCG-III), responses to L-glutamate, D- and L-aspartate were markedly enhanced. The enhancement lasted for a period of several hours without a further application of L-CCG-III.

6 L-CCG-III also caused a depolarization, but it seemed unlikely that the potentiation of the glutamate response was directly related to the depolarization evoked by L-CCG-III.

7 The potentiation might be due to inhibition of uptake processes, but L-CCG-III was superior to L-(–)-threo-3-hydroxyaspartate, a potent uptake inhibitor of L-glutamate and L-aspartate, in enhancing the response to L-glutamate in terms of amplitude and duration of responses.

8 CCG isomers should provide useful pharmacological tools for analysis of glutamate neurotransmitter systems.

Introduction

cis- and *trans*-L- α -(carboxycyclopropyl)glycine (CCG) have been isolated from young fruits of the plants, *Aesculus parviflora* and *Blighia sapida* (Fowden *et al.*, 1969). Both natural CCGs cause a depolarization of neurones in the dorsal horn of the rat spinal cord when administered iontophoretically (McLennan *et al.*, 1982). CCG is a conformationally restricted glutamate analogue in which the cyclo-

propyl group fixes the glutamate chain in an extended or folded form. The glutamate molecule is relatively flexible and so the carbon chain of glutamate is able to conform completely with that of CCG. Thus, it is reasonable to assume that an acyclic transmitter molecule has different conformations and is capable of fitting to the different types of receptors. Therefore, glutamate is supposed to be able to bind to different types of glutamate receptors: kainate-, quisqualate- and N-methyl-D-

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Figure 1 Chemical structures of eight diastereomers of α -(carboxycyclopropyl)glycine (CCG). L-CCG-I and D-CCG-I are abbreviated to L-I and D-I, respectively, and so forth.

aspartate (NMDA)-type receptors. However, the interaction of glutamate with its receptors is not well understood with respect to their preference for particular conformers of the glutamate molecule.

At present, there is no way to determine directly the conformation of transmitter molecules activating their receptors. The only possible technique to analyze the active conformations of transmitter molecules is by chemical modification of agonists and the characterization of the different actions of the various analogues. CCG would be expected to be a good compound for this study. Ohfune and his colleagues (Kurokawa & Ohfune, 1985; Yamanoi *et al.*, 1988) synthesized four isomers of L-CCG expecting that these compounds would provide useful information about the interaction between the conformation of glutamate molecules and activation of glutamate receptor subtypes. In our initial experiments (Shinozaki & Ishida, 1988; Shinozaki *et al.*, 1989), all isomers of L-CCG caused a depolarization of the rat isolated spinal motoneurone with various depolarizing activities. The (2S, 3R, 4S) isomer of L-CCG (L-CCG-IV), a folded form of L-CCG, was the most potent among these isomers, and had a preferred action at the NMDA-type receptor. In addition, the (2S, 3S, 4S) isomer (L-CCG-I), an extended form of L-CCG, was more potent than L-glutamate (Shinozaki & Ishida, 1988; Shinozaki *et al.*, 1989), and L-CCG-I had a preferred action at the non-NMDA-type receptors, although it was much less potent than either kainate or quisqualate. Thus,

L-CCG isomers gave us an insight into the conformation of glutamate molecules for activating their receptors.

CCG has eight stereoisomers theoretically, including L- and D-forms (Figure 1). Some of these isomers of CCG would be expected to provide additional information about the interaction of glutamate molecules with excitatory amino acid receptor subtypes, and thus to be potentially valuable compounds for analyzing mechanisms of the physiological effect of glutamate. In experiments in the rat isolated spinal cord, we have now examined the different responses to these CCG isomers arising from their conformational variation. The potent NMDA-like actions, marked potentiation of glutamate responses and a new-type depolarization were observed with some of the compounds and of particular interest.

Methods

The newborn rat spinal cord has been widely used for experiments on the mammalian CNS because of the ease of changing drug concentrations of the bathing solution (Otsuka & Konishi, 1974; Ault *et al.*, 1980; Agrawal & Evans, 1986). The methods used for the electrophysiological experiments in the newborn rat isolated spinal cord (1–5 day-old Wistar rats, $n = 94$) were essentially similar to those described previously (Ishida & Shinozaki, 1988a,

1988b; Shinozaki & Ishida, 1988). Under ether anaesthesia, the lumbar-sacral spinal cord was isolated, hemisected sagittally and placed in a 0.15 ml bath perfused at a fixed flow rate of 5–6 ml min⁻¹ with artificial cerebrospinal fluid (composition in mM: NaCl 138.6, KCl 3.4, CaCl₂ 1.26, NaHCO₃ 21.0, NaH₂PO₄ 0.58, glucose 10.0, and tetrodotoxin (TTX) 0.5 μ M, pH 7.4) which was oxygenated with a gas mixture of 95% O₂ and 5% CO₂. TTX was added to the bathing solution in order to block spontaneous depolarization and indirect drug effects. In some cases, MgCl₂ was added to the perfusing fluid in a concentration of 1.0 mM to examine the depolarizing activity of NMDA-type agonists (Mayer *et al.*, 1984; Nowak *et al.*, 1984). The potential changes generated in the motoneurons were recorded extracellularly from the L₃–L₅ ventral roots with a suction electrode. The tip of the suction electrode was a glass capillary, with an inner diameter that fitted tightly onto the recorded ventral roots in order to enhance the isolation impedance, which severely affected recording of the responses. After selecting only those spinal cord preparations, which demonstrated the polysynaptic reflexes of above 2 mV in amplitude in the Mg²⁺ (1 mM)-containing, TTX-free saline, the perfusing solution was replaced by normal Mg²⁺-free and TTX-containing solution. Excitatory amino acids and other test compounds were applied to the preparation either by perfusion or by brief pulse injection into the perfusion system. The temperature of the perfusing fluid was kept at 27°C.

Drugs

Eight stereoisomers of α -(carboxycyclopropyl)glycine (CCG) [the (2S, 3S, 4S) isomer (L-CCG-I), the (2S, 3R, 4R) isomer (L-CCG-II), the (2S, 3S, 4R) isomer (L-CCG-III), the (2S, 3R, 4S) isomer (L-CCG-IV), the (2R, 3R, 4R) isomer (D-CCG-I: m.p. 240–242°C, [α]²⁵D –94.7° (c 0.50, H₂O)), the (2R, 3S, 4S) isomer (D-CCG-II: m.p. 254–258°C, [α]²⁵D +21.6° (c 0.50, H₂O)), the (2R, 3R, 4S) isomer (D-CCG-III: m.p. 202–203°C, [α]²⁵D –15.6° (c 0.50, H₂O)) and the (2R, 3S, 4R) isomer (D-CCG-IV: m.p. 172–180°C, [α]²⁵D –86.7° (c 0.50, H₂O))]. D-CCG isomers were newly synthesized according to the previously reported procedure with (2R)-2-amino-3-butenol used as the starting material (Yamanoi *et al.*, 1988). Spectroscopic data of D-CCGs were completely identical with those of the respective corresponding L-CCGs. Enantiometric purity of these compounds found to be >98% was ascertained by high performance liquid chromatography (h.p.l.c.) analysis using an optically active column (Daicel Chemical Industries Ltd., CROWNPACK CR(+), pH 2.0 aqueous HClO₄ as an eluent).

The following compounds were used: ($\pm$)-2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, Tocris), D-(–)-2-amino-5-phosphonovaleric acid (D-APV, Tocris), D- and L-aspartic acid (Nacalai Tesque), 3-[($\pm$)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP, Tocris), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris), L-cysteic acid (Sigma), dihydrokainic acid (Tocris), 6,7-dinitro-quinoxaline-2,3-dione (DNQX, Tocris), D-glutamic acid (Nacalai Tesque), sodium L-glutamate mono (Wako), L-homocysteic acid (Sigma), D-(+)- and L-(–)-threo-3-hydroxyaspartic acid (Tocris), L- α -kainic acid (Sigma), N-methyl-D-aspartic acid (NMDA, Sigma), 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, Merck), picrotoxin (Tokyo Kasei), L-quisqualic acid (purity >99.7%) isolated from the plant, *Quisqualis indica* L, and strychnine hemisulphate salt (Sigma).

Results

Various depolarizing activities of CCGs

When eight CCG stereoisomers used in the present study (see Figure 1) were added to the bathing solution for various times, all CCG isomers demonstrated a depolarization of the spinal motoneurons with a large variation in their depolarizing activity (Figure 2). When these compounds were added to the perfusing fluid for a period of more than 30 s, the amplitude of responses to L-CCG-I, IV and D-CCG-IV was not maintained but gradually declined despite the presence of these agonists (Figure 2a). This decrease in amplitudes of responses to these CCGs during their application appears to be due to the desensitization of receptors for excitatory amino acids. The decline was observed even in small amplitudes of the responses in spite of the fact that small amplitudes of responses to L-glutamate did not decline during its application. The depolarizing responses were generally reproducible in any preparations and isomers and would be roughly divided into two groups showing fast and slow responses, which was evident in the case of a short period application (Figure 2b). NMDA, L-CCG-I, D-CCG-I, II and III showed slow responses (the prolonged time course of action), while L-CCG-II, III, IV and D-CCG-IV gave fast responses. In particular, L-CCG-I had a very long time course of action. It may be noted, in passing, that kainate produces slow responses, but quisqualate and L-glutamate produce fast responses in this preparation (Shinozaki *et al.*, 1989). D-CCG-II seemed to be the most potent among the CCG isomers when compared in terms of

Figure 2 Sample records of responses to eight α -(carboxycyclopropyl)glycine (CCG) isomers, L-glutamate (Glu) and N-methyl-D-aspartate (NMDA). Responses to each excitatory amino acid were recorded from the ventral root extracellularly in Mg^{2+} -free, tetrodotoxin ($0.5 \mu M$)-containing solution. All test samples were added to the bathing solution for a period of 2 min (a) or 10 s (b). Numerals under each trace represent the concentrations (μM) of test compounds. All records in each group were obtained from the respective preparation. L-CCG-I and D-CCG-I are abbreviated to L-I and D-I, respectively, and so forth. Scale: 0.5 mV, 5 min.

peak amplitudes of their responses. As shown in our previous paper (Shinozaki *et al.*, 1989), L-CCG-IV also demonstrated a high depolarizing activity, but it was apparently less potent than D-CCG-II.

Several factors, such as desensitization of receptors, equilibrium constants of agonist-receptor complexes and differences in rates of uptake of agonists, would affect the peak amplitude of depolarizing responses, which sometimes varied in accordance with the duration of application of agonists. Therefore, it seemed to be technically difficult to determine exactly the relative potency of excitation induced by CCGs in terms of the peak amplitude. For the experiment shown in Figure 3, peak amplitudes of responses were plotted against the concentrations of excitatory agonists, including representative excitatory amino acids, which were added to the per-

Figure 3 The concentration-response curves of eight stereoisomers of α -(carboxycyclopropyl)glycine (CCG) and representative excitatory amino acids. Experimental conditions were similar to those in Figure 2. Peak amplitudes of depolarization (mV) are plotted against the concentrations (μM): (a) 2 min application, (b) 10 s application. Glu: L-glutamate, Asp: L-aspartate, K: kainate, Q: quisqualate. L-CCG-I and D-CCG-I are abbreviated to L-I and D-I, respectively, and so forth. Vertical bars represent s.e.mean (n at least 4).

fusing fluid for a period of 10 s or 2 min. It was noted that the order of depolarizing activities of CCGs in these two cases did not vary much except for L-CCG-IV and D-CCG-IV, which produced fast desensitizing responses (Figure 2a), that appeared relatively less potent from 2 min applications.

Pharmacological classification of responses to CCGs

When the Mg^{2+} -free solution was changed to one containing 1 mM Mg^{2+} to test the NMDA-type depolarizing activity of all CCGs (all 10 s applications), responses to L-CCG-IV and all stereoisomers of D-CCG were markedly reduced, those to L-CCG-I (0.1 mM) were insensitive to 1 mM Mg^{2+} ions and those to L-CCG-III (1 mM) were slightly reduced. After the preparation was exposed to MK-801 ($2 \mu M$) for more than 30 min (Wong *et al.*, 1986), repetitive responses to NMDA ($20 \mu M$), L-CCG-IV ($10 \mu M$) and D-CCG-II ($5 \mu M$) gradually decreased in a use-dependent manner, while the responses to kainate ($3 \mu M$) or quisqualate ($2 \mu M$)

Figure 4 Sample records of depolarizing responses to N-methyl-D-aspartate (NMDA), L-CCG-IV and all D-isomers in the absence and presence of a considerably high concentration of D-(−)-2-amino-5-phosphorvaleric acid (D-APV). All responses were almost completely abolished after application of D-APV (0.05–0.1 mM) and the effect of the antagonists was fully reversible upon washout. Agonists (NMDA: 10 μ M, L-CCG-IV: 10 μ M, D-CCG-I: 0.1 mM, D-CCG-II: 2 μ M, D-CCG-III: 1 mM, D-CCG-IV: 0.2 mM) were added to the perfusing fluid for 30 s. D-APV was added to the perfusing fluid 90 s before the administration of agonists for 3 min, as indicated by rectangles beneath trace. Numerals under each trace represent concentrations of D-APV in mM.

remained unchanged and those to L-CCG-I (0.1 mM) were only slightly reduced or unchanged. Amplitudes of responses to L-glutamate (1 mM) were reduced by about 85% of the control. Neither strychnine (10 μ M) nor picrotoxin (10 μ M) affected the depolarizations induced by D-CCG-II and L-CCG-IV.

The above experiments using non-competitive NMDA blockers suggest that L-CCG-IV and all stereoisomers of D-CCG preferentially activate NMDA-type receptors. Next, effects of competitive NMDA blockers were examined (Davies *et al.*, 1981; 1986). The blockers were added from 90 s before the application of agonists to the end of the agonist actions in a wide range of concentrations to block NMDA responses fully. When responses to all CCGs were examined in the presence of CPP (1–100 μ M) and D-APV (10–100 μ M), both blockers reduced responses to L-CCG-IV and all D-CCGs in a dose-dependent manner. Figure 4 shows that high concentrations of D-APV almost completely abolish responses to NMDA, L-CCG-IV and all D-isomers of CCG. Responses to D-CCG-II (2 μ M) were completely abolished by 50 μ M D-APV. Likewise, responses to L-CCG-IV (10 μ M), D-CCG-III (1 mM) and D-CCG-IV (0.2 mM) were also completely abolished. Residual amplitudes of responses to L-CCG-I and II were about 70% of the control in the presence of these NMDA blockers (see below). Responses to L-CCG-III (1 mM) were reduced by about 50% in the presence of 10 μ M D-APV, and by about 30% or less in the presence of 30–100 μ M CPP or 100 μ M D-APV, but residual amplitudes were still observed even in higher concentrations of NMDA blockers. The effects of these NMDA competitive blockers were fully reversible upon washout. Figure 5 represents the relationship between the agonist

concentration and the peak amplitude of responses in the absence and presence of CPP (2, 5, 10 μ M). This figure demonstrates that CPP shifts the dose-response curve of D-CCG-II, L-CCG-IV and NMDA in an almost parallel manner. The pA_2 values were 5.99 ± 0.08 ($n = 4$), 5.90 ± 0.03 ($n = 4$) and 5.86 ± 0.04 ($n = 4$) for CPP versus D-CCG-II, L-CCG-IV and NMDA, respectively. In all cases the pA_2 values were not significantly different from 6.0.

As mentioned above, L-CCG-IV and all D-isomers demonstrated almost exclusive preference for NMDA-type receptors. Here, the preference of remaining CCGs (L-CCG-I, II and III) for each receptor subtype was examined by a single or combined application of various blockers for excitatory amino acids. CNQX (10–300 μ M) and DNQX (10–300 μ M) were used for these experiments in addition to D-APV and CPP (0.1 mM). CNQX and DNQX have been characterised as a broad spectrum excitatory amino acid antagonist. They are potent competitive antagonists at non-NMDA receptors and have non-competitive actions at NMDA receptors, although they do not distinguish between the receptors for quisqualate and kainate (Birch *et al.*, 1988; Honore *et al.*, 1988). The blockers were added from 90 s before the application of agonists for a period of 3 min. At a concentration of 10 μ M, CNQX and DNQX considerably reduced responses to L-glutamate (0.3–0.7 mM), kainate (2–5 μ M), NMDA (5–10 μ M), quisqualate (0.3–2 mM) and AMPA (2–5 μ M) and at 0.1 mM they completely abolished responses to kainate (2–5 μ M), NMDA (5–10 μ M) and AMPA (2–5 μ M) (Figure 6e, f, h). However, responses to L-glutamate (0.3–0.7 mM) and quisqualate (0.3–2 mM) were still observed even in the presence of CNQX or DNQX (up to 0.3 mM) and D-APV (0.1 mM) (Figure

Figure 5 Dose-response curves of (a) L-CCG-IV, (b) D-CCG-II and (c) N-methyl-D-aspartate (NMDA) in the absence and presence of 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP). Agonists were added to the perfusing fluid for a period of 10 s. CPP was added to the bathing solution in concentrations of 2 (●), 5 (▲), 10 (■) μM from 90 s prior to the agonist application and was present to the end of the agonist action; (○) control response. Peak amplitudes of response to excitants were plotted against their logarithmic concentrations. Vertical bars represent s.e.mean (n at least 4).

6d, g, h). Residual amplitudes of responses to L-glutamate (0.5 mM) and quisqualate (1 μM) were $28 \pm 2\%$ and $21 \pm 1\%$ of the control, respectively ($n = 4$), in the presence of 0.1 mM of both D-APV and CNQX. Responses to L-CCG-I (50 μM) and L-CCG-II (1 mM) were merely decreased slightly by the further addition of non-NMDA blockers (Figure 6a, b). Responses to L-CCG-I (50 μM) were reduced by $76 \pm 4\%$ ($n = 4$) in the presence of 0.1 mM D-APV, and by $77 \pm 2\%$ ($n = 4$) in the combination of

D-APV and CNQX (0.1 mM). It was of great interest that responses to L-CCG-I and L-CCG-II were not reduced by even high concentrations of CNQX in combination with CPP or D-APV. L-CCG-I and L-CCG-II might belong to a new category of excitatory amino acids. Residual components of responses to L-CCG-III in the presence of D-APV or CPP (about 30%) were markedly reduced by CNQX (0.1 mM) (Figure 6c). Similar results were obtained when DNQX (0.1 mM) was applied. The pharmacological property of responses to L-CCG-III will be mentioned in a later paragraph because of potentiation of its own and L-glutamate responses.

Glycine in micromolar concentrations has been reported to augment the excitatory action of NMDA in cultured cortical and diencephalic neurones (Johnson & Ascher, 1987). This potentiation is insensitive to strychnine, and seems to be one of the characteristic properties of the NMDA response in the mammalian central nervous system. Since D-CCG-II and L-CCG-IV were shown in the present study to be potent NMDA-type agonists, we examined the action of glycine on responses to these agonists in the rat isolated spinal cord, but we were unable to demonstrate that superfusion with glycine (1 μM–1 mM) facilitated significantly depolarizations evoked by CCG-isomers in strychnine-containing (10 μM) solution. This is interpreted as meaning that the endogenous levels of glycine were already maximal for this effect in the rat isolated spinal cord. In order to demonstrate the potentiation of the NMDA-type response by glycine in the rat spinal cord, other experimental conditions would be required (Fletcher & Lodge, 1988).

Marked potentiation of glutamate responses by L-CCG-III

As shown above, L-CCG-III caused a depolarization of the rat spinal motoneurone. In addition to its own depolarizing action, it caused marked potentiation of responses to L-glutamate and L-aspartate in the rat isolated spinal cord. This potentiation lasted for a long time. After a single application of L-CCG-III (1 mM, 10–15 s), the preparation was thoroughly washed by the normal bathing solution, and the depolarizing response was restored very quickly. When the periodic addition of L-glutamate to the bathing solution was resumed without simultaneous application of L-CCG-III, the amplitude of their responses was markedly enhanced (Figure 7a, c). When L-glutamate was applied for a long time, a decline in amplitude, probably due to receptor desensitization, was observed during application of L-glutamate (Figure 7c), in striking contrast to the control response. The potentiation of the glutamate response lasted for a period of more than several

Figure 6 Responses to L-CCG-I, L-CCG-II and L-CCG-III in the presence of high concentrations of 3-[($\pm$)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP), D-(–)-2-amino-5-phosphonopentanoic acid (D-APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Agonists were added to the perfusing fluid for a period of 30 s except L-CCG-III, and antagonists were added for a period indicated by columns in a single or in combination with CNQX. Numerals represent the concentrations of antagonists (mM). (a) L-CCG-I (50 μ M); (b) L-CCG-II (1 mM); (c) L-CCG-III (1 mM, 20 s); (d) L-glutamate (0.5 mM); (e) kainate (2 μ M); (f) N-methyl-D-aspartate (NMDA) (5 μ M); (g) quisqualate (1 μ M); (h) Q: quisqualate (1 μ M), L-I: L-CCG-I (30 μ M), G: L-glutamate (0.5 mM), K: kainate (5 μ M), N: NMDA (10 μ M), A: 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (5 μ M). Note that residual amplitudes of responses to quisqualate are at an almost similar level 90 s and 40 min after the addition of CNQX.

hours without a further application of L-CCG-III (Figure 7a). Responses to L-aspartate were also enhanced by L-CCG-III in a similar manner to L-glutamate, and the potentiation of responses to L-glutamate or L-aspartate was reinforced by repeated application of L-CCG-III (Figure 7b). The above case is one example of the effects observed after a single application of L-CCG-III; however, marked potentiation is seen even when both L-glutamate and L-CCG-III are added simultaneously to the bathing solution (Figure 7d). In this case, the minimum effective concentration of L-CCG-III causing the potentiation seemed to be significantly less than 50 μ M. At low concentrations of L-CCG-III, the potentiation did not continue for a long time, in spite of the fact that the response amplitude was considerably augmented at the first stage (Figure 7d). Other isomers of CCG did not demonstrate such potentiation of responses to L-glutamate or L-aspartate.

When L-glutamate or L-aspartate was applied in relatively high or low concentrations, L-CCG-III (1 mM) did not augment significantly responses to them, but in dose-ranges where log-log plots of responses to L-glutamate or L-aspartate against the concentration represent almost a straight line, L-CCG-III significantly augmented these responses, as if the dose-response curve was shifted to the left in a parallel manner (Figure 8).

We examined the responses to a variety of amino acid excitants which were affected by L-CCG-III. Responses to L-glutamate, D- and L-aspartate, L-cysteate and L-CCG-III were potentiated by L-CCG-III (1 mM), but those to kainate, quisqualate, NMDA, D-glutamate, L-CCG-I, IV, D-CCG-I, II and IV were hardly affected by L-CCG-III (1 mM).

As shown above, L-CCG-III seems unlikely to be a pure NMDA-type agonist. To test whether the potentiation of the glutamate response is directly

Figure 7 Potentiation of responses to L-glutamate and L-aspartate by L-CCG-III. Glu: L-glutamate, Asp: L-aspartate. (a) After single application of L-CCG-III (○, 1 mM, 15 s), marked potentiation of responses to L-glutamate (▲, 1 mM, 10 s) was observed, and it lasted for a long time. Even 3 h later, a larger response was still observed. (b) Responses to L-aspartate (△) were also augmented by L-CCG-III (○) in a similar manner to those to L-glutamate (▲). (c) Responses evoked by a prolonged application (2 min) of agonists. Numerals represent the concentration (mM) of L-glutamate and L-CCG-III. (d) Simultaneous application of L-CCG-III and L-glutamate. L-CCG-III (0.05 and 0.1 mM) was added with L-glutamate (1 mM, 10 s) for a period indicated by horizontal bars. Relatively rapid restoration was observed.

related to the L-CCG-III-evoked depolarization, the potentiation was examined under conditions where the depolarization was extremely reduced by Mg^{2+} , CPP and DNQX. The responses to L-glutamate (1 mM) were slightly reduced by changing from Mg^{2+} -free to Mg^{2+} -containing normal solution, but, even in the Mg^{2+} -containing solution, the marked potentiation of responses to L-glutamate was still observed after application of L-CCG-III (1 mM). When L-CCG-III (1 mM) was added to the bathing solution in combination with CPP (10 μ M) or DNQX (0.1 mM) in order to reduce the peak amplitude of responses to L-CCG-III, the glutamate response was greatly augmented after the application of L-CCG-III. Even when both CPP (10 μ M) and DNQX (20 μ M) were added simultaneously to the bathing solution in order to reduce further the L-CCG-III response, the marked potentiation was still observed, and the duration of potentiation remained unchanged.

Uptake inhibitors and L-CCG-III

As a possible mechanism for the potentiation, it is reasonable to assume that L-CCG-III blocks uptake of excitatory amino acids from the vicinity of their receptors by the surrounding neurone and glia (Watkins & Evans, 1981). Some amino acids, such as D-(+)- and L-(-)-*threo*-3-hydroxyaspartate, dihydrokainate and L-cysteate, have been known to inhibit the uptake of glutamate and aspartate in the mammalian central nervous system (Balcar & Johnston, 1972; Johnston *et al.*, 1979; 1980). So, we compared the actions of L-CCG-III and these uptake inhibitors on the potentiation of the glutamate response. Both D-(+)- and L-(-)-*threo*-3-hydroxyaspartate caused a slight depolarization of the rat spinal motoneurone to a similar degree, but the L-(-)-isomer was superior to the D-(+)-isomer in causing the potentiation of the glutamate response. L-(-)-*threo*-3-hydroxyaspartate (1 mM) augmented

Figure 8 Dose-response curves of L-glutamate before and after application of L-CCG-III. L-Glutamate (10 s) was added to the bathing solution in various concentrations before (○) and after (●) three single applications of L-CCG-III (1 mM, 15 s). Ordinate scale: amplitude of the glutamate response (mV). Abscissa scale: logarithmic concentrations of L-glutamate.

the response to L-glutamate (1 mM) in a very similar manner to L-CCG-III. However, the duration of its action was considerably shorter than that of L-CCG-III (1 mM), demonstrating that L-CCG-III was more potent than L-(−)-*threo*-3-hydroxyaspartate (Figure 9). In the case of *threo*-3-hydroxyaspartate, both L- and D-isomers demonstrate an inhibitory effect on uptake of L-glutamate or L-aspartate, although the

L-isomer is approximately twice as potent as the D-isomer with respect to the uptake of either L-glutamate or L-aspartate (Balcar *et al.*, 1977). However, in contrast to L-CCG-III, D-CCG-III hardly affected the responses to these excitatory amino acids. Dihydrokainate was a weak agonist in the rat isolated spinal cord, and caused a slow depolarization of the motoneurons. Dihydrokainate did not affect the response to L-glutamate or L-aspartate in the rat isolated spinal cord. L-Cysteate caused a depolarization of the motoneurone to a similar degree to L-aspartate, but did not enhance the response to L-glutamate.

The spinal reflex evoked by electrical stimulation (duration 100 μ s, 10 V, at intervals of about 1 min) of dorsal root (L_4 or L_5) was hardly affected by the one-shot application of L-CCG-III (1 mM) or L-(−)-*threo*-3-hydroxyaspartate (1 mM). When L-CCG-III was added to the bathing solution for a period of 3 min in a concentration of 0.1 mM, potentiation of the spinal reflex was not observed.

Discussion

NMDA-like actions

The isomers of L- and D-CCG were synthesized in order to gain some insight into possible conformations of L-glutamate molecules during activation of each glutamate receptor subtype. As a result, it was found that all D-isomers of CCG and a folded form of L-CCG (L-CCG-IV) activated selectively the NMDA-type receptor. Binding assays of CCG

Figure 9 Comparison of the potentiation of the glutamate response induced by L-CCG-III and L-(−)-*threo*-3-hydroxyaspartate. L-Glutamate was added to the bathing solution for a period of 10 s in a concentration of 1 mM. The potentiating effect of L-CCG-III (L-III, ○, 1 mM, 15 s) was more pronounced and lasted for a longer time than that of L-(−)-*threo*-3-hydroxyaspartate (THLA, ○, 1 mM, 15 s).

isomers using labelled CPP in the rat hippocampus neurone have supported our present results (Kawai & Ishihara, unpublished observation). The structure of L-CCG-IV, as mentioned above, mimics very closely the folded conformation of L-glutamate, therefore, the present results suggest that an active conformation of the L-glutamate at the NMDA-type receptor is a folded form. It is of great interest that all D-isomers of CCG, which include both the folded and extended forms, cause a depolarization due to the selective activation of the NMDA-type receptor in the rat isolated spinal cord. Among D-isomers, D-CCG-II demonstrated an extremely potent depolarizing activity. Although the data concerning the depolarizing activity of other NMDA-type agonists were not shown in Figure 3, the depolarizing activity of other NMDA-type agonists was not always superior to that of NMDA. The depolarizing activity of L-homocysteate was almost similar to that of NMDA in the rat isolated spinal cord preparation, L-cysteate being less potent than NMDA. Thus, of those compounds we examined on the rat isolated spinal cord, D-CCG-II is one of the most potent NMDA-type agonists. While D-CCG-II and L-CCG-IV are markedly potent NMDA-type agonists, there is no apparent structural similarity between them. As shown in the Figure 1, the cyclopropyl ring of CCGs fixes its carbon chains in extended or folded forms close to those adopted by L-glutamate. L-CCG-IV corresponds to a folded form of L-glutamate from the point of view of its configuration as well as conformation, on the other hand, D-CCG-II corresponds to an extended form of glutamate in its conformation but not in configuration. The polar functional groups of D-CCG-II and L-CCG-IV, which are probably binding sites with the receptors, never accurately superimpose. The carbon chain of aspartate is one atom shorter than that of glutamate and the ionizable groups of aspartate and NMDA cannot match those of glutamate. The NMDA-type receptor might have high affinity for substrates with the D-configuration by unknown mechanisms. The conformational arrangement of the D-isomer when it interacts with receptors remains to be solved (Watkins & Olverman, 1987; Magnuson *et al.*, 1988).

A novel agonist, L-CCG-I

L-CCG-I is more potent than L-glutamate in causing a depolarization in the rat isolated spinal cord. Responses to L-CCG-I and II were merely decreased slightly by Mg^{2+} and competitive NMDA blockers. Residual amplitudes of responses to these CCGs in the presence of competitive NMDA antagonists were almost insensitive to CNQX or DNQX. Their conformation is the extended form. At the crayfish neuromuscular junction, ibotenic acid demonstrated

an increase in Cl^- permeability which was blocked by picrotoxin (Shinozaki & Ishida, 1980), likewise, L-CCG-I showed a quite similar action to ibotenic acid at this junction (unpublished observation). It is of great interest to examine the similarity between L-CCG-I and ibotenic acid in detail from the chemical viewpoint. Our results demonstrate a clear preference of L-CCG-I for non-NMDA, non-kainate and non-quisqualate-type receptors. Similar results were obtained in the case of L-CCG-II. Such results may suggest that L-glutamate has an additional mechanism for exciting spinal neurones (Honore *et al.*, 1988). The development of this new and potent agonist opens the possibility for extensive studies of the glutamate receptor subtypes. Further studies will be required to clarify the receptor subtype to which L-CCG-I binds.

Potentiation of glutamate responses

Only L-CCG-III was found to potentiate markedly the response to L-glutamate and D- and L-aspartate. In contrast to L-CCG-IV, which possessed potent depolarizing activity, L-CCG-III showed considerably lower depolarizing activity despite the fact that it has a similar folded structure to L-CCG-IV, and the configurations at β - and γ -carbon atoms are the only difference between them. Activation of the NMDA-type receptor and the potentiation of the response to L-glutamate are certainly differentiated by the steric role of the cyclopropyl group of CCG. In spite of the fact that the depolarization evoked by L-CCG-III is mainly due to the activation of the NMDA-type receptor, the potentiation of response to L-glutamate does not seem to be directly related to the activation of NMDA-type receptors.

One may consider some possibilities for the mechanism underlying the potentiation of the glutamate response, such as inhibition of uptake of agonists from the neuronal environment, an increase in transmitter release, changes of properties of receptors which might be related to glutamate neurotoxicity, and so on. It is also worth considering whether the potentiation in our case is similar to the glycine-induced potentiation of the NMDA response (Johnson & Ascher, 1987). However, according to binding assays on hippocampal neurones, it seems unlikely that L-CCG-III binds to these glycine receptors (Kawai & Ishihara, unpublished observation). It would seem more plausible that L-CCG-III blocks the uptake process for excitatory amino acids. According to Johnston *et al.* (1980), under conditions where *threo*-3-hydroxyaspartate had little or no effect on the spontaneous firing rate of neurones in the cat spinal cord, excitation induced by the administration of L-glutamate was clearly potentiated. In the present study, spinal reflexes were not affected by

L-CCG-III and neither dihydrokainate nor L-cysteate potentiated responses to L-glutamate or L-aspartate, but only *threo*-3-hydroxyaspartate and L-CCG-III enhanced them. Responses to kainate, quisqualate, NMDA and D-glutamate were hardly affected by L-CCG-III. L-Glutamate, D-aspartate and L-cysteate are able to utilize the glutamate transport system efficiently, but D-glutamate, NMDA, and kainate are poor substrates for the glutamate transport system (Stallcup *et al.*, 1979). It was of great interest that the response to several compounds, which were believed to be taken up from the vicinity of their receptors, was often enhanced by L-CCG-III and/or *threo*-3-hydroxyaspartate in the rat isolated spinal cord. Although the potentiation of the glutamate response by L-CCG-III might be due to the inhibition of the uptake process, the extremely long duration of the potentiation is a question to be solved. Further investigations will be required in order to validate or negate such a relationship between the potentiation of the glutamate response and the inhibition of the uptake of excitatory amino acids.

Neuronal damage is supposed to be aggravated by the potentiation of responses to L-glutamate (McBean & Roberts, 1985), and may be related to

the activation of NMDA-type receptors (Rothman & Olney, 1987; Finkbeiner & Stevens, 1988). Since L-CCG-III, a NMDA-preferring agonist, significantly potentiates the glutamate response for a long time, it is possible that L-CCG-III may provide useful information about glutamate neurotoxicity. In addition, the long-term potentiation (LTP) in hippocampal neurones has captured the imagination of neuroscientists as a model with which to probe the cellular mechanisms of learning and memory in the mammalian brain. Many researchers are interested in the relationship between NMDA-type receptors and LTP (Collingridge & Bliss, 1987). Although the present data were obtained in the rat spinal cord, the actions of CCG-isomers are expected to be similar in other regions of the mammalian central nervous system. Whatever the mechanism for the potentiation of the glutamate response, L-CCG-III is expected to be very useful as a pharmacological tool for analyzing the mechanisms underlying glutamate's transmitter functions.

This work was supported in part by a Grant in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. MK-801 was a gift of Merck, Sharp and Dohme.

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(Received March 28, 1989)

Revised July 21, 1989

Accepted August 7, 1989)