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Interaction of group I mGlu and NMDA receptor agonists within the dorsal horn of the spinal cord of the juvenile rat

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1 The modulatory effects of mGlu receptors on NMDA-induced potential changes in spinal motoneurones were studied *in vitro*.

2 Selective activation of mGlu5 receptors by 10 μM (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG; $EC_{50} = 280 \pm 24$ μM) did not produce any change in the ventral root potential. However, the same concentration of CHPG (10 min perfusion) significantly attenuated the NMDA-induced ventral root depolarization (VRD). The effect persisted for 10 min after washout. NMDA-induced responses returned to control in 30 min. Brief co-application of CHPG and NMDA did not alter the NMDA-induced response indicating lack of direct receptor interaction.

3 The attenuating effect of CHPG on the NMDA-induced VRD was inhibited by the mGluR5 receptor antagonist, 2-methyl-6-phenyl-ethynylpyridine (MPEP).

4 In the presence of CGP56433A, a $GABA_B$ receptor antagonist, the NMDA-induced VRD was unchanged. However, NMDA-induced responses were potentiated after 10 min co-application of CHPG and CGP56433A.

5 (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate ((2R,4R)-APDC), a group II mGlu receptor agonist did not attenuate the NMDA-induced response.

6 Under normal physiological conditions group I mGlu receptor agonists activate at least two populations of neurones: (1) GABA-ergic cells, which could release GABA and inhibit dorsal horn neurones, and (2) deep dorsal horn neurones/motoneurones which express NMDA receptors. Therefore, activation of mGlu5 receptors located on GABA-ergic interneurones could influence any direct potentiating interaction between mGlu5 and NMDA receptors in spinal cord and result in depression of the VRD. In the presence of a $GABA_B$ receptor antagonist, the direct synergistic interaction is unmasked. These data suggest that group I mGlu receptors provide a complex modulation of spinal synaptic processes.

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Abbreviations: AMPA, a-amino-3-hydroxy-5-methylisoxasole-propionic acid; (2R,4R)-APDC, (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate; CGP 56433A, [3-[1-(S)-[[3-cyclohexylmethyl)-hydroxyphosphinyl]-2-(S); -hydroxypropyl]amino]ethyl]-benzoic acid; CHPG, (RS)-2-Chloro-5-hydroxyphenylglycine; (S)-3,5-DHPG, (S)-3,5-dihydroxyphenylglycine; GABA, gamma amino butyric acid; GABA(subscript: B), receptor gamma amino butyric acid-B receptor; GAD, glutamic acid decarboxylase; mGlu receptor, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; NMDA, N-methyl-D-aspartate; TTX, Tetrodotoxin; VRD, ventral root depolarization

Introduction

Glutamate is one of the most ubiquitous transmitters in the central nervous system, including the spinal cord. It activates the ionotropic and metabotropic receptors and therefore it is equally essential for fast excitatory synaptic transmission and modulation of synaptic transmission (Urban *et al*., 1994). In the spinal dorsal horn, in lamina II neurones, glutamate primarily activates the non-N-methyl-D-aspartic acid (non-NMDA) type AMPA (α-amino-3-hydroxy-5-methylisoxasole-propionic acid) and kainate receptors (Yoshimura & Jessell, 1990) while in superficial (lamina I) and deep dorsal horn neurones both non-NMDA and NMDA receptor activation occurs in control conditions (Willis & Westlund, 1997; Millan, 1999). While activation of the NMDA receptor produces enhanced synaptic transmission in the dorsal horn of the spinal cord, activation of the metabotropic glutamate receptors produces various effects on synaptic transmission. Firstly, mGlu receptors do not produce synaptic fast potentials and their effects could be measured primarily as slow changes in the membrane potential or modulation of synaptic activity evoked by fast transmitters (Morisset & Nagy, 1996).

The metabotropic glutamate receptor family is subdivided into three groups: I, II and III on the basis of their receptor homology, pharmacology and coupling to second messenger systems (Nakanishi, 1992; Conn & Pin, 1997). Group I mGlu receptors include mGlu1 and mGlu5. Group II comprises

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mGlu2 and mGlu3, while mGlu4, mGlu6 and mGlu7 receptors belong to group III. They are all G-protein coupled receptors. Group I receptors are linked to protein kinase C (PKC) and their activation increases intracellular Ca^{2+} levels, whereas group II mGlu receptors are coupled to adenylate cyclase (AC) through Gi/Go and decrease cyclic AMP accumulation. In the spinal cord, transcripts of mGluR1 and mGlu5 receptors and their corresponding proteins are heavily concentrated in the superficial dorsal horns (Berthele *et al*., 1999; Boxall *et al*., 1998b).

Contribution of mGlu receptor activation to nociceptive transmission in the spinal cord is well documented (Neugebauer *et al*., 1994; Young *et al*., 1994). Positive potentiation of NMDA- and AMPA-induced depolarization or current in spinal neurones by mGlu receptors has been reported by several groups (Cerne & Randic, 1992; Bond & Lodge, 1995; Jones & Headley, 1995, Bordi & Ugolini, 2000; Ugolini *et al*., 1997; 1999). The effect is induced primarily *via* activation of group I mGlu receptors (Ugolini *et al*., 1997). Recent evidence also suggests that group II mGlu receptors depress synaptic transmission in the dorsal horn (Gerber *et al*., 2000) as well as in motoneurones (Jane *et al*., 1995). Consistent with these findings, there is published evidence that group I and II mGlu receptor activation has also been shown to induce long-term depression-like responses in the spinal cord *in vitro* (Chen & Sandkühler, 2000; Zhong *et al*., 2000).

Recent immunohistochemical data indicate that mGlu5 receptors are found on a large number of inhibitory interneurones in lamina II of the spinal cord (Davies *et al*., 2000; Jia *et al*., 1999). The inhibitory neurotransmitter GABA, its synthesizing enzyme glutamic acid decarboxylase (GAD) and one of its receptor classes, the $GABA_B$ receptor are clustered in the superficial laminae of the spinal cord (Todd *et al*., 1996). Therefore, activation of mGlu5 receptors on these interneurones may cause the release of one or more inhibitory neurotransmitters and could explain the negative modulatory effects of group I mGlu receptors in the spinal cord.

In this study, we used selective pharmacological agents for mGlu receptors and the $GABA_B$ receptor for the investigation of the effects of group I mGlu receptors on synaptic transmission in the isolated spinal cord of the juvenile rat.

Methods

Details of the hemisected spinal cord preparation of the juvenile rat has been described previously (Thompson *et al*., 1995). Briefly, under deep, terminal enflurane anaesthesia rat pups (Sprague Dawley, 9–12 days; 25–30 g) were decapitated. The spinal cord was rapidly removed with dorsal and ventral roots intact in ice-cold (4°C) aerated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) containing: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10.0; all concentrations given in mM). The dural sheath was removed and the cord was hemisected along the ventral midline. The hemisected cord was transferred to a perspex bath at 23°C and perfused at a flow rate of 8 ml min^{-1}. The hemisected cords were left for at least 1 h before superfusion of drugs. Recordings were made from a ventral root (L5 or L6) which was isolated from the spinal cord by a vaseline and a Perspex barrier.

Responses were amplified (Axon Instruments), digitized (1401 Plus) and displayed using the Spike2 software (Cambridge Electronic Design). Statistical analysis was carried out on peak amplitude (mV) measurements using ANOVA followed by *post hoc* Dunnett test.

Solutions of drugs were made freshly and superfused directly to the spinal cord by use of peristaltic pumps (P-1, Pharmacia). NMDA was superfused for 30 s with a cycle time of 10 min while mGlu receptors agonists were applied for 20 min. Superfusion of the antagonists started 10 min prior to application of the agonists and continued during the agonist application. All drugs were purchased from Tocris except CGP56433A and 2-methyl-6-(phenylethynyl)-pyridine (MPEP) which were synthesized in-house (Novartis Pharma AG, Basel).

Results

Effect of CHPG on the ventral root depolarization in the hemisected spinal cord of the juvenile rat

Brief, 30 s superfusion of CHPG resulted in a concentration-dependent depolarization of the ventral root, with an estimated 280 ± 24 μM EC_{50} value ($n=4$; Figure 1). The subthreshold concentration of CHPG was determined at 10 μM, which consistently remained ineffective even when the superfusion period exceeded 30 s. The concentration–response curve was significantly shifted to the right hand side in the presence of 30 μM MPEP, the selective mGlu5 receptor antagonist.

Effect of CHPG on NMDA responses

Brief superfusion of sub-threshold concentration of CHPG (10 μM; 30 s; $n=4$) did not produce any depolarization of spinal motoneurones and did not affect NMDA-induced ventral root responses (10 μM; 30 s) evoked at 10 min intervals, 1 min after CHPG application (not shown). However, while 10 min superfusion of the same concentration of CHPG did not produce change in the ventral root potential, it inhibited the NMDA-induced ventral root response by $34 \pm 9\%$ and $32 \pm 10\%$ ($n=10$) of control at the end of the 10 min perfusion time and 10 min after washout, respectively (Figure 2). The NMDA-induced ventral

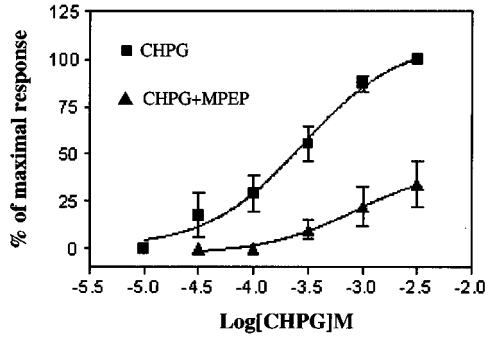

Figure 1 Concentration-response relationship of CHPG in the hemisected spinal cord preparation. The calculated EC_{50} of CHPG was 280 ± 24 μM; $n=4$. In the presence of 30 μM MPEP, a significant right hand shift of the CHPG concentration–response curve was observed.

root depolarization recovered to the control value after a 30 min washout period. One striking effect of CHPG was the reversible inhibition of the spontaneous synaptic activity (see traces in Figures 2 and 4). MPEP (2-methyl-6-(phenylethynyl)-pyridine; 100 nM; $n=4$), the selective mGlu5 receptor antagonist, superfused for 20 min, did not affect the spontaneous activity (Figure 3) and NMDA-induced ventral root depolarisation (Figure 3B). However, it blocked the CHPG-induced (30 μM; 30 s) ventral root depolarization (Figure 3A).

The same concentration of MPEP (100 nM; 20 min; $n=3$) blocked the CHPG-induced (10 min) depression of the ventral root depolarization evoked by 30 s perfusion of 10 μM NMDA (Figure 4). This effect of MPEP was completely reversible after a 30 min washout.

Effect of CGP 56433A on CHPG-induced modulation of NMDA responses

The specific, high affinity $GABA_B$ receptor antagonist CGP 56433A (10 μM; 10 min; $n=8$; Getova *et al*., 1997; Patel *et al*., 2001) caused some increase in the spontaneous firing rate and produced a slight depolarization of the baseline potential associated with increased bursting activity in some preparations. However, it did not significantly affect the amplitude or duration of the NMDA-induced ventral root response when applied for 10 min prior to perfusion of 10 μM NMDA for 30 s. When CGP 56433A and CHPG were co-perfused for 10 min prior to NMDA application the NMDA-induced ventral root response was significantly potentiated by $64\pm12\%$ ($n=8$; also see histogram in Figure 5). This returned to control value following 10 min of washout (Figure 5A). Figure 5 demonstrates the attenuating effects of CHPG on the NMDA-induced ventral root potential in the absence of CGP56433A, obtained from the same preparation (Figure 5B).

The effects of the selective group II agonist, (2R,4R)-APDC

Superfusion of 10 μM (2R,4R)–APDC ($n=4$), the selective group II mGlu receptor agonist, for 10 min, did not affect the baseline potential but significantly blocked the spontaneous activity (see Figure 6). On the other hand, it did not have any significant effect on the amplitude and duration of the NMDA-induced ventral root response (10 μM, 30 s). The spontaneous activity re-appeared about 10 min following washout.

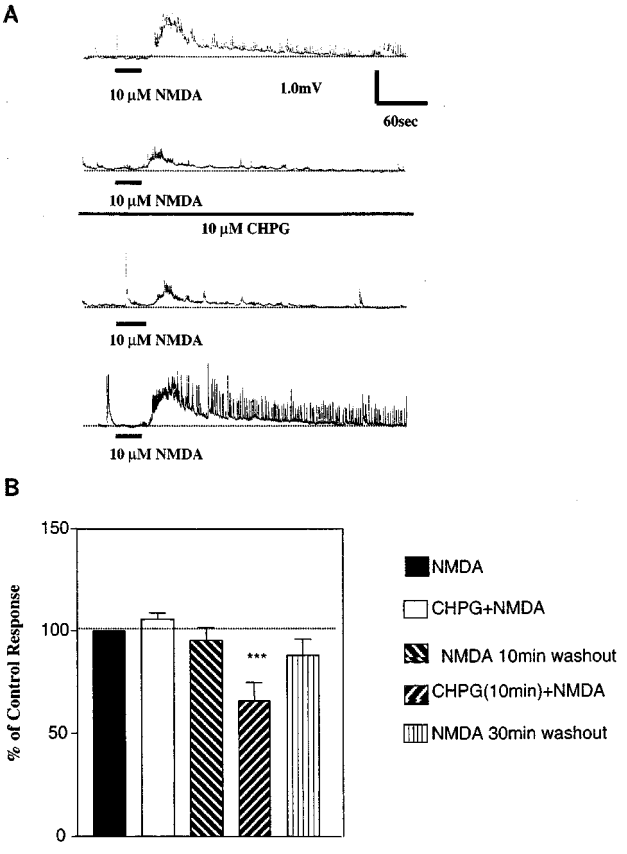

Figure 2 The selective mGlu5 receptor agonist, CHPG, attenuates the NMDA-induced ventral root depolarization. (A) Ventral root responses evoked by NMDA (first trace: control; 10 μM; 30 s) were attenuated by a 10 min superfusion of 10 μM CHPG. NMDA was applied at the end of the 10 min application of CHPG (second trace), 10 (third trace) and 30 min (fourth trace) after CHPG application. Note the effect of 10 μM CHPG on the spontaneous activity. All traces are from the same preparation. (B) Quantitative representation of the effects of CHPG on NMDA-induced ventral root depolarization, based on $n=10$ experiments. All data are presented as mean $\pm$ s.e.mean.

Discussion

Activation of the mGlu5 and/or mGlul receptors, positively modulates the NMDA receptor in the thalamus (Salt &

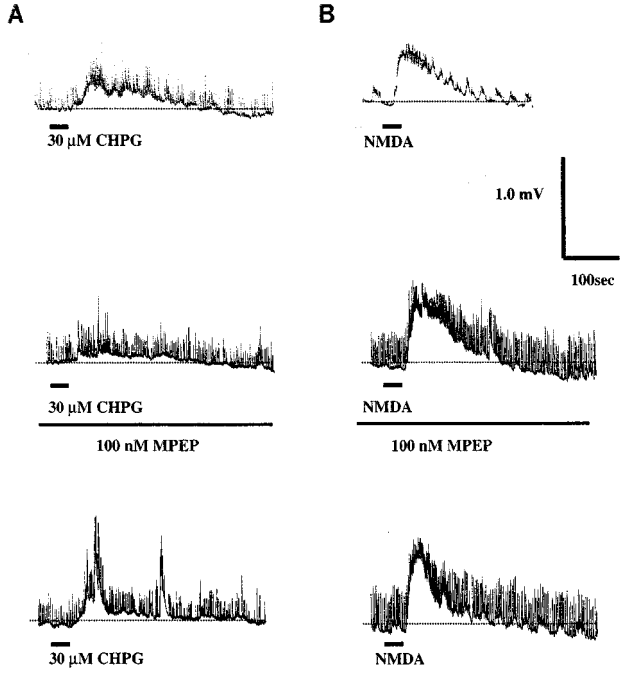

Figure 3 MPEP (100 nM; 20 min superfusion), the selective mGluR5 antagonist (A) blocks the ventral root depolarization evoked by 30 s superfusion of 30 μM CHPG but (B) did not affect the NMDA-induced (10 μM, 30 s) ventral root depolarization. The interval between drug applications was 30 min. All recordings are taken from the same preparation.

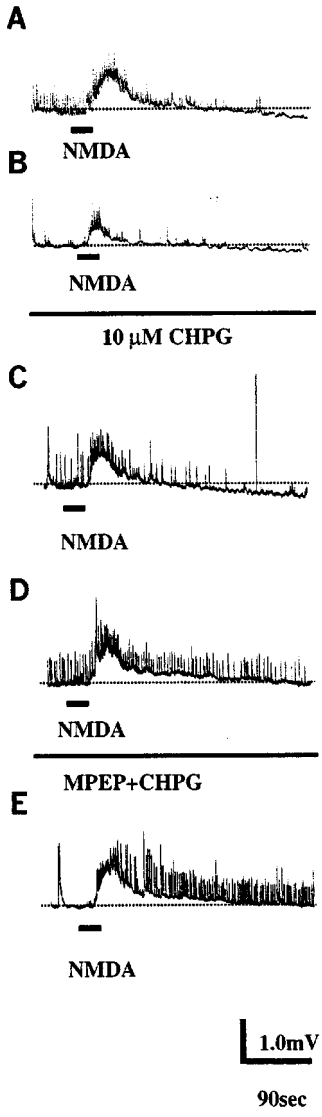

Figure 4 MPEP (100 nM; 20 min superfusion) blocks the synergistic effects of CHPG (10 μM, 10 min superfusion) on NMDA-induced (10 μM, 30 s) ventral root depolarization. (Note that the concentration of MPEP is the same as used in the experiment, illustrated in Figure 2.) (A) Control response of NMDA. (B) CHPG reduced the amplitude of the NMDA-induced ventral root depolarization. (C) After 30 min washout the NMDA-induced ventral root depolarization returned to its control level. (D) The CHPG-induced attenuation of the ventral root depolarization evoked by NMDA was prevented by co-perfusion of 100 nM MPEP. (E) The NMDA-induced ventral root depolarization returned to control level after 30 min washout. Note the reduction of spontaneous activity in the presence of 10 μM CHPG. All traces represent data from the same experiment.

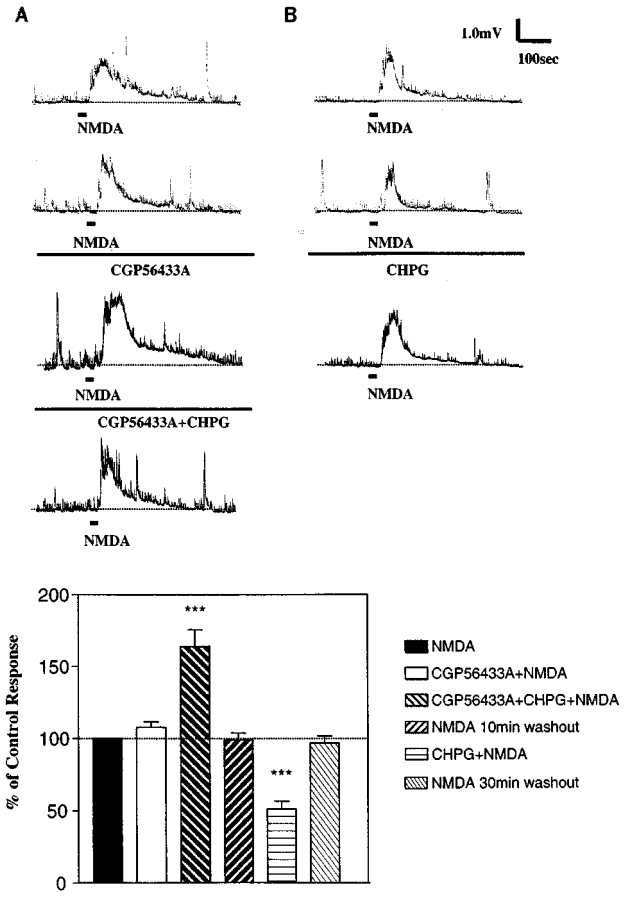

Figure 5 The selective $GABA_B$ receptor antagonist CGP56433A reverses the effects of CHPG on NMDA-induced ventral root depolarization. (A) The NMDA-induced ventral root depolarization was not affected by the selective $GABA_B$ receptor antagonist, CGP56433A (10 μM, 10 min superfusion; second trace). However, the combination of CGP56433A and CHPG (both 10 μM, 10 min; third trace) produced an increase of the NMDA response. After a 30 min washout period the NMDA response returned to control level (fourth trace). (B) The inhibitory effects of CHPG in the absence of CGP56433A is demonstrated in the same preparation. Third and fourth trace were recorded 10 and 30 min after CHPG superfusion, respectively. All traces are from the same preparation. (C) Quantitative analysis of the effects of CGP56433A and CHPG on the NMDA-induced ventral root depolarization ($n=13$). All data are presented as mean $\pm$ s.e.mean.

Binns, 2000; Salt & Turner, 1998). This interaction is similar to that previously described between neurokinin and NMDA receptors in the spinal cord (Fox *et al*., 1996; Rusin *et al*., 1992). There is evidence for positive modulatory effects of group I mGlu receptors in the isolated and *in vivo* preparations of spinal cord (Bordi & Ugolini, 2000; Ugolini *et al*., 1997; 1999; Boxall *et al*., 1998a). Some of these studies (Ugolini *et al*., 1997; 1999) examined the direct effects of various group I mGlu receptor ligands on motoneurones in the presence of TTX and assumed clear positive modulation of NMDA responses measured by voltage changes in the ventral root potential.

In the present study, selective activation of mGlu5 receptors with a sub-threshold concentration of CHPG, resulted in a long lasting depression of the NMDA-induced ventral root response which recovered only following 30 min of washout. CHPG alone produced a concentration–dependent depolarization of the ventral root with an EC_{50} of 280 μM in the same preparation. The sensitivity of the preparation to CHPG is difficult to explain as we needed a considerably lower concentration in the present study than reported earlier by others. Ugolini *et al*. (1999) used a 1.0 mM CHPG, however in this study authors did not examine the effect of sub-threshold CHPG and they used neonatal rats. The reduction of spontaneous activity of the ventral root potential was also a common observation during these

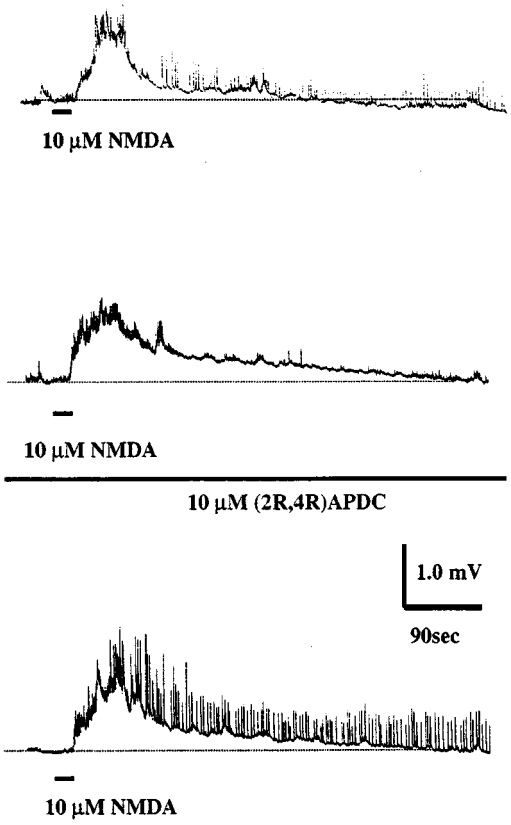

Figure 6 Lack of effect of the group II mGluR receptor agonist (2R,4R)–APDC on NMDA-induced ventral root depolarization. Prolonged (10 min) superfusion of 10 μM (2R,4R)–APDC, a selective group II mGluR agonist, did not affect the amplitude or duration of the NMDA-induced (10 μM; 30 s) ventral root depolarization. The spontaneous activity of the ventral root potential was attenuated by (2R,4R)–APDC.

experiments. The effect of CHPG was prevented by the use of the mGlu5 receptor specific antagonist, MPEP. MPEP is a highly potent inhibitor of qisqualate-induced PI hydrolysis ($IC_{50}=3$ nM) in L(tk-) cells expressing the human mGlu5a receptors, and in rat hippocampal slices (Gasparini *et al*., 1999). MPEP was also shown to inhibit (S)-3,5-DHPG-enhanced release of electrically-induced [3H]D-Asp in rat brain slices (Thomas *et al*., 2001). The concentration of MPEP for the present study was defined in the experiments represented in Figure 3, and was within the range of that used in the brain slice preparation ($IC_{50}=240$ nM; Thomas *et al*., 2001). Our data indicate that activation of mGlu5 receptors is at least partly responsible for the depression of the NMDA-induced ventral root depolarization. While activation of mGlu5 receptors produces an increase in responsiveness of various neurones in the nervous system (Fitzjohn *et al*., 1996; Jones & Headley, 1995; Pisani *et al*., 1997; Salt *et al*., 1999) including mechanically or chemically (TTX) isolated spinal cord neurones (Cerne & Randic, 1992; Ugolini *et al*., 1999), the attenuating effects of CHPG on the ventral root potential could be only explained by the consideration of a spinal circuitry involving inhibitory elements.

Recent evidence suggests that mGlu5 receptors are located in abundance on inhibitory interneurones in lamina II of the spinal dorsal horn (Davies *et al*., 2000). The inhibitory neurotransmitter GABA, its synthesizing enzyme glutamic acid decarboxylase (GAD) and one of its receptor classes, the $GABA_B$ receptor are clustered in the superficial laminae of the spinal cord. About 50% of $GABA_B$ receptors in lamina II are on unmyelinated C-fibres and are thought to be presynaptic (Malcangio & Bowery, 1996; Teoh *et al*., 1996). Activation of $GABA_B$ receptors result in decreased release of transmitters from nerve terminals and a decrease in postsynaptic excitability.

We tested the hypothesis that functional mGlu5 receptors might be located on GABA-ergic interneurones (Hongge *et al*., 1999). Superfusion of CGP56433A, the specific, high affinity $GABA_B$ receptor antagonist for 10 min did not alter the ventral root response to NMDA superfusion, although by itself it induced an increase in the rate and amplitude of spontaneous activity in addition to a small increase in the baseline ventral root potential. However, prolonged, combined superfusion of CGP56433A and CHPG to the hemisected spinal cord potentiated the response of NMDA.

One possible interpretation of our observations is that mGlu5 receptors are expressed on both GABA-ergic and excitatory neurones in the superficial dorsal horn of the spinal cord. Their activation would, on one hand, potentiate GABA release and activate $GABA_B$ receptors on deep dorsal horn neurones to attenuate NMDA-induced responses. On the other hand, during $GABA_B$ receptor blockade, the positive modulation of NMDA responses by activation of mGlu5 receptors in the same neurones would result in hyperexcitability. This explanation is also in good agreement with earlier finding, which demonstrated that the interaction of group I mGlu and NMDA receptors is always facilitatory in single spinal neurones (Cerne & Randic, 1992). The above hypothesis is further supported by histological evidence showing the presence of mGlu5 receptors on GABA-ergic cells in lamina II (Davies *et al*., 2000). In the present series of experiments we used NMDA, instead of glutamate, the natural transmitter. This made it possible to avoid the activation of the majority of neurones in lamina II through their most dominant non-NMDA type glutamate receptor (Yoshimura & Jessell, 1990).

Alternatively, our results could be explained by the loss of GABA-ergic modulation of motoneuronal excitability. Activation of mGlu5 receptors on inhibitory neurones can also modulate the GABAergic inhibitory tone of the motoneurones, hence when the $GABA_B$ receptor antagonist was applied together with CHPG, a positive modulation of NMDA responses in the motoneurones was revealed. This is supported by the findings that in the presence of TTX both group I mGlu receptor and NMDA receptor agonists produced ventral root depolarization (Cao *et al*., 1997; Chen *et al*., 2000; Ugolini *et al*., 1997).

When the group II specific agonist (2R,4R)–APDC was superfused for a prolonged period of time, it did not change the NMDA response, although the spontaneous activity of the ventral root was abolished. A recent study by Gerber *et al*. (2000) found that both group II and III mGluRs can negatively modulate synaptic transmission in the dorsal horn. They found that both excitatory and inhibitory postsynaptic potentials evoked by primary afferent stimulation were inhibited by the selective group II and III mGlu receptor agonist, (2S,1′R,2′R,3′R)-2-(2′,3′-dicarboxy-cycloprpropyl)

glycine. The mixed effects of group II and III agonists on excitatory and inhibitory transmission might have balanced the net influence of (2R,4R)-APDC on the NMDA-induced ventral root potential in the present experiments.

In conclusion, activation of mGlu5 receptors on GABA-ergic interneurones, either in lamina II of the spinal dorsal horn or in the ventral horn, evokes release of GABA, which acts, in part through $GABA_B$ receptors to suppress group I mGlu receptor-induced modulation of NMDA responses. The inhibitory effect is long lasting and masks the potentiation of NMDA-induced responses by mGlu5 receptor activation. Blockade of $GABA_B$ receptors with its specific high affinity $GABA_B$ receptor antagonist CGP56433A reveals the synergistic effect of mGlu5 receptor activation on NMDA responses. Loss of GABA in neuropathic pain may contribute to spinal hyper-excitability and increased sensitivity to pain (Sivilotti & Woolf, 1994).

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