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Potentiation by DL-α-aminopimelate of the inhibitory action of a novel mGluR agonist (L-F_2CCG-I) on monosynaptic excitation in the rat spinal cord

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1 Neuropharmacological actions of all the possible stereoisomers of 3′,3′-difluoro-2-(carboxycyclopropyl)glycine (3′,3′-difluoro-CCG) were compared with those of the corresponding 2-(carboxycyclopropyl)glycine (CCG) isomers in the isolated spinal cord of newborn rats. (2**S**,1′**S**,2′**S**)- and (2**S**,1′**R**,2′**S**)-2-(2-carboxy-3,3-difluorocyclopropyl)glycine (L-F_2CCG-I and L-F_2CCG-IV) were the most potent in causing depolarization, their threshold concentrations being approximately 1 μM.

2 The depolarization evoked by L-F_2CCG-I (30 μM) was depressed by (+)-α-methyl-4-carboxyphenylglycine (MCPG, 1 mM ($n=4$)) to 17±3% of the control: this depolarizing action was not decreased by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 100 μM), and only slightly decreased by high concentrations of D-2-amino-5-phosphonopentanoic acid (D-AP5, 100 μM), suggesting that L-F_2CCG-I activates mainly metabotropic glutamate receptors.

3 L-F_2CCG-I preferentially depressed the monosynaptic component of the spinal reflex approximately 3 times more effectively than (2**S**,1′**S**,2′**S**)-2-(carboxycyclopropyl)glycine (L-CCG-I). The depressant action of L-F_2CCG-I (0.2 μM–0.7 μM) on monosynaptic excitation was antagonized by (2**S**,1′**S**,2′**S**)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG, 0.3 mM–1 mM) and (**S**)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4, 0.3 mM).

4 DL-α-Aminopimelate (10 and 100 μM) selectively potentiated the depression of monosynaptic excitation caused by L-CCG-I (0.2 μM) and L-F_2CCG-I (0.1 μM). The actions of (2**S**,1′**R**,2′**R**,3′**R**)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) (50 nM–0.2 μM), L-2-amino-4-phosphonobutanoic acid (L-AP4) (0.3–1 μM), (1**S**,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1**S**,3**R**)-ACPD) (1–7 μM) and baclofen (0.1–0.7 μM) were unaffected by DL-α-aminopimelate. The threshold concentration for the potentiating actions of DL-α-aminopimelate was 3 μM.

5 The depolarization induced by quisqualate (3 μM, 10 s application) was increased to 115±2% and 137±5% of the control values during combined application of quisqualate with either 30 μM or 100 μM DL-α-aminopimelate, respectively.

6 Following the application and subsequent washout of L-F_2CCG-I, DL-α-aminopimelate (3–100 μM) decreased the amplitude of the monosynaptic component of spinal reflexes in a concentration-dependent manner, indicating a 'priming' effect of L-F_2CCG-I.

Keywords: Metabotropic glutamate receptor; α-aminopimelate; L-F_2CCG-I; 2-(carboxycyclopropyl)glycine (CCG); potentiation; priming; monosynaptic reflexes

Introduction

2-(Carboxycyclopropyl)glycine (CCG) is a conformationally restricted analogue of glutamate (Ohfune & Shinozaki, 1993). CCG and its derivatives demonstrate unique neuropharmacological actions (Shinozaki *et al*., 1989; Ishida *et al*., 1990; 1993a; 1994; 1995; Genazzani *et al*., 1993; Bruno *et al*., 1994; 1995; Miyamoto *et al*., 1994; 1997; Poncer *et al*., 1995; Kamiya *et al*., 1996; Nicoletti *et al*., 1996; Kwak *et al*., 1996). Some CCG derivatives, in which the glutamate skeleton takes a conformationally extended form, such as (2**S**,1′**S**,2′**S**)-2-(carboxycyclopropyl)glycine (L-CCG-I), (2**S**,1′**R**,2′**R**,3′**R**)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), (2**S**,1′**S**,2′**R**,-3′R)- and (2**S**,1′**S**,2′**R**,3′**S**)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine (*cis*- and *trans*-MCG-I, respectively), have been shown to be potent agonists at metabotropic glutamate receptors (mGluRs) (Ishida *et al*., 1990; 1993a; 1994; 1995; Nakagawa *et al*., 1990). At present, mGluRs are subdivided into three groups (group I, II and III) on the basis of their structural homology and transduction pathways. L-CCG-I preferentially activates group II mGluRs, but also stimulates group I and III mGluRs (Hayashi *et al*., 1992). Substituents at the 3′-position of CCG seem to exert a marked effect on the selectivity of action at mGluR subgroups. For example, DCG-IV is a highly potent and selective agonist for group II mGluRs with no substantial activity at other mGluRs (Ishida *et al*., 1993a; Hayashi *et al*., 1993), while *cis*- and *trans*-MCG-I (3′-methoxymethyl-CCG) effectively depressed monosynaptic excitation in the spinal reflex of newborn rats (Ishida *et al*., 1994; 1995), also suggestive of activity at mGluR subgroups II and/or III (Watkins & Collingridge, 1994). In addition, 2-(2-carboxy-3-phenylcyclopropyl)glycine is a useful potential ligand for several classes of excitatory amino acid receptors, including mGluRs and ionotropic glutamate receptors (iGluRs) as well as Na^+-dependent and Ca^{2+}/Cl^--dependent glutamate transport systems (Pellicciari *et al*., 1996).

In an attempt to obtain more useful compounds for investigating physiological functions of mGluRs (Schoepp & Conn, 1993), we have introduced fluorine at the 3′-position of

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CCG (Figure 1). By substituting fluorine for both the hydrogen atoms at the 3′-position of CCG, the conformation of the glutamate skeleton within the CCG molecules becomes more restricted by electrostatic factors and hydrogen bonds, furthermore, the modification would be expected to increase the acidity of the cyclopropyl group of CCG (Shibuya *et al*., 1996). Therefore, it is of particular interest to examine the pharmacological activity of these compounds in relation to their non-fluorinated counterparts.

Methods

The methods used for the electrophysiological experiments in the isolated spinal cord of newborn rats (1–7 days-old Wistar rats, $n=143$) were essentially similar to those described previously (Shinozaki *et al*., 1989). Under deep 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 (ACSF, composition in mM: NaCl 138.6, KCl 3.4, $CaCl_2$ 1.26, $MgCl_2$ 1.15, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10.0, and pH 7.4) which was oxygenated with a gas mixture of 95% O_2 and 5% CO_2. In some cases, tetrodotoxin (TTX, 0.5 μM) was added to the bathing solution in order to block spontaneous depolarization and indirect drug effects. The spinal reflex was repetitively evoked at 1 min intervals by repeated electrical stimulation of the L_4 or L_5 dorsal root of the newborn rat in TTX-free and Mg^{2+}-containing ACSF. Single stimuli were delivered to dorsal roots and the potential changes of motoneurones (DR-VRP) were extracellularly recorded from the ipsilateral ventral roots of the corresponding segment. In some experiments, the dorsal root potential (DRP) and the potential change (DR-DRP) corresponding to the stimulation of the dorsal root, which were derived from the dorsal root (L_4 or L_5) adjacent to the stimulated root, were extracellularly recorded. mGluR agonists and other test compounds were applied to the spinal cord preparation by inclusion in the ACSF and perfusion at a constant rate. The temperature of the perfusing fluid was maintained at $27\pm0.2^{\circ}C$.

Drugs

The eight stereoisomers of 3′,3′-difluoro-2-(carboxycyclopropyl)glycine (CCG) [the (2**S**,1′**S**,2′**S**)-isomer (L-F_2CCG-I), the (2**S**,1′**R**,2′**R**)-isomer (L-F_2CCG-II), the (2**S**,1′**S**,2′**R**)-isomer (L-F_2CCG-III), the (2**S**,1′**R**,2′**S**)-isomer (L-F_2CCG-IV), the (2**R**,1′**R**,2′**R**)-isomer (D-F_2CCG-I), the (2**R**,1′**S**,2′**S**)-isomer (D-F_2CCG-II), the (2**R**,1′**R**,2′**S**)-isomer (D-F_2CCG-III) and the (2**R**,1′**S**,2′**R**)-isomer (D-F_2CCG-IV)] (Shibuya *et al*., 1996) were generously provided by Prof. T. Taguchi (Tokyo Pharmaceutical College).

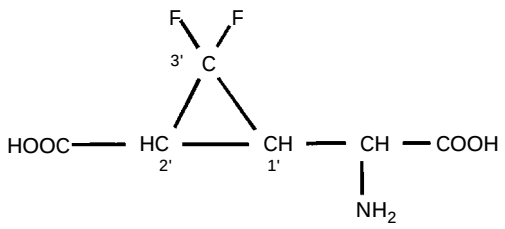

Figure 1 Chemical structure of 3′,3′-difluoro-2-(carboxycyclopropyl)glycine (3′,3′-difluoro-CCG). Theoretically, there are 8 stereoisomers of 3′,3′-difluoro-CCG (see Shinozaki *et al*., 1989), including L- and D-forms. Due to electrostatic factors, hydrogen bonds and increased acidity, the conformational variability of the glutamate skeleton of 3′,3′-difluoro-CCG would become more restricted than that of CCG.

The following compounds were used: (1**S**,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1**S**,3**R**)-ACPD, Tocris), (**S**)-2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, Tocris), (**RS**)-1-aminoindan-1,5-dicarboxylic acid (AIDA, Tocris), (**S**)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4, Tocris), L-2-amino-4-phosphonobutanoic acid (L-AP4, Tocris), D-2-amino-5-phosphonopentanoic acid (D-AP5, Tocris), DL-α-aminopimelic acid (Nacalai tesque), baclofen (Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris), kainic acid (Sigma), N-methyl-D-aspartic acid (NMDA, Sigma), (2**S**,1′**S**,2′**S**)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG, Tocris), (+)-α-methyl-4-carboxyphenylglycine (MCPG, Tocris), (**RS**)-α-methyl-4-tetrazolylphenylglycine (MTPG, Tocris), 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX, Tocris), quisqualic acid (Sigma) and tetrodotoxin (TTX, Seikagaku-kougyo).

The eight stereoisomers of CCG, with the configurations [(2**S**,1′**S**,2′**S**) (L-CCG-I), (2**S**,1′**R**,2′**R**) (L-CCG-II), (2**S**,1′**S**,2′**R**) (L-CCG-III), (2**S**,1′**R**,2′**S**) (L-CCG-IV), (2**R**,1′**R**,2′**R**) (D-CCG-I), (2**R**,1′**S**,2′**S**) (D-CCG-II), (2**R**,1′**R**,2′**S**) (D-CCG-III), (2**R**,1′**S**,2′**R**) (D-CCG-IV)], and also (2**S**,1′**R**,2′**R**,3′**R**)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), (2**S**,1′**S**,2′**R**,3′**S**)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine (*trans*-MCG-I) and (2**S**,1′**S**,2′**R**,3′**R**)-2-(2-carboxy-3-methoxymethylcyclopropyl)glycine (*cis*-MCG-I) were generous gifts from Prof. Y. Ohfune (Osaka City Univ.).

Results

Pharmacological activities of the eight difluoro CCG stereoisomers

The depolarizing activities of the eight 3′,3′-difluoro-CCGs were compared with those of the corresponding CCG stereoisomers in the isolated spinal cord of newborn rats. In Mg^{2+}-free and TTX-containing (0.5 μM) medium the 3′,3′-difluoro-CCGs caused depolarization in a concentration-dependent manner with a large variation between the threshold concentrations required. The concentration-response relationships of the 3′,3′-difluoro-CCGs are shown in Figure 2. L-F_2CCG-IV showed the greatest depolarizing activity among the eight 3′,3′-difluoro-CCGs, followed by L-F_2CCG-I. After the preparation was exposed to an NMDA antagonist, D-2-amino-5-phosphonopentanoic acid (D-AP5, 100 μM), for at least 5 min, depolarizing responses to NMDA (30 μM, 10 s, $n=4$), L-F_2CCG-III [the (2**S**,1′**S**,2′**R**)-isomer] (0.7 mM, 10 s, $n=4$), L-F_2CCG-IV [the (2**S**,1′**R**,2′**S**)-isomer] (10 μM, 10 s, $n=4$), D-F_2CCG-I [the (2**R**,1′**R**,2′**R**)-isomer] (100 μM, 10 s, $n=4$) and D-F_2CCG-II [the (2**R**,1′**S**,2′**S**)-isomer] (20 μM, 10 s, $n=4$) were almost completely abolished, while the depolarizing responses to L-F_2CCG-I (10–30 μM, 10 or 20 s, $n=6$) were not significantly altered. The depolarizing action of L-F_2CCG-II was weak (threshold concentration about 0.3 mM) (Figure 2), with the depolarization reduced to $52\pm6\%$ ($n=4$) of the control by a high concentration (100 μM) of D-AP5, and the residual depolarization remaining unaffected by a high concentration (100 μM) of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA/kainate receptor antagonist (see below). In contrast, the depolarization was almost completely blocked by (+)-α-methyl-4-carboxyphenylglycine (MCPG, 1 mM, $n=4$), a relatively non-selective mGluR antagonist

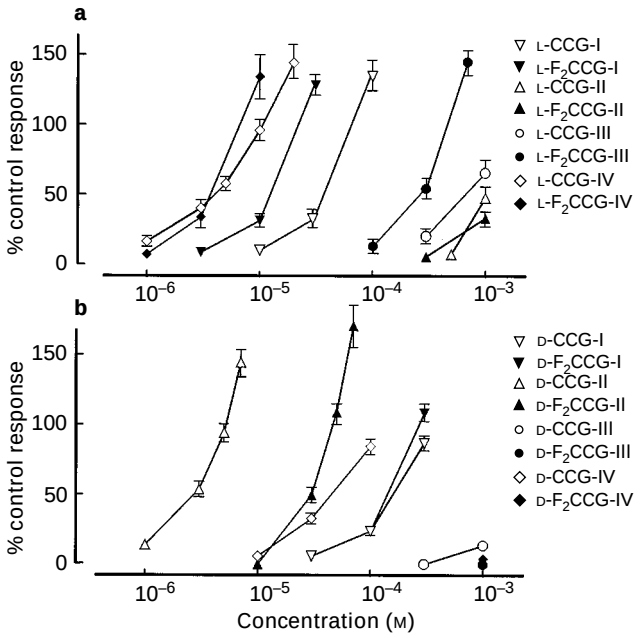

Figure 2 The concentration-depolarization response relationships for various stereoisomers of 2-(carboxycyclopropyl)glycine (CCG) and 3′,3′-difluoro-2-(carboxycyclopropyl)glycine (3′,3′-difluoro-CCG); (a) L-isomer and (b) D-isomers. Peak amplitudes of depolarizing responses to test compound (10 s application) were plotted against their concentrations (results were normalized to that of 1 mM L-glutamate). Vertical lines represent s.e.mean (n=at least 4). Abbreviations and experimental conditions are as described in the text.

(Watkins & Collingridge, 1994). The depolarization of motoneurones caused by prolonged application of L-F_2CCG-I (higher than 3 μM, longer than 3 min) was not maintained but gradually declined in the presence of the agonist. This was similar to the effects seen with L-CCG-I. The 3′,3′-difluorination of L-CCG-III and L-CCG-IV delayed the decay of depolarizing responses; the half decay times ranging from 11.5 ± 0.5 s ($n=4$) to 32.0 ± 2.3 s ($n=4$), and from 8.0 ± 0.6 s ($n=4$) to 38.4 ± 1.2 s ($n=4$), respectively. It has been shown that L-CCG-III markedly augmented the depolarizing response to L-glutamate by blocking Na^+-dependent uptake of L-glutamate (Robinson *et al*., 1993; Nakamura *et al*., 1993). After treatment with L-CCG-III, depolarizing responses to L-glutamate were markedly augmented and this effect remained following washout. However, no effects on L-glutamate-induced depolarization (1 mM) were observed after L-F_2CCG-III application (0.1 mM – 1 mM) ($n=4$), suggesting that L-F_2CCG-III did not possess the ability to augment the glutamate responses. Thus, the depolarizing responses to the 3′,3′-difluoro-CCGs show a similar pattern to the responses of the corresponding CCGs (cf. Shinozaki *et al*., 1989), except for the marked change of depolarizing activities and the lack of inhibition of Na^+-dependent uptake of L-glutamate in the case of L-F_2CCG-III. This suggests that the 3′,3′-difluorination does not greatly influence the pharmacological profiles of the parent CCGs.

Pharmacological properties of L-F_2CCG-I

Depolarization As shown in Figure 2, the depolarizing activity of L-CCG-I was increased up to approximately 3 fold by the 3′,3′-difluorination. In the present paper, we examined the pharmacological properties of L-F_2CCG-I, in comparison with those of the corresponding unfluorinated compound, L-CCG-I, a potent mGluR agonist. The L-F_2CCG-I (30 μM) induced depolarization was decreased by MCPG (1 mM) to $17\pm3\%$ ($n=4$) of the control, and was only slightly depressed by high concentrations of D-AP5 (0.1 mM, $87\pm7\%$, $n=4$), but CNQX (0.1 mM) in combination with D-AP5 (0.1 mM) did not affect the depolarization ($105\pm8\%$, Figure 3a). (**RS**)-1-Aminoindan-1,5-dicarboxylic acid (AIDA, 1 mM) (Pellicciari *et al*., 1995) slightly decreased the depolarization induced by L-F_2CCG-I (30 μM, 20 s application; $87\pm7\%$ ($n=4$) of the control). Agonists for group I mGluRs induce depolarization of neurones by the stimulation of phosphoinositide hydrolysis, therefore, it seems likely that L-F_2CCG-I activates mGluRs including group I mGluRs.

Depression of the DR-VRP monosynaptic component L-F_2CCG-I preferentially and reversibly depressed the DR-VRP monosynaptic component much more effectively than L-CCG-I with a threshold concentration of about 0.03 μM (Figure 3b), and in analogy with the action of the unfluorinated CCG, this was probably due to a presynaptic inhibition of transmitter release. The depression of the DR-VRP monosynaptic component by some mGluR agonists was decreased in 1 day-old, relative to older neonatal rats (Ishida *et al*., 1993b), the EC_{50} for L-F_2CCG-I being 0.20 ± 0.05 μM ($n=4$) in 1 day-old rats and 1.04 ± 0.37 μM ($n=4$) in 7 day-old rats.

Several effective antagonists for mGluRs have been described, such as (2**S**,1′**S**,2′**S**)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG, a group II mGluR antagonist), (**RS**)-α-methyl-4-tetrazolylphenylglycine (MTPG, a group II mGluR antagonist) and (**S**)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4, a group III mGluR antagonist) (Jane *et al*., 1994; 1995; Roberts, 1995). The L-F_2CCG-I-induced depression of the DR-VRP monosynaptic component was effectively reversed by MCCG, MTPG and MAP4 (Figure 3c, d). When L-F_2CCG-I (0.3 μM) depressed the DR-VRP monosynaptic component to $34.6\pm3.9\%$ of the control, addition of MCCG (0.3 mM, $n=4$), MTPG (0.3 mM, $n=4$) or MAP4 (0.3 mM, $n=4$) reversed the amplitude of the DR-VRP monosynaptic component to 68.0 ± 8.7, 67.2 ± 7.0 and $63.6\pm8.2\%$ of the control, respectively.

Depression of polysynaptic responses Polysynaptic components of the spinal reflex were also decreased by L-F_2CCG-I under the conditions that the DR-VRP monosynaptic component was decreased by this substance to less than half of the control amplitude. The long-lasting after-potentials with slow time courses (Akagi & Yanagisawa, 1987) were markedly decreased by L-F_2CCG-I (data not shown).

Depression of the DR-DRP The resting level of the DRP was not affected by L-F_2CCG-I (0.1 – 0.5 μM), but the amplitude of the DR-DRP was decreased in a concentration-dependent manner to a maximal extent of about 50%. At concentrations higher than 1 μM, L-F_2CCG-I caused a slight depolarizing change in the DRP.

Therefore, L-F_2CCG-I seems to activate all mGluR subtypes (group I, II and III) as a non-selective mGluR agonist, although the threshold concentration for activating each mGluR subtype appears to vary. The EC_{50} values of L-F_2CCG-I and DCG-IV for activation of mGluR2 expressed in CHO cells were approximately 0.09 μM and 0.34 μM, respectively (Okada *et al*., personal communication). Table 1 shows the IC_{50} values for the depression of the monosynaptic component of the DR-VRP by some CCG related mGluR agonists. Thus, it can been seen that the pharmacological properties of L-F_2CCG-I are similar to those of L-CCG-I.

Enhancement of responses to L-F_2CCG-I by DL-α-aminopimelate

At relatively high concentrations (>0.3 mM), DL-α-aminopimelate caused the depolarization of the spinal motoneurones of newborn rats which was almost completely blocked by 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX 10 μM). At a concentration of 0.3 mM, DL-α-aminopimelate did not affect or only slightly decreased amplitudes of the DR-VRP monosynaptic component (87±12% of the control, $n=4$), this effect being sometimes associated with a small depolarization of the motoneurones (0.5±0.1 mV, $n=4$). The DR-VRP polysynaptic components were apparently decreased to 56±7% ($n=4$) of the control, presumably due mainly to the NMDA receptor blocking action of the D-form of this compound; D-α-aminopimelate (0.1mM) depressed depolarization induced by NMDA (10 μM) (61±8% of the control, $n=3$), but DL-α-aminopimelate (0.3 mM) did not affect responses to AMPA (3 μM) or (1**S**,3**R**)-ACPD (50 μM).

At concentrations of less than 0.1 mM, DL-α-aminopimelate neither affected spinal reflexes nor resting membrane potentials of motoneurones. L-F_2CCG-I (0.1 μM) did not depolarize motoneurones, but decreased the amplitude of the DR-VRP monosynaptic component to 79±6% ($n=4$) of the control value. Simultaneous administration of L-F_2CCG-I and DL-α-aminopimelate caused a more marked decrease in the DR-VRP monosynaptic component than the sole application of L-F_2CCG-I (Figure 4). When DL-α-aminopimelate was simultaneously applied at concentrations of 10 μM and 0.1 mM with L-F_2CCG-I (0.1 μM), the DR-VRP monosynaptic component was markedly depressed to 36±6% and 8±5% of the control, respectively ($n=4$), but no detectable depolarization of motoneurones was observed (Figure 4b). With increasing concentrations of L-F_2CCG-I, the extent of the potentiation induced by DL-α-aminopimelate seemed to become larger.

Table 1 Pharmacological characterization of CCG related mGluR agonists

Agonists	*NMDA receptor*	*Group I mGluR*	*Group II mGluR*	*Group III mGluR*	*MSR IC_{50} (μM)*
L-CCG-I	±	+	+	+	0.63±0.10 ($n=9$)
L-F_2CCG-I	±	+	+	+	0.20±0.04 ($n=6$)
DCG-IV	+	–	+	–	0.08±0.02 ($n=4$)
cis-MCG-I	–	–	+	–	3.81±0.81 ($n=5$)
trans-MCG-I	–	–	+	–	0.32±0.05 ($n=4$)

Depolarizing activities and inhibition of monosynaptic components of the spinal reflex were examined in the presence of a high concentration of NBQX (100 μM), D-AP5 (100 μM), MCPG (1 mM), MCCG (0.3 mM) and MAP4 (0.3 mM). +: activation; –: no effect. MSR IC_{50}: the IC_{50} concentration of monosynaptic reflexes in the spinal cord of newborn rats (1–3 days-old).

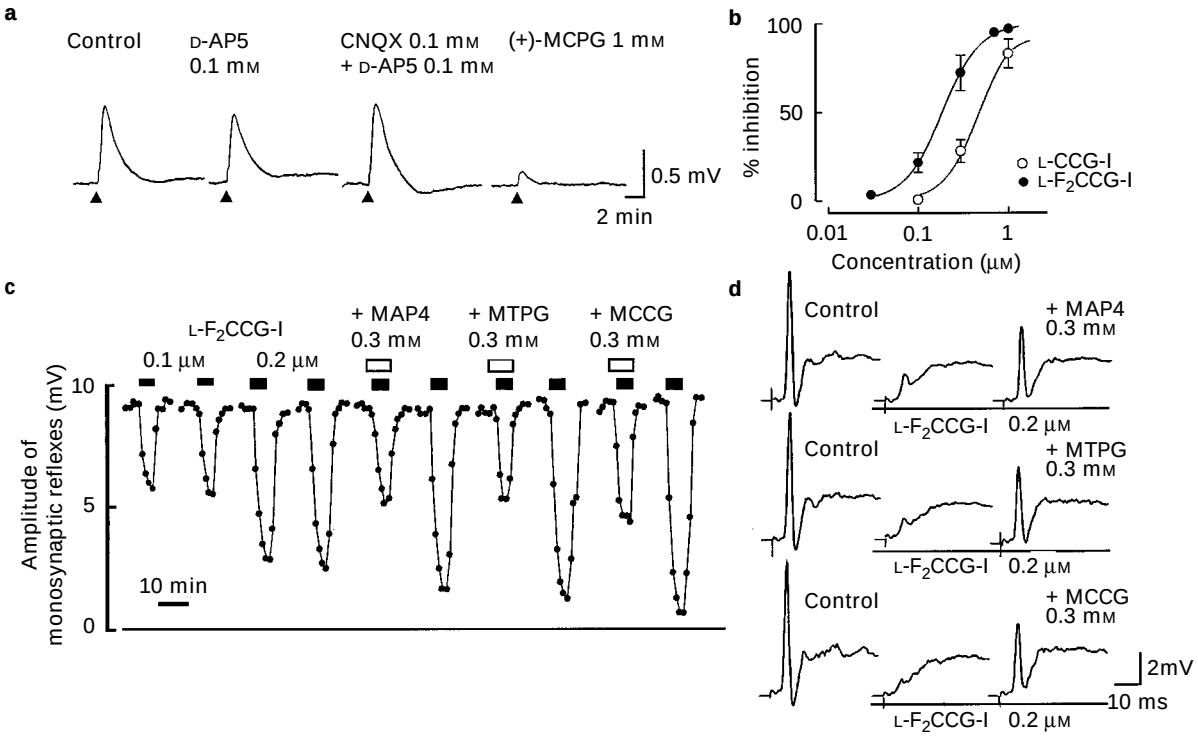

Figure 3 (a) Pharmacology of L-F_2CCG-I-induced depolarization. Each trace (representing a depolarization induced by L-F_2CCG-I (30 μM, 20 s)) was obtained from a preparation from a 5 day-old rat pup following 30 min exposure to tetrodotoxin (TTX, 0.5 μM) containing, Mg^{2+}-free bathing solution. The experiments were carried out in the presence and absence of D-2-amino-5-phosphonopentanoic acid (D-AP5, 0.1 mM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 0.1 mM) and (+)-α-methyl-4-carboxyphenylglycine (MCPG, 1 mM). (b) Comparison of the inhibitory action of L-F_2CCG-I and L-CCG-I on the DR-VRP monosynaptic component (2 day-old rat). Vertical lines represent s.e.mean (n=at least 3). (c) Pharmacology of the L-F_2CCG-I-induced inhibition of the DR-VRP monosynaptic component (L_4, 2 day-old rat). Amplitudes of the DR-VRP monosynaptic component were plotted at 1 min intervals. L-F_2CCG-I (0.2 μM) was added to the normal perfusing solution for a period of 5 min, at intervals of 30 min before and after the addition of (**S**)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4, 0.3 mM),(**RS**)-α-methyl-4-tetrazolylphenylglycine (MTPG, 0.3 mM) and (2**S**,1′**S**,2′**S**)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG, 0.3 mM). Antagonists were applied 2 min before the application of L-F_2CCG-I. These antagonists effectively reversed the action of L-F_2CCG-I. (d) Sample records of spinal reflexes of which amplitudes were shown in (c).

When L-F_2CCG-I was applied at concentrations greater than 0.7 μM it caused a slight depolarization and DL-α-aminopimelate (0.1 mM) markedly potentiated this depolarization of the motoneurones and inhibited the DR-DRP. In addition, the resting level of the DRP did not change even when DL-α-aminopimelate (0.1 mM) and L-F_2CCG-I (0.5 μM) were simultaneously applied. Therefore, it seems unlikely that DL-α-aminopimelate accentuates the depolarization of the presynaptic terminal to depress further the transmitter release.

DL-α-Aminopimelate (0.1 mM) markedly potentiated the depressant effect of L-CCG-I (0.2–1 μM), L-F_2CCG-I (0.1–1 μM) and *trans*-MCG-I (0.1–1 μM) on the DR-VRP monosynaptic component in a concentration-dependent manner. When L-CCG-I (0.2 μM) depressed the DR-VRP monosynaptic component to 78 ± 5% of the control, DL-α-aminopimelate at a concentration of 10, 30 and 100 μM potentiated the inhibition of the DR-VRP monosynaptic component to 60 ± 8, 49 ± 5 and 29 ± 0.3 % of the control, respectively ($n=4$). On the other hand, DL-α-aminopimelate (0.1 mM) did not affect the inhibition caused by DCG-IV (50 nM–0.2 μM, $n=4$), L-2-amino-4-phosphonobutanoic acid (L-AP4, Cao *et al*., 1995) (0.3 μM–1 μM, $n=4$),(1**S**,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid((1**S**,3**R**)-ACPD, 1–7 μM, $n=4$), baclofen (0.1–0.7 μM, $n=4$) and other 3′,3′-difluoro-CCGs (at least $n=3$). When the depolarization induced by representative iGluR agonists, such as kainic acid (3–10 μM, $n=5$), AMPA (1–3 μM, $n=5$), NMDA (5–10 μM, $n=4$) and quisqualic acid (3 μM, $n=4$), was determined in the presence of DL-α-aminopimelate (0.1 mM), only the quisqualate-induced depolarization was considerably augmented. Depolarization induced by quisqualate (3 μM, $n=4$) was augmented by DL-α-aminopimelate (30 and 100 μM) up to 115 ± 2.3% and 137 ± 4.5% of the control, respectively.

As mentioned above, high concentrations of DL-α-aminopimelate depolarized motoneurones pharmacologically in a similar manner to AMPA. Therefore, we investigated whether simultaneous application of L-F_2CCG-I and low concentrations of AMPA had an effect on the DR-VRP monosynaptic component. Low doses (3–30 nM) of AMPA did not augment the inhibition of the DR-VRP monosynaptic component caused by L-F_2CCG-I (0.1–1 μM), although a variable degree of depolarization of motoneurones was usually observed.

L-F_2CCG-I priming

After L-F_2CCG-I had been applied to the spinal cord preparation, DL-α-aminopimelate decreased the amplitude of the DR-VRP monosynaptic component in a concentration-dependent manner, even after L-F_2CCG-I had been thoroughly washed out; this showed the 'L-F_2CCG-I priming effect' (Figure 5a). Before the application of L-F_2CCG-I, DL-α-aminopimelate did not have any detectable pharmacological action at all concentrations lower than 0.1 mM. The threshold concentrations of DL-α-aminopimelate after L-F_2CCG-I priming were 3–10 μM. When DL-α-aminopimelate (0.1 mM) was repetitively administered after the L-F_2CCG-I treatment (2 μM) at a fixed interval of 30 min, the inhibition of the DR-VRP monosynaptic component caused by DL-α-aminopimelate seemed to decay almost exponentially, and

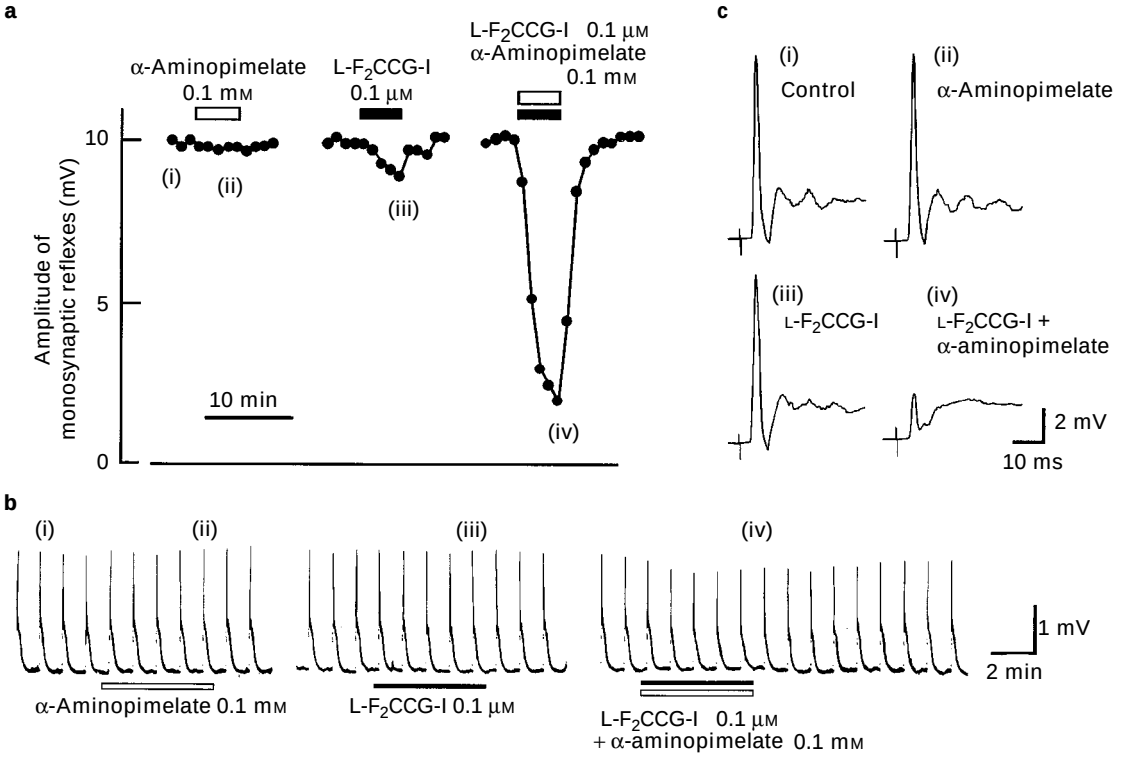

Figure 4 Marked potentiation by DL-α-aminopimelate of the inhibition of the DR-VRP monosynaptic component caused by L-F_2CCG-I. (a) Amplitudes of the DR-VRP monosynaptic component were plotted every 1 min in the presence of DL-α-aminopimelate (0.1 mM) and/or L-F_2CCG-I (0.1 μM). Test compounds were applied for a period of 5 min at an interval of 30 min. (b) Sample records of spinal reflexes on a pen writer extracellularly recorded from the L_4 ventral root by a d.c. amplifier, corresponding to the data shown in (a). The most rapid component of the DR-VRP was not able to be recorded due to the frequency characteristic of the pen writer, the trace showing mainly the amplitudes of the DR-VRP polysynaptic component and the resting level of potential changes. (c) Sample records of spinal reflexes of which amplitudes were shown in (a) and (b).

appeared to be use-dependent (Figures 5b and 6). Additional treatment with L-F_2CCG-I regenerated the ability of DL-α-aminopimelate to cause inhibition of the DR-VRP monosynaptic component (Figure 5b). The DR-VRP inhibition caused by DL-α-aminopimelate lasted for several hours. At longer intervals between the DL-α-aminopimelate additions (greater than 2 h), the exponential decay of the DR-VRP inhibition was still observed. When DL-α-aminopimelate (0.1 mM) was applied for a prolonged period of 1 h, the marked inhibition of the DR-VRP monosynaptic component seen after the L-F_2CCG-I treatment (2 μM) was observed immediately after the application of DL-α-aminopimelate, but the inhibition was not maintained and was exponentially decreased to the control level (Figure 5c). Further repeated applications of the same dose of DL-α-aminopimelate did not cause significant inhibition at this stage. When doses of DL-α-aminopimelate (0.1 mM) and L-F_2CCG-I (various doses) were repeatedly applied, the inhibitory action of DL-α-aminopimelate on the DR-VRP monosynaptic component appeared to depend on the concentration of L-F_2CCG-I applied (Figure 5d). When L-F_2CCG-I (1 μM) was repeatedly applied, the inhibition of the DR-VRP monosynaptic component was

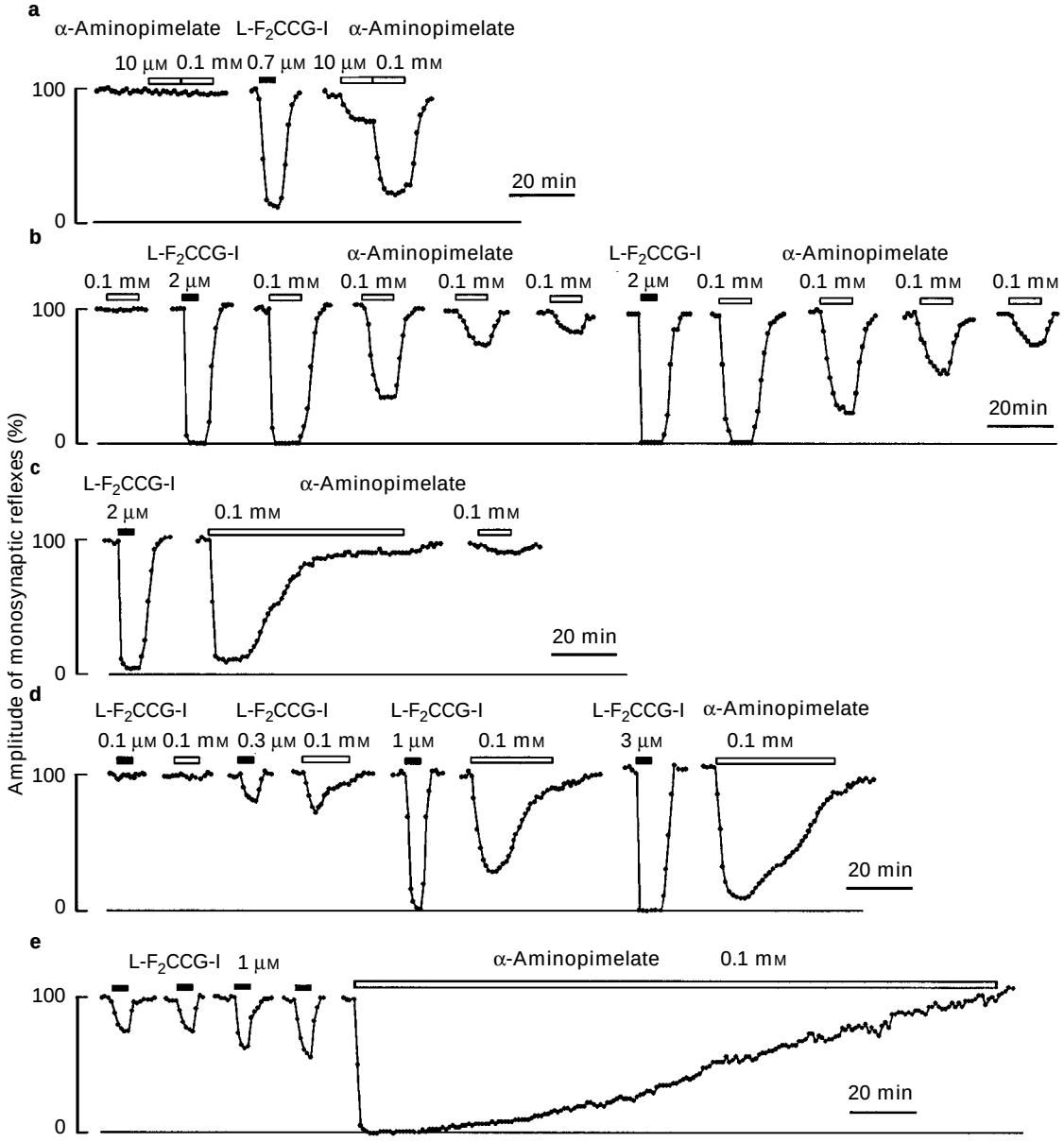

Figure 5 L-F_2CCG-I priming: % amplitudes of the DR-VRP monosynaptic component were plotted every 1 min in the absence and presence of DL-α-aminopimelate or L-F_2CCG-I. All compound additions were made at an interval of 30 min. Open bar: DL-α-aminopimelate, solid bar: L-F_2CCG-I. (a) DL-α-Aminopimelate acquired the ability to cause inhibition of the DR-VRP monosynaptic component after treatment with L-F_2CCG-I in a concentration-dependent manner (1 day-old rat). (b) The inhibition of the DR-VRP monosynaptic component caused by repetitive application of DL-α-aminopimelate seen after the L-F_2CCG-I treatment (2 μM) decayed exponentially (1 day-old rat). The repeated treatment with L-F_2CCG-I regenerated the D-α-aminopimelate-induced depression of the DR-VRP monosynaptic component. (c) Effect of prolonged application of DL-α-aminopimelate seen after L-F_2CCG-I treatment (1 day-old rat). (d) The inhibition of the DR-VRP monosynaptic component caused by DL-α-aminopimelate seen after the L-F_2CCG-I treatment depended on the applied concentration of L-F_2CCG-I (5 day-old rat). (e) Effect of a prolonged application of DL-α-aminopimelate after repetitive treatment with L-F_2CCG-I (1 μM) (7 day-old rat).

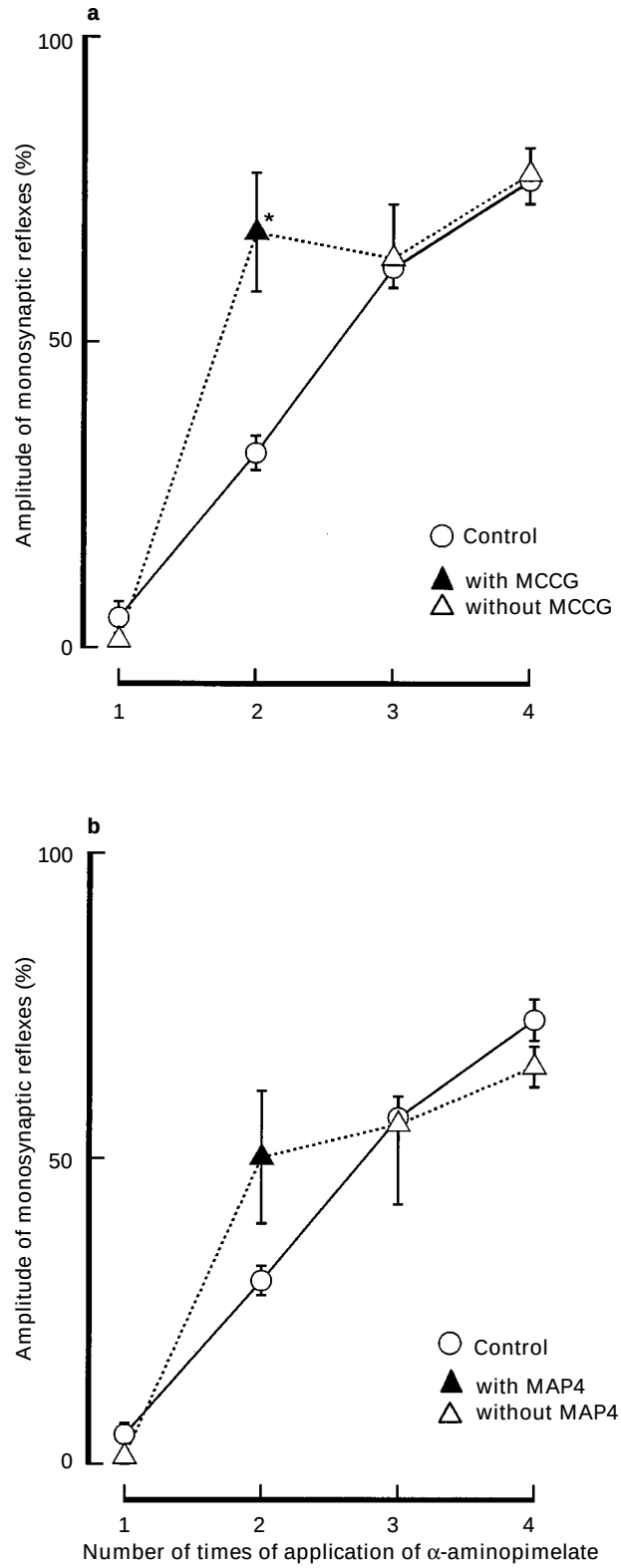

Figure 6 The effect of antagonists for metabotropic glutamate receptors on the inhibition of the DR-VRP monosynaptic component induced by DL-α-aminopimelate. DL-α-Aminopimelate (0.1 mM, 10 min application) was repeatedly applied, 30 min after the L-F_2CCG-I treatment (2 μM, 5 min application) in the absence and presence of MCCG (a) and MAP4 (b) (see Figure 5b). MCCG (0.3 mM) and MAP4 (0.3 mM) were added to the perfusing fluid 2 min before the second application of DL-α-aminopimelate. Mean percentage amplitudes of the DR-VRP monosynaptic component were plotted against the number of times DL-α-aminopimelate had been applied. MCCG, but not MAP4, reduced the DL-α-aminopimelate-induced depression of the monosynaptic DR-VRP. Vertical lines represent s.e.mean (n = at least 5). Asterisk indicates statistically significant difference ($P<0.05$) from control by Student's t test.

gradually increased, and, subsequently, DL-α-aminopimelate caused significant and long-lasting inhibition of the DR-VRP monosynaptic component (Figure 5e). The inhibitory effect of DL-α-aminopimelate (0.1 mM) on the DR-VRP monosynaptic component seen after L-F_2CCG-I treatment (1–2 μM) was effectively reversed by MCCG (0.3 mM) but only slightly and inconsistently altered by MAP4 (0.3 mM) (Figure 6). Other CCGs did not behave like L-F_2CCG-I. After treatment with L-CCG-I (3 μM) and *trans*-MCG-I (3 μM), DL-α-aminopimelate caused a depression of the DR-VRP monosynaptic component, but the extent of the depression was much less than that caused after L-F_2CCG-I pretreatment. As shown in Figure 6, the DR-VRP monosynaptic component was markedly decreased to 3±1% ($n=6$) of the control upon the first application of DL-α-aminopimelate (0.1 mM) and following treatment with L-F_2CCG-I (2 μM). However, after treatment with L-CCG-I (3 μM) and *trans*-MCG-I (3 μM), the DR-VRP monosynaptic component was decreased to only to 79±8 and 57±6% of the control, respectively ($n=4$). The inhibition of the DR-VRP monosynaptic component was still observed with 1 μM concentration of L-F_2CCG-I (to 26±3% of control, $n=23$).

Discussion

The pharmacological properties of CCG stereoisomers (Shinozaki *et al*., 1989) were largely unaffected by the 3′,3′-difluorination, except that an increase in the depolarizing activities of some isomers was observed. Fluorination of CCG stereoisomers increased the depolarizing activity of L-CCG-I and L-CCG-IV, and L-F_2CCG-I activated mGluRs (group I) about 3 times more effectively than L-CCG-I. Therefore, it seems likely that L-F_2CCG-I has a more active conformation for activating mGluRs than L-CCG-I. One of the most marked pharmacological changes induced by the 3′,3′-fluorination was the removal of the Na^+-dependent uptake of glutamate by L-CCG-III. The slight conformational change of the glutamate skeleton by the fluorination appears to be responsible for such an effect. This may provide additional information about the structure-activity relationship in the Na^+-dependent uptake of glutamate (Robinson *et al*., 1993). Within the difluoro-CCG series, only L-F_2CCG-I was further investigated in the present study, but the pharmacological actions of other 3′,3′-difluoro-CCG and 3′-monofluoro-CCG are also of great interest. 3′-Monofluoro-CCG may provide additional information about the relationship between receptor selectivity and molecular conformation, because 3′-monofluorination would cause a much more subtle conformational change to the glutamate skeleton of CCG compounds than 3′,3′-difluorination.

DL-α-Aminopimelate appeared to activate AMPA receptors at relatively high concentrations, but low concentrations of AMPA did not potentiate the inhibitory actions of L-F_2CCG-I on the DR-VRP monosynaptic component, suggesting that the activation of AMPA receptors was not related to the potentiation. As shown in Figure 4, the inhibition of the DR-VRP monosynaptic component was markedly potentiated without an apparent depolarization of motoneurones in many cases. While non-linear summation of the inhibitory actions of both DL-α-aminopimelate and L-F_2CCG-I or other mGluR agonists on the DR-VRP monosynaptic component is a possible mechanism underlying the potentiation, the extent of the potentiation seemed to become larger with an increase in concentration of L-F_2CCG-I.

At low concentrations, DL-α-aminopimelate markedly potentiated the inhibition of the DR-VRP monosynaptic

component caused by L-CCG-I, L-F_2CCG-I and *trans*-MCG-I, but it did not affect the responses to DCG-IV, L-AP4, (1**S**,3**R**)-ACPD and baclofen. L-F_2CCG-I and L-CCG-I activate all known mGluRs, whereas *trans*-MCG-I is a preferential agonist for group II mGluRs relative to group I mGluRs (Ishida *et al*., 1995). Thus, it seems unlikely that there is direct relationship between receptor selectivity and the potentiation of the inhibition of the DR-VRP monosynaptic component. To elucidate the mechanism underlying the potentiation of the DR-VRP monosynaptic component by DL-α-aminopimelate, previous studies on quisqualate priming (Robinson *et al*., 1986; Koerner & Johnson, 1992; Price *et al*., 1994; Sheardown & Thomsen, 1996) may be of use. DL-α-Aminopimelate, and both *cis*- and *trans*-MCG-I augmented depolarizing responses to quisqualate in a concentration-dependent manner, but the latter two compounds did not cause any detectable depolarization *per se* even at high concentrations, in contrast to DL-α-aminopimelate (Ishida *et al*., 1995). However, there are marked differences between the quisqualate priming and the L-F_2CCG-I priming. The quisqualate priming is a phenomenon of depolarization, while the L-F_2CCG-I priming is related mainly to the inhibitory action on the DR-VRP monosynaptic component. Nevertheless, compounds such as L-2-amino-6-phosphonohexanoic acid (L-AP6) (Koerner & Johnson, 1992), *cis*-MCG-I (Ishida *et al*., 1995) and DL-α-aminopimelate are able to induce both the quisqualate priming and the L-F_2CCG-I priming. The L-F_2CCG-I priming was also observed in epileptiform activity in rat cortical slices (unpublished observation). The inhibition of the uptake system of glutamate may also be considered as a mechanism of the potentiation. In our preliminary experiment, neither L-F_2CCG-I (up to 100 μM) nor DL-α-aminopimelate (up to 1 mM) inhibited the Na^+-dependent uptake of L-[^{3}H]-glutamate (unpublished observations). Further experiments will thus be required to elucidate the mechanism of this potentiating effect of DL-α-aminopimelate, including the examination of the priming or the Ca^{2+}/Cl^--dependent uptake of glutamate.

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