Actions of LY341495 on metabotropic glutamate receptor-mediated responses in the neonatal rat spinal cord

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1 The group II metabotropic glutamate (mGlu) receptor antagonist (2*S*,1'*S*,2'*S*)-2-(2-carboxycyclopropyl)-2-(9*H*-xanthen-9-yl)glycine (LY341495) also has activity at group I and III mGlu receptors at higher concentrations and can be used to discriminate between mGlu receptor subtypes. We report the antagonist action of LY341495 on glutamate receptors expressed in the neonatal rat spinal cord preparation and the use of this antagonist to investigate the group III mGlu receptor subtypes responsible for mediating the depression of synaptic transmission in the spinal cord mediated by the group III mGlu receptor agonists (*S*)-2-amino-4-phosphonobutanoic acid ((*S*)-AP4) and (1*S*,3*R*,4*S*)-1-aminocyclopentane-1,2,4-tricarboxylic acid (ACPT-I).

2 LY341495 antagonised mGlu receptor agonist-induced responses in the spinal cord with a rank order of potency of group II > group III > group I, which is the same as that observed in human cloned mGlu receptor cell lines. Antagonism of group II and III mGlu receptor-mediated effects were time dependent when low-nanomolar concentrations of LY341495 were used. Although the rank order of potency of LY341495 was the same on native rat and cloned human mGlu receptors, there was a compression in the selectivity between group II and III mGlu receptors, expressed in the spinal cord.

3 In agreement with a previous study on cloned ionotropic glutamate receptors 100 μ M LY341495 had little or no effect on *N*-methyl-*D*-aspartate, (*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionic acid or kainate receptor-mediated responses on motoneurons.

4 LY341495 exhibited low-nanomolar potency antagonist activity against (*S*)-AP4 and ACPT-I suggesting that these agonists are activating predominantly mGlu8 and that mGlu4 receptors do not play a role in modulating synaptic transmission in the pathways stimulated in the experiments described here.

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Abbreviations: (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; (1*S*,3*S*)-ACPD, (1*S*,3*S*)-1-aminocyclopentane-1,3-dicarboxylic acid; ACPT-I, (1*S*,3*R*,4*S*)-1-Aminocyclopentane-1,2,4-tricarboxylic acid; (2*R*,4*R*)-APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid; (*S*)-AP4, (*S*)-2-amino-4-phosphonobutanoic acid; (*R*)-AP5, (*R*)-2-amino-5-phosphonopentanoic acid; CRC, concentration–response curve; (*S*)-3,5-DHPG, (*S*)-3,5-dihydroxyphenylglycine; fDR-VRP, fast component of the dorsal root-evoked ventral root potential; LY341495, (2*S*,1'*S*,2'*S*)-2-(2-carboxycyclopropyl)-2-(9*H*-xanthen-9-yl)glycine

Introduction

To date, eight metabotropic glutamate (mGlu) receptors have been identified and classified into three distinct groups, based on their signal transduction mechanism, agonist selectivity, and sequence homology (Pin & Duvoisin, 1995; Schoepp *et al.*, 1999). When expressed in non-neuronal cell lines, group I mGlu receptors (mGlu1 and 5) are positively coupled to phosphoinositide hydrolysis that leads to an increase in intracellular $[Ca^{2+}]$. In contrast, group II (mGlu2 and 3) and group III (mGlu4, 6, 7, and 8) mGlu receptors are negatively coupled to adenylyl cyclase turnover leading to a decrease in intracellular cAMP levels. In neurones, mGlu receptors have been shown to couple to Ca^{2+} and K^{+} channels thereby

modulating neuronal excitability (for a review see Pin & Duvoisin, 1995).

In the neonatal rat hemisectioned spinal cord preparation activation of group I mGlu receptors with (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid ((1*S*,3*R*)-ACPD) results in a depolarisation of motoneurons (Jane *et al.*, 1993; Watkins & Collingridge, 1994). This provides a system for evaluation of novel agonists and antagonists of group I mGlu receptors. This preparation also allows the characterisation of group II and III mGlu receptor selective compounds, as selective agonists of group II ((1*S*,3*S*)-1-aminocyclopentane-1,3-dicarboxylic acid ((1*S*,3*S*)-ACPD) (Pook *et al.*, 1992) and (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid ((2*R*,4*R*)-APDC)) (this report) and group III (e.g. (*S*)-2-amino-4-phosphonobutanoic acid ((*S*)-AP4) (Evans *et al.*, 1981, 1982)) produce a concentration-dependent reduction in the fast component of

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Figure 1 Structures of some of the mGlu receptor agonists and antagonists used in this study. The acronyms are defined in the text.

Table 1 Activity of (S)-AP4 and LY341495 on human mGlu receptor subtypes

Human mGlu receptor	EC_{50} μM (K_i) (S)-AP4 ^a	K_i or K_D μM (IC_{50}) LY341495
mGlu1a	> 1000	7.0 ± 1.2^b (6.8 ± 1.1) ^c
mGlu5b	> 1000	7.6 ± 2.6^b , (8.2 ± 2.0) ^c
mGlu2	448 ± 80	0.023 ± 0.005^c , 0.00167 ± 0.00020^d
mGlu3	> 1000	0.0036 ± 0.0003^c , 0.00075 ± 0.00043^d
mGlu4a	0.32 ± 0.03	$(22.0 \pm 1.3)^c$
mGlu6	0.055 ± 0.004 (6.8 ± 3.1) ^c	0.0316 ± 0.0068^f
mGlu7a	175 ± 39 (211 ± 43) ^c	0.0727 ± 0.0220^f , (0.990 ± 0.070) ^c
mGlu8	0.06 ± 0.01 (1.6 ± 0.3) ^c	0.057 ± 0.012^c , 0.0140 ± 0.0011^f

^a EC_{50} values for inhibition of forskolin-stimulated cAMP production, Thomas *et al.* (2001). ^b K_i values from Schild plots of functional assays, Fitzjohn *et al.* (1998). ^c K_i values from Schild plots of functional assays (in some cases IC_{50} values were measured these are given in parenthesis), Kingston *et al.* (1998). ^d K_D values for binding of [³H]LY341495, Johnson *et al.* (1999). ^e K_i for displacement of [³H]LY341495 binding by (S)-AP4, Wright *et al.* (2000). ^f K_D values for binding of [³H]LY341495, Wright *et al.* (2000).

the dorsal root-evoked ventral root potential (fDR-VRP). This reduction in amplitude is probably because of the activation of presynaptic group II or III mGlu receptors leading to a reduction of glutamate release and depression of synaptic transmission (Jane *et al.*, 1994). However, it is currently unknown which subtypes of the group II and III mGlu receptors mediate this depression. These presynaptic group III mGlu receptors are likely to act as autoreceptors with a similar function to that proposed for group II mGlu receptors (Cartmell & Schoepp, 2001; Schoepp, 2001).

Recently, Ornstein *et al.* (1998a,b) and Kingston *et al.* (1998) have identified an antagonist with low-nanomolar potency for mGlu receptors. This compound (2*S*,1'*S*,2'*S*)-2-(2-carboxycyclopropyl)-2-(9*H*-xanthen-9-yl)glycine (LY341495) (Figure 1) exhibits 10-fold selectivity for mGlu2 and three over mGlu8 receptors. Furthermore, LY341495 is ~six-fold selective for mGlu8 receptors when compared with mGlu7 or mGlu6 receptors. LY341495 also displays micromolar potency as an antagonist at cloned mGlu1, 4, and 5 receptors (see Table 1) (Schoepp *et al.*, 1999). To investigate whether LY341495 shows the same degree of selectivity on native mGlu and ionotropic glutamate (iGlu) receptors, we have characterised its actions on glutamate receptor subtypes expressed in the spinal cord.

In this report, we describe the pharmacological characterisation of LY341495 on group II and III mGlu receptors on primary afferent terminals and group I mGlu receptors, *N*-methyl-D-aspartate (NMDA), (S)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) and kainate

receptors expressed on neonatal rat motoneurons. In particular, we were interested in determining which of the subtypes within group III are involved in controlling synaptic transmission in the spinal cord. As part of this project the actions of LY341495 on the depression of synaptic transmission in the spinal cord mediated by the group III selective agonists (S)-AP4 and (1*S*,3*R*,4*S*)-1-aminocyclopentane-1,2,4-tricarboxylic acid (ACPT-I) (Acher *et al.*, 1997; De Colle *et al.*, 2000) were studied.

Preliminary reports of part of this work have been published (Jane & Thomas, 1999; Howson & Jane, 2001).

Methods

The neonatal rat spinal cord preparation is a convenient source of a number of glutamate receptor subtypes (Watkins & Evans, 1981; Tölle *et al.*, 1993; Schoepp *et al.*, 1999; Stegenga & Kalb, 2001). Stimulation of the dorsal root allows the dorsal root-evoked ventral root potential (DR-VRP) to be recorded from the corresponding ventral root. The fDR-VRP is mediated chiefly by the activation of postsynaptically expressed AMPA receptors (More *et al.*, 2002), while the slow component is mediated mainly by NMDA receptors (Evans *et al.*, 1982).

The hemisectioned spinal cords from 1- to 5-day-old rats were used according to the method of Evans *et al.* (1982). Briefly, recordings were made from a ventral root in the lumbar region of the spinal cord following supramaximal stimulation of the

corresponding dorsal root ($16 \times$ threshold V, 2 stimulations min^{-1} , pulse width 0.2 ms). The standard superfusion medium contained (mM): NaCl 118, NaHCO_3 25, KCl 3, CaCl_2 2.5, D-glucose 12, gassed with 95% O_2 /5% CO_2 .

Characterisation of mGlu receptors on primary afferent terminals

We have previously shown that a number of group II and III mGlu receptor agonists depress the fDR-VRP (Jane *et al.*, 1994; Thomas *et al.*, 2001). The broad-spectrum mGlu receptor antagonist LY341495 was investigated for the ability to reverse (1S,3S)-ACPD-, (2R,4R)-APDC-, (S)-AP4-, and ACPT-I-induced depressions of the fDR-VRP in neonatal rat spinal cord.

Isolation of the fDR-VRP, which is mediated primarily by AMPA receptors (More *et al.*, 2002), was achieved by addition of 2 mM MgSO_4 and 50 μM (R)-2-amino-5-phosphonopentanoic acid ((R)-AP5) into the standard medium (30 min preincubation) to block any NMDA receptor-mediated component. All solutions were superfused over the preparation at a rate of 1 ml min^{-1} . Agonist-containing medium was superfused over the preparation for 5 min at a range of concentrations producing a 5–85% depression of the fDR-VRP compared to control. It was determined that the group II and III mGlu receptor agonists used in this study were able to abolish completely the fDR-VRP (results not shown). Averages of the peak amplitudes of two consecutive responses in the absence of the agonist and when the minimum response size was achieved after agonist application were used to calculate the percentage depression of the fDR-VRP.

Timecourse experiments were performed by applying concentrations of (1S,3S)-ACPD (5–6 μM), (2R,4R)-APDC (2–4 μM) and (S)-AP4 (3 μM) that caused approximately a 50% reduction of the fDR-VRP in the absence of LY341495. The same concentration of each agonist was then reapplied in the presence of 5 nM LY341495 ((1S,3S)-ACPD), 10 nM LY341495 ((2R,4R)-APDC), and 20 nM LY341495 ((S)-AP4). Agonist application was continued until a plateau of antagonism was reached. In addition, timecourse experiments were also performed against (S)-AP4 using increasing concentrations of LY341495 (20, 50 and 100 nM). In all the timecourse experiments, the percentage antagonism was calculated in the following manner: $\% \text{ antagonism} = (\% \text{ depression of fDR-VRP}_{\text{absence}} - \% \text{ depression fDR-VRP}_{\text{presence}}) / (\% \text{ depression of fDR-VRP}_{\text{absence}}) \times 100$.

After the equilibration time of LY341495 was obtained from the timecourse experiments, nonsequential, noncumulative concentration–response curves (CRCs) were constructed to (2R,4R)-APDC, (S)-AP4, and ACPT-I in the absence and presence of LY341495. CRCs to (S)-AP4 and ACPT-I were constructed in the presence of different concentrations of LY341495 and Schild plots constructed.

Characterisation of glutamate receptors on motoneurons

In order to assess the selectivity of LY341495 it was characterised on group I mGlu (mGlu1 and mGlu5) and NMDA, AMPA, and kainate receptors expressed on motoneurons. Experiments on postsynaptic glutamate receptors were carried out in the presence of tetrodotoxin (TTX: 10 μM for 2 min, then 0.1 μM continuously), but in the absence of

MgSO_4 /(R)-AP5 and electrical stimulation of the ventral root. Agonists were applied for 1 min in these experiments. In all experiments described in this section, the peak amplitude of agonist-induced depolarisation of motoneurons was measured from d.c. shifts in ventral root polarity after agonist application.

Noncumulative, nonsequential CRCs to the group I mGlu receptor agonist (1S,3R)-ACPD were constructed in the absence and presence of 5 μM LY341495.

NMDA (8 μM), AMPA (0.8 μM), kainate (4 μM) and the selective group I mGlu receptor agonist (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG, 20 μM) were applied at concentrations that produced equal-sized depolarisations. The agonists were then reapplied in the presence of 100 μM LY341495 (15 min preincubation). Agonists were again applied after a 30 min washoff of LY341495.

Materials

LY341495, (S)-AP4, (1S,3S)-ACPD, (2R,4R)-APDC, ACPT-I, (S)-3,5-DHPG, NMDA, AMPA, kainate, (R)-AP5, and TTX were obtained from Tocris Cookson, Langford, Bristol, U.K. All other chemicals were of analytical grade or above.

Data analysis

All CRCs were analysed by iterative nonlinear regression (GraphPAD Prism v 2.0) to obtain EC_{50} values. Apparent K_D values for antagonists were obtained using the Gaddum–Schild equation, $\text{DR} = 1 + ([\text{Antag}]/K_D)$, where for this study the dose ratio (DR) is defined as the EC_{50} in the presence of the antagonist divided by the EC_{50} in the absence of the antagonist. Where Schild plots were constructed pA_2 values were obtained. In addition, if the slope of the Schild plot did not significantly differ from unity, the slope was restricted to one and a pK_B value calculated.

Results

Timecourse experiments

The effects of LY341495, against group II and III mGlu receptor-mediated depressions of the fDR-VRP were time dependent. To examine this phenomenon timecourse experiments were performed. (1S,3S)-ACPD, at a concentration that produced a 50% reduction of the fDR-VRP (5–6 μM) in the absence of LY341495, was applied to the spinal cord already perfused in 5 nM LY341495. Under these conditions, the degree of antagonism caused by LY341495 increased with time. This time-dependent antagonism reached a plateau 140 min after LY341495 application. The degree of antagonism reached was approximately 75% (Figure 2a, $n = 3$). It is known that (1S,3S)-ACPD also has agonist activity at group I mGluRs and mGlu4 receptors (for a review see Schoepp *et al.*, 1999). To ensure that this effect was not peculiar to (1S,3S)-ACPD a more selective group II mGlu receptor agonist, (2R,4R)-APDC (Schoepp *et al.*, 1995), was used (2–4 μM), in this case against 10 nM LY341495. In agreement with the experiments using (1S,3S)-ACPD, time-dependency of the antagonism of (2R,4R)-APDC-induced responses by LY341495 was observed. The degree of antagonism reached

Figure 2 Time-dependent antagonism of LY341495 in the neonatal rat spinal cord preparation. Time-dependent antagonism was observed using three different agonists; (1*S*,3*S*)-ACPD (a), (2*R*,4*R*)-APDC (b) and (S)-AP4 (c) against different concentrations of antagonist; 5 nM (a), 10 nM (b), and 20, 50 and 100 nM (c). Against (S)-AP4 the time taken to reach a plateau was shortened and the percentage of antagonism reached was higher with increasing concentrations of (S)-AP4. Values represent percentage of antagonism $\pm$ s.e.m. and are from three independent preparations.

was again approximately 75%, although the time taken for this antagonism to develop was reduced to 100 min (Figure 2b, $n=3$). Similar time-dependency experiments were carried out using (S)-AP4 ($3\mu\text{M}$) as the agonist against three different concentrations of LY341495 (20, 50, and 100 nM). Again a time-dependent effect was observed. This time-dependent effect was also concentration dependent with a greater degree of antagonism and plateau being reached earlier in higher concentrations of LY341495 (Figure 2c, all $n=3$).

Table 2 K_D or pA_2 values for the antagonism by LY341495 of group I, II or III mGlu receptor agonist-induced effects in the neonatal rat spinal cord

Agonist	$EC_{50}\ \mu\text{M}^a$	Apparent K_D	pA_2
(1 <i>S</i> ,3 <i>R</i>)-ACPD	29.7 ± 1.8 (3)	$2.6 \pm 0.6\ \mu\text{M}$ (3)	—
(2 <i>R</i> ,4 <i>R</i>)-APDC	2.8 ± 0.5 (3)	$2.3 \pm 0.3\ \text{nM}$ (3)	—
(S)-AP4	2.5 ± 0.5 (4)	—	8.02
ACPT-I	7.1 ± 0.6 (7)	—	8.27

Values represent mean $\pm$ s.e.m. (number of independent determinations in parenthesis). ^a EC_{50} values for agonist-induced effects in the neonatal rat spinal cord.

Figure 3 Concentration-response curves for (2*R*,4*R*)-APDC in the absence and presence of 10 nM LY341495. LY341495 produced a rightward shift of the concentration response curve. Values represent mean % depression $\pm$ s.e.m. and are from three independent preparations.

LY341495 is an antagonist of group II and group III mGlu receptors

CRCs were constructed to (2*R*,4*R*)-APDC in the absence and presence of 10 nM LY341495. An incubation time of 100 min was used, as indicated by the timecourse experiments. In the absence of LY341495, (2*R*,4*R*)-APDC was a potent and full agonist and completely abolished the fDR-VRP with an EC_{50} of $2.8 \pm 0.5\ \mu\text{M}$ ($n=3$). In the presence of LY341495 the CRC was approximately parallel but shifted to the right (Figure 3) allowing an apparent K_D value of $2.3 \pm 0.3\ \text{nM}$ ($n=3$) to be calculated (see Table 2 for a summary of the data).

LY341495 was also tested as an antagonist against two group III mGlu receptor agonists; (S)-AP4 and ACPT-I. Both agonists were able to abolish completely the fDR-VRP with EC_{50} values of $2.5 \pm 0.5\ \mu\text{M}$ ($n=4$) and $7.1 \pm 0.6\ \mu\text{M}$ ($n=7$) for (S)-AP4 and ACPT-I, respectively. The CRCs to the agonists were shifted rightward in the presence of 20 nM LY341495 (Figures 4a and b). CRCs to (S)-AP4 were then constructed in the absence and presence of six concentrations of LY341495 (10 nM (3), 20 nM (4), 30 nM (3), 50 nM (3), 100 nM (4), and 200 nM (3), n =number of independent experiments in parenthesis). A Schild plot of $\log [\text{LY341495}]$ vs $\log (\text{DR}-1)$ was then constructed (Figure 5a). Linear regression produced a pA_2 value of 8.02 and a slope of 0.75 ± 0.11 . Similarly, CRCs to ACPT-I were constructed in the absence and presence of four concentrations of LY341495 (10, 20, 30, and 50 nM, all $n=3$). Again a Schild plot was constructed (Figure 5b) that

Figure 4 Concentration–response curves for (S)-AP4 (a) and ACPT-1 (b) in the absence and presence of 20 nM LY341495. In both cases, LY341495 produced a rightward shift in the CRC. Values represent mean % of maximum depolarisation to (1S,3R)-ACPD in the absence of LY341495 $\pm$ s.e.m. from three independent preparations, (b): results are from three independent preparations.

Figure 5 Schild plots to LY341495 vs (S)-AP4 (A) and LY341495 vs ACPT-1 (B). See text for details.

Figure 6 CRCs for (1S,3R)-ACPD in the absence and presence of 5 μ M LY341495. LY341495 produced a rightward shift of the CRC. Values represent mean % of maximum depolarisation to (1S,3R)-ACPD in the absence of LY341495 $\pm$ s.e.m. from three independent preparations.

had a pA_2 value of 8.27 and a slope of 1.10 ± 0.15 . In the case of the Schild plot for LY341495 vs ACPT-1 the slope was restricted to unity, this produced a pK_B value of 8.33.

LY341495 as an antagonist of group I mGlu receptors on motoneurones

Depolarisation of spinal motoneurones was achieved using the group I mGlu receptor selective agonist (1S,3R)-ACPD. CRCs to (1S,3R)-ACPD were constructed with an EC_{50} value of $29.7 \pm 1.8 \mu$ M ($n = 3$). A second CRC to (1S,3R)-ACPD constructed in the presence of 5 μ M LY341495 was shifted rightward allowing an apparent K_D value of $2.6 \pm 0.6 \mu$ M (Figure 6, $n = 3$) to be calculated.

Selectivity of LY341495 for mGlu receptors vs iGlu receptors

Concentrations of NMDA (8 μ M), AMPA (0.8 μ M), kainate (4 μ M), and (S)-3,5-DHPG (20 μ M) were applied to the spinal cord so that equieffective depolarisations occurred. In the presence of 100 μ M LY341495, the agonists were reapplied. The responses to NMDA, AMPA, and kainate were largely unchanged in the presence of LY341495 at 127.9 ± 39.4 , 90.7 ± 25.1 , and $82.7 \pm 11.7\%$ of control values, respectively (combined results from three independent preparations, Figure 7). In contrast, the response to the control agonist, (S)-3,5-DHPG, was completely abolished in the presence of 100 μ M LY341495. After washout of the LY341495 for 30 min the response to (S)-3,5-DHPG returned to $75.1 \pm 18.0\%$ of control.

Discussion

A range of iGlu and mGlu receptor subtypes are expressed in the neonatal rat spinal cord making it an excellent preparation for testing the activity of compounds on native glutamate receptors. The main aim of this work was to characterise pharmacologically the broad-spectrum mGlu receptor antagonist LY341495 on mGlu and iGlu receptors expressed in the neonatal rat spinal cord preparation. Once the selectivity of LY341495 was confirmed on native mGlu receptors, the

Figure 7 A trace from a typical experiment showing the antagonist effect of $100\ \mu\text{M}$ LY341495 on depolarisations generated directly in motoneurons by $8\ \mu\text{M}$ NMDA (N), $0.8\ \mu\text{M}$ AMPA (A), $4\ \mu\text{M}$ kainate (K) and $20\ \mu\text{M}$ (S)-3,5-DHPG (D).

antagonist could be used to investigate the subtypes of group III mGlu receptors, which contribute to agonist-induced depression of synaptic transmission in the spinal cord.

Pharmacological characterisation of LY341495 on mGlu and iGlu receptors expressed in the rat spinal cord

LY341495 has been characterised on human mGlu receptor subtypes expressed in cell lines, where it was shown to be a competitive antagonist with differential potency at all eight mGlu receptors (Table 1 and Kingston *et al.*, 1998; Johnson *et al.*, 1999; Wright *et al.*, 2000, 2001). Thus, LY341495 has the following group selectivity group II > group III > group I with the exception being mGlu4 in group III as LY341495 has the lowest potency at this receptor.

The present work shows that LY341495 antagonises a range of mGlu receptor subtypes expressed in the rat spinal cord preparation. Characterisation of LY341495 as an antagonist of selective group I, II, or III mGlu receptor agonist-induced responses in the spinal cord has revealed the following rank order of potency: group II > group III > group I. This is in agreement with previously reported studies using cloned human mGlu receptor subtypes (see Table 1). The apparent K_D values obtained for antagonism by LY341495 of group III mGlu receptor-mediated effects in the spinal cord were generally lower than the K_i values from functional assays on

cloned receptors that have been previously reported (see Tables 1 and 2), and therefore the selectivity of LY341495 between group II and III is less in the native system. These discrepancies may be because of protocol and species differences. The antagonism of group II or III mGlu receptor-mediated responses in the spinal cord by low-nanomolar concentrations of LY341495 is both time- and concentration-dependent and is possibly because of the sequestration of the lipophilic antagonist into fatty tissue and/or poor tissue penetration at low antagonist concentrations. It is unlikely that slow kinetics are a factor in the time-dependency as in binding studies LY341495 has been shown to display very rapid kinetics for binding to group II and III mGlu receptor subtypes (Johnson *et al.*, 1999; Wright *et al.*, 2000). It has been reported that (1S,3S)-ACPD shows little discrimination between mGlu2/3 *vs* mGlu4 (for a review see Schoepp *et al.*, 1999). The low-nanomolar potency of the antagonist effect of LY341495 on (1S,3S)-ACPD-induced depression of the fDR-VRP suggests that this agonist is activating group II but not mGlu4 receptors in the spinal cord. In addition, as no depolarisations were observed in the concentration range of (1S,3S)-ACPD used in these studies it is unlikely that this agonist was activating group I mGlu receptors. Responses to the selective group II mGlu receptor agonist, (2R,4R)-APDC (Schoepp *et al.*, 1995) were antagonised by LY341495 with similar potency to that for antagonism of (1S,3S)-ACPD responses. Thus, under the conditions of the experiments performed in this study, (1S,3S)-ACPD was acting as a selective group II mGlu receptor agonist.

LY341495 is more potent at mGlu3 over mGlu2 receptors (Kingston *et al.*, 1998; Johnson *et al.*, 1999). The high potency of LY341495 in this study suggests that (2R,4R)-APDC mediates its effects via mGlu3 receptors. However, LY341495 does not exhibit enough selectivity for mGlu3 over mGlu2 receptors to draw a definite conclusion. It is worth noting that in the spinal cord high levels of mRNA for mGlu3 but not mGlu2 receptors are found (Berthele *et al.*, 1999). LY341495 antagonised group I mGlu receptors expressed on motoneurons with a similar potency to that observed with cloned human mGlu1 and mGlu5 receptors (see Tables 1 and 2). (1S,3R)-ACPD has been reported to activate presynaptically located group II mGlu receptors as well as group I mGlu receptors located on motoneurons (Pook *et al.*, 1992). Generally, we have observed that K_D values for group I mGlu receptor antagonists obtained using (1S,3R)-ACPD as the agonist challenge on the spinal cord preparation are similar to those obtained using cloned group I mGlu receptor assays (Schoepp *et al.*, 1999). In addition, we have observed that comparable K_D values are obtained for group I mGlu receptor antagonists when either (1S,3R)-ACPD or the more selective group I mGlu receptor agonist (S)-3,5-DHPG is used as the agonist challenge (Thomas, Miller, and Jane, unpublished observations). This is likely because of the use of TTX in such experiments to block action potential-dependent glutamate release, which would limit any contribution by presynaptic group II mGlu receptors to the postsynaptically mediated (1S,3R)-ACPD-induced response (Jane *et al.*, 1994). Interestingly, continuous application of (1S,3R)-ACPD to a hemisected cord that has not been treated with TTX led to a complete loss of the depolarisation response on motoneurons because of desensitisation, while the depression of the

fDR-VRP was maintained suggesting that these two responses are not causally associated (Pook *et al.*, 1992). Importantly, 100 μM LY341495 produced little or no effect on depolarisations of motoneurons by NMDA, AMPA, or kainate thus confirming the antagonist specificity for mGlu over iGlu receptors reported previously (Kingston *et al.*, 1998).

Identification of the mGlu receptor subtypes responsible for mediating the depression of the fDR-VRP induced by two selective group III mGlu receptor agonists

Both group II and III mGlu receptors are thought to be expressed on primary afferent terminals that synapse with motoneurons (Pook *et al.*, 1992; Jane *et al.*, 1994; Ohishi *et al.*, 1995; Cao *et al.*, 1997a, b) where they play a role in regulating synaptic transmission. There is evidence for a solely presynaptic location of group II and group III mGlu receptors. Thus, selective group II or group III mGlu receptor agonists or antagonists have no hyperpolarising or depolarising effect when applied to a TTX-treated cord at concentrations where they have been shown to depress the fDR-VRP (Evans *et al.*, 1982; Pook *et al.*, 1992; Jane *et al.*, 1994, 1996). In addition, iGlu or group I mGlu receptor agonist-induced depolarisations of rat spinal motoneurons are not affected by group II or III mGlu receptor agonists or antagonists (Pook *et al.*, 1992; Jane *et al.*, 1994, 1996).

(S)-AP4 has potent agonist activity at mGlu4, 6 and 8 receptors with only weak activity at the mGlu7 receptor (Table 1 and for a review see Schoepp *et al.*, 1999). As mGlu6 seems to be exclusively expressed in retinal ON bipolar cells (Nomura *et al.*, 1994) (S)-AP4 could possibly be mediating its effects on the fDR-VRP through one or more of the remaining group III mGlu receptor subtypes (mGlu4, mGlu7 and mGlu8). It is unlikely that (S)-AP4 is predominantly activating mGlu7 as this agonist has a low potency (EC_{50} value $175 \pm 39 \mu\text{M}$, Table 1) and affinity (K_i value $211 \pm 43 \mu\text{M}$, Wright *et al.*, 2000) at mGlu7 and (S)-AP4 can almost completely depress the fDR-VRP at a concentration of 10 μM . Thus, mGlu4 and/or mGlu8 may be activated by (S)-AP4 leading to the depression of fDR-VRP. Although LY341495 exerts its most potent antagonist activity on group II mGlu receptors, within group III this antagonist has differential activity on individual subtypes with a rank order of potency of $\text{mGlu8} > \text{mGlu6} > \text{mGlu7} \gg \text{mGlu4}$ (see Table 1). LY341495 has been shown to be 380- to 1500-fold selective for mGlu8 over mGlu4 (Kingston *et al.*, 1998; Wright *et al.*, 2000, see Table 1) and can therefore discriminate between mGlu4 and mGlu8 receptor-mediated responses. In the present study, LY341495 antagonised (S)-AP4-induced depression of the fDR-VRP, producing a pA_2 value of 8.02. The high potency of LY341495 against (S)-AP4-mediated effects suggests that the effect of (S)-AP4 on the fDR-VRP is mediated via mGlu8 rather than mGlu4 receptors. However, the slope of the Schild plot deviated significantly from unity suggesting that in this system LY341495 is not acting in a simple, competitive manner against (S)-AP4 at a single receptor. Owing to this unexpected result, LY341495 was tested against another group III mGlu receptor agonist, ACPT-I (Acher *et al.*, 1997; De Colle *et al.*, 2000). ACPT-I has approximately equipotent agonist activity on mGlu8 and mGlu4 receptors (EC_{50} values of 8.2 and 7.2 μM , respectively, De Colle *et al.*, 2000).

LY341495 was a potent antagonist of ACPT-I responses giving a pA_2 value of 8.27 suggesting that this agonist is predominantly activating mGlu8. Interestingly, the slope of the Schild plot was not significantly different from unity, indicating competitive antagonism at a single receptor. This result also suggests that activation of the mGlu4 receptor does not contribute to the depression of the fDR-VRP, as a slope of unity would not be expected if two receptors were being activated. It is therefore reasonable to conclude that activation of mGlu8 receptors by ACPT-I depresses the fDR-VRP. This is in agreement with a study demonstrating that mGlu8 receptors were expressed in lamina IX of the neonatal rat spinal cord using mGlu8-specific antibodies (Miller *et al.*, 2001) and that the mGlu8 selective agonist, (S)-3,4-DCPG is a potent depressant of the fDR-VRP (Thomas *et al.*, 2001).

It is as yet unknown why (S)-AP4 vs LY341495 did not produce a Schild plot with a slope of unity. Two reasons why a Schild plot can have a slope of less than unity are that another receptor is activated at higher concentrations of (S)-AP4 or that there is a saturable removal mechanism for the agonist (Lutz & Kenakin, 1999). The mGlu7 receptor, at which (S)-AP4 has weak agonist activity, has been shown to be expressed on primary afferent terminals using immunohistochemical techniques (Ohishi *et al.*, 1995). In the absence of antagonist (S)-AP4 can completely depress the fDR-VRP at a concentration of $\sim 10 \mu\text{M}$. As the EC_{50} for (S)-AP4 on cloned mGlu7 receptors was 175 μM (Table 1), it is unlikely that mGlu7 receptors play a major role in the depression of the fDR-VRP under these conditions. However, for the higher concentrations of LY341495 used to construct the Schild plot, the maximum concentration of (S)-AP4 used was 100 μM , a concentration that may activate mGlu7 receptors possibly accounting for the slope of less than unity in the Schild plot. However, uptake of (S)-AP4 cannot be ruled out as a possible reason for the nonideal Schild plot.

ACPT-I, however, does produce a Schild plot with a slope of unity against LY341495 suggesting that ACPT-I and LY341495 are acting competitively at a single receptor, at least at the concentrations used in this study. ACPT-I is now the agonist of choice for activation of mGlu8 receptors expressed in the hemisected neonatal rat spinal cord preparation as it produces a monophasic concentration response curve and can completely depress the fDR-VRP. The mGlu8 selective agonist (S)-3,4-DCPG is less useful as it produces a biphasic concentration response curve for depression of the fDR-VRP (Thomas *et al.*, 2001).

Concluding remarks

We have demonstrated that LY341495 has a similar selectivity on native rat mGlu receptors expressed in the spinal cord to that found on human mGlu receptors expressed in a cell line. The potent antagonist action of (S)-AP4 and ACPT-I suggests that these agonists are activating predominantly mGlu8 receptors in the spinal cord and that mGlu4 receptors do not play a role in modulating synaptic transmission in the pathways in the spinal cord stimulated under the conditions of the experiments in this study. Schild analysis of the antagonism of (S)-AP4-induced effects by LY341495 gave a slope of less than unity suggesting the possibility that mGlu7 receptors in the spinal cord may be activated under conditions

where high concentrations of (*S*)-AP4 are used. Thus, ACPT-I would appear to be the agonist of choice for activating mGlu8 in the spinal cord.

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