British Journal of Pharmacology (1996) 117, 1735–1743

Differences in agonist and antagonist activities for two indices of metabotropic glutamate receptor-stimulated phosphoinositide turnover

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1 The abilities of the four diastereoisomers of 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) to stimulate, and the metabotropic glutamate receptor (mGluR) antagonist (±)-α-methylcarboxyphenylglycine (MCPG) to inhibit, phosphoinositide turnover in neonatal rat cerebral cortex have been studied. Two indices of phosphoinositide cycle activity were assessed; inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) mass accumulation, and total inositol phosphate [^{3}H]-$InsP_x$ accumulation (in the presence of Li^+) in *myo*-[^{3}H]-inositol prelabelled slices.

2 The diastereoisomers of ACPD stimulated each response with a rank order of potency of 1S, 3R > 1R, 3R > 1S, 3S > > 1R, 3S. The response to 1R, 3R-ACPD was largely prevented by pre-addition of the NMDA-receptor antagonist, MK-801, or omission of extracellular Ca^{2+}, suggesting that this isomer acts indirectly on phosphoinositide responses through activation of NMDA-type ionotropic glutamate receptors. In contrast, the responses to 1S, 3R- and 1S, 3S-ACPD were unaffected by prior addition of MK-801, but were blocked by MCPG.

3 The concentration of 1S, 3R-ACPD required to half-maximally stimulate the $Ins(1,4,5)P_3$ response ($-\log EC_{50}$ (M), -4.09 ± 0.10) was significantly higher than that required to exert a similar effect on [^{3}H]-$InsP_x$ accumulation ($-\log EC_{50}$ (M), -4.87 ± 0.07; $P < 0.01$; $n = 4$). A similar marked 8–9 fold discrepancy between these two values was observed for the 1S, 3S isomer, which elicited similar maximal responses to those caused by 1S, 3R-ACPD.

4 Significant differences were also observed with respect to the ability of (±)-MCPG (1 mM) to cause a rightward shift in the concentration-response relationships for 1S, 3R-ACPD-stimulated $Ins(1,4,5)P_3$ (5.59 ± 0.24 fold shift) and [^{3}H]-$InsP_x$ (3.04 ± 0.34 fold shift; $P < 0.01$; $n = 4$) responses, giving rise to K_d values of 218 and 490 μM for (±)-MCPG antagonism of the respective responses.

5 The potency difference between the 1S, 3R-ACPD-stimulated $Ins(1,4,5)P_3$ and [^{3}H]-$InsP_x$ responses was reduced when experiments were performed in nominally calcium-free medium ($[Ca^{2+}]_e = 2-5$ μM) and EC_{50} values were almost identical when extracellular calcium was reduced further by EGTA addition ($[Ca^{2+}]_e \leqslant 100$ nM). Similarly, the K_d value for (±)-MCPG antagonism of the 1S, 3R-ACPD-stimulated [^{3}H]-$InsP_x$ response decreased under $[Ca^{2+}]_e$-free conditions, approaching those obtained for the 1S, 3R-ACPD-stimulated $Ins(1,4,5)P_3$ response in the presence of normal $[Ca^{2+}]_e$.

6 These data suggest that estimates of the activities of mGluR agonists and antagonists, derived by measuring phosphoinositide turnover, can differ significantly depending on whether $Ins(1,4,5)P_3$ mass or [^{3}H]-$InsP_x$ responses are measured. In particular, the possibility that the mGluR-mediated [^{3}H]-$InsP_x$ response may not simply reflect direct receptor/G protein/phosphoinositidase C (PIC) activation, but may also be the consequence of stimulation of a facilitatory Ca^{2+}-influx pathway is discussed.

Keywords: Metabotropic glutamate receptor; inositol 1,4,5-trisphosphate; phosphoinositide turnover; phosphoinositidase C; 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) diastereoisomers; (±)-α-methylcarboxyphenylglycine; cerebral cortex (neonatal rat)

Introduction

Since the initial reports of a distinct class of glutamate receptors which link to intracellular signalling pathways (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986) enormous advances have been made in establishing the structure, pharmacology and possible physiological roles of the metabotropic glutamate receptor (mGluR) family (see Schoepp & Conn, 1993; Nakanishi, 1994; Pin & Duvoisin, 1995). Progress with respect to the mGluRs which preferentially couple to phosphoinositide turnover has been facilitated by the cloning and expression of mGluR1 and mGluR5 (Abe *et al.*, 1992; Aramori & Nakanishi, 1992; Thomsen *et al.*, 1993) and their splice variants (Pickering *et al.*, 1993; Joly *et al.*, 1995). Studies where a single mGluR subtype has been transfected into a simple model cell system devoid of endogenous glutamate receptors have demonstrated that mGluR1α (but not mGluR1β; Pickering *et al.*, 1993) and mGluR5 stimulate phosphoinositide turnover via a partially pertussis toxin-sensitive mechanism (Abe *et al.*, 1992; Aramori & Nakanishi, 1992) and have been invaluable in unambiguously defining the potency rankings of agonists (Hayashi *et al.*, 1992; Pickering *et al.*, 1993; Joly *et al.*, 1995) and selectivity of putative mGluR antagonists (Hayashi *et al.*, 1994; Joly *et al.*, 1995).

Despite the clear advantages of such model systems for establishing the activities of putative mGluR agonists and antagonists, they provide little insight into the physiological roles of mGluRs in the brain. Furthermore, there is some evidence that recombinant mGluRs expressed in non-excitable homogeneous cell populations do not entirely recapitulate the properties exhibited by receptors expressed endogenously in neurones (Pin & Duvoisin, 1995). Although such differences

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may in part be accounted for by the mixed mGluR populations expressed in heterogeneous (or near-homogeneous (Prézeau *et al.*, 1994; Santi *et al.*, 1994)) neuronal preparations (Schoepp & Conn, 1993; Littman *et al.*, 1993; Testa *et al.*, 1994), it is also possible that additional effector targets (e.g. receptor- and/or voltage-gated ion channels) or different G proteins/phospholipase C isoenzyme complements can significantly modify the observed responses in neurones, negating the extrapolation/application of information derived from simple model, non-neuronal cell systems (Ikeda *et al.*, 1995).

In the present study, the effects of 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) diastereoisomers on phosphoinositidase C (phosphoinositide-specific phospholipase C; PIC) activation and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) mass accumulation, and the ability of (±)-α-methylcarboxyphenylglycine (MCPG) to antagonize these responses, have been compared in neonatal rat cerebral cortex slices. We demonstrate that a significantly higher concentration of **1S, 3R**-ACPD is required to half-maximally activate $Ins(1,4,5)P_3$ accumulation compared to total [^{3}H]-inositol phosphate ([^{3}H]-$InsP_x$) accumulation. Similar discrepancies are revealed for apparent mGluR antagonist affinities with respect to inhibition of agonist-stimulated $Ins(1,4,5)P_3$ mass and [^{3}H]-$InsP_x$ accumulations. We propose that the discrepancies between derived pharmacological parameters using these widely used indices of phosphoinositide turnover arise through a differential dependence of the two responses on direct or indirect mGluR-evoked Ca^{2+}-influx facilitating mGluR-stimulated PIC activity.

Methods

Incubation methods

Cerebral cortex from 7–8 day old neonatal rats (Wistar strain, either sex) was cross-cut (350 × 350 μm) with a McIwain chopper and dispersed in a large volume of modified Krebs-Henseleit buffer (KHB; composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 1.3, $MgSO_4$ 1.2, HEPES 5, glucose 10, pH 7.4 following equilibration with O_2/CO_2 (95:5)). Following incubation at 37°C for 45–60 min with multiple buffer changes, slices were allowed to sediment and 25 μl aliquots transferred to flat-bottomed polypropylene tubes containing 250 μl KHB. Where inositol phospholipid radiolabelling was required, [^{3}H]-inositol was introduced at this stage (0.5 μCi [^{3}H]-inositol per vial; i.e. 1.67 μCi ml^{-1}) and incubations continued for 60 min at 37°C with regular purging with O_2/CO_2. Unless otherwise stated in the Results section, LiCl (5 mM final concentration) was added at the end of the labelling period in radiolabelling experiments, whilst for studies involving $Ins(1,4,5)P_3$ mass determinations, LiCl was omitted from the protocol.

In experiments where the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) was altered, slices were first incubated in normal KHB for 60 min as described above and if necessary, radio-labelled in 20 ml KHB containing 1.67 μCi [^{3}H]-inositol ml^{-1} for 60 min. Following this period slices were washed (6 × 20 ml) with either KHB ($[Ca^{2+}] = 1.3$ mM) or nominally Ca^{2+}-free KHB ($[Ca^{2+}] = 2-5$ μM) over a period of 10–15 min at 37°C. Following washing, slices were allowed to pack under gravity and 25 μl aliquots transferred to flat-bottomed polypropylene tubes containing 250 μl of the appropriate buffer (KHB, KHB-Ca, or KHB-Ca containing 100 μM EGTA ($[Ca^{2+}] \leqslant 100$ nM)), 5 mM LiCl and 0.5 μCi [^{3}H]-inositol.

Where indicated antagonists (MK-801 ((+)-5-methyl-10, 11-dihydro-5*H*-dibenzo[a,d]-cyclhepten-5,10-imine maleate), (±)-, (+)-, (−)-α-methylcarboxyphenylglycine) were added 20 min prior to agonist challenge. In all cases incubations were terminated by addition of an equal volume (300 μl) of ice-cold 1 M trichloroacetic acid (TCA) and immediate transfer to an ice-bath.

Analysis of phosphoinositide cycle intermediates

Following a period of 20–30 min on ice, during which samples were intermittently vortex-mixed, samples were centrifuged (4000 *g*, 20 min, 4°C) and TCA extracted from the supernatant by repeated washing with water-saturated diethylether (4 × 3 vol). For total [^{3}H]-inositol phosphate ([^{3}H]-$InsP_x$) determination, 450 μl aliquots of the neutralized samples were taken and 50 μl 60 mM $NaHCO_3$ added; samples were stored at 4°C prior to column separation being carried out within 48 h. For $Ins(1,4,5)P_3$ mass determinations, 50 μl $NaHCO_3$ and 50 μl EDTA were added to 200 μl neutral extract and samples were stored at 4°C prior to $Ins(1,4,5)P_3$ assay within 48 h.

[^{3}H]-$InsP_x$ were batch-recovered by ion exchange chromatography on Dowex-1 (Cl^- form) columns (Challiss *et al.*, 1994a). $Ins(1,4,5)P_3$ mass was determined as described previously (Challiss & Nahorski, 1993). To allow [^{3}H]-$InsP_x$ and $Ins(1,4,5)P_3$ values to be expressed as d.p.m. mg^{-1} protein and pmol mg^{-1} protein respectively, the slice pellet was routinely digested in NaOH overnight for subsequent protein determination (Lowry *et al.*, 1951).

Materials

The four diastereoisomers of 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and (±); (+)-and (−)-α-methylcarboxylphenylglycine (MCPG) were purchased from Tocris Cookson Ltd. (Langford, U.K.). All other agents were purchased from the suppliers listed previously (Challiss *et al.*, 1994a, b).

Data analysis

All data are presented as means ± s.e.mean for the indicated number of separate experiments which were performed in triplicate unless otherwise stated. Agonist concentration-response curves were analysed with a commercially available programme (InPlot, GraphPad Software, San Diego CA, U.S.A.) and used to generate EC_{50}/IC_{50} values. Antagonist equilibrium dissociation constants (K_d) were estimated in the absence and presence of a fixed concentration of antagonist assuming parallel log concentration-response curves using the equation:

$$K_d = A/(DR-1)$$

where A is the antagonist concentration and DR represents the ratio of the concentrations required to evoke 50% of maximal responses in the presence and absence of the antagonist. K_d values were also estimated with the method described by Lazareno & Birdsall (1993) where concentration-inhibition curves are constructed against a single agonist concentration which evokes >50% of the maximal response. Statistical comparisons were performed by Student's *t* test for unpaired observations.

Results

Phosphoinositide responses evoked by ACPD diastereoisomers

Concentration-response relationships were established for the four diastereoisomers of ACPD with respect to [^{3}H]-$InsP_x$ accumulation (in the presence of LiCl at 15 min) and $Ins(1,4,5)P_3$ mass accumulation (at 15 s) in cerebral cortex slices (Figure 1). **1S, 3R**-ACPD stimulated an approximate 6 fold increase in [^{3}H]-$InsP_x$ at 300 μM, with a half-maximal activation of this response occurring at a concentration of 14 μM (log EC_{50} (M) = −4.87 ± 0.07; $n = 4$; Figure 1a). Although at concentrations of 3–30 μM **1R, 3R**-ACPD appeared only slightly less potent than **1S, 3R**-ACPD with respect to [^{3}H]-$InsP_x$ accu-

mulation, at higher concentrations the magnitude of the response decreased. Time-course experiments showed that whilst the [^{3}H]-InsP$_x$ response stimulated by 30 μM 1**R**, 3**R**-ACPD (and 300 μM 1S, 3**R**-ACPD; see Figure 5) was approximately linear over a 20 min time-course, the response to 300 μM was linear only over the initial 2–3 min, the rate of [^{3}H]-InsP$_x$ generation declining markedly during the 5–20 min period of exposure to 1**R**, 3**R**-ACPD (data not shown). Although 1S, 3S-ACPD (log EC_{50} = 4.21 ± 0.03; n = 4) was clearly 4–5 fold less potent than 1S, 3**R**-ACPD, it appeared to evoke a similar maximal response. In contrast, 300 μM 1**R**, 3S-ACPD caused only a slight increase in [^{3}H]-InsP$_x$ accumulation amounting to only 2–3% of the response to 300 μM 1S, 3**R**-ACPD (Figure 1a).

In general the concentration-response curves for the diastereoisomers of ACPD were significantly rightward-shifted with respect to the initial peak Ins(1,4,5)P$_3$ mass accumulation compared to the [^{3}H]-InsP$_x$ response (Figure 1b). Thus, 300 μM 1S, 3**R**-ACPD caused a 5–6 fold increase in Ins(1,4,5)P$_3$ accumulation with a half-maximal activation of this response occurring at a concentration of 82 μM (log EC_{50} = −4.09 ± 0.10; n = 4); 1S, 3S-ACPD was about 7 fold less potent than the 1S, 3**R** diastereoisomer (log EC_{50} = −3.26 ± 0.04), whilst 1**R**, 3S-ACPD (at 1 mM) was entirely without effect. In contrast to the [^{3}H]-InsP$_x$ response evoked by 1**R**, 3**R**-ACPD, the initial magnitude of the Ins(1,4,5)P$_3$ response increased up to the maximum concentration tested (1 mM) and reached a level ~85% (log EC_{50} = −4.03 ± 0.09; n = 4) of that achieved in the presence of 1 mM 1S, 3**R**-ACPD.

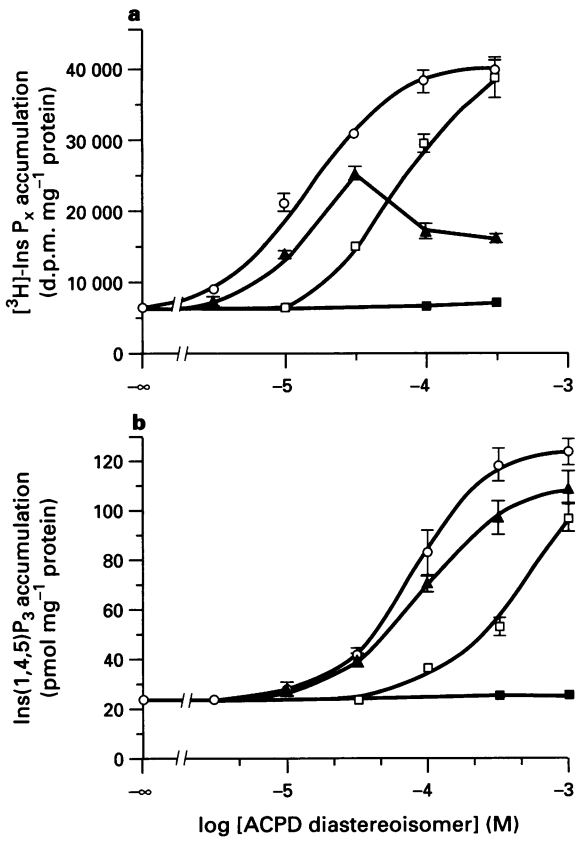

Figure 1 Concentration-dependence of [^{3}H]-InsP$_x$ and Ins(1,4,5)P$_3$ mass accumulations stimulated by the four diastereoisomers of ACPD. In (a), neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60 min. LiCl was added (final concentration 5 mM) and slices were stimulated by addition of various concentrations of 1S, 3R- (○), 1R, 3S- (■), 1S, 3S- (□) or 1R, 3R- (▲) ACPD. Incubations were terminated after 15 min. In (b) similar ACPD diastereoisomer additions were made to unlabelled neonatal cerebral cortex slices in the absence of LiCl. In this case incubations were terminated after 15 s. Samples were processed for [^{3}H]-InsP$_x$ and Ins(1,4,5)P$_3$ mass assay respectively as described in the Methods section. Values are presented as means ± s.e.mean for four separate experiments each performed in triplicate.

Inhibition of [^{3}H]-InsP$_x$ response to ACPD diastereoisomers by metabotropic glutamate receptor and NMDA receptor antagonists

Pre-incubation of cerebral cortex slices with either MK-801 (1 μM) or (±)-MCPG (1 mM) for 20 min prior to challenge resulted in differential inhibition of the [^{3}H]-InsP$_x$ response stimulated by ACPD diastereoisomers (Figure 2). The metabotropic glutamate receptor antagonist (±)-MCPG substantially inhibited the [^{3}H]-InsP$_x$ responses evoked by 1S, 3**R**-ACPD (30 μM) and 1S, 3S-ACPD (30 μM); in contrast this agent had no effect on the response evoked by 1**R**, 3**R**-ACPD (30 μM). Reciprocally, the uncompetitive NMDA receptor antagonist, MK-801, inhibited the [^{3}H]-InsP$_x$ response evoked by 30 μM 1**R**, 3**R**-ACPD by >95%, but was without significant effect on the responses to 1S, 3**R**-ACPD and 1S, 3S-ACPD (Figure 2). The small [^{3}H]-InsP$_x$ response evoked by 300 μM 1**R**, 3S-ACPD also appeared to be MK-801-sensitive/(±)-MCPG-insensitive. These data suggest that whilst 1S, 3**R**- and 1S, 3S-ACPD exert their effects on phosphoinositide turnover via metabotropic glutamate receptor activation, 1**R**, 3**R**-ACPD (and perhaps 1**R**, 3S-ACPD) acts predominantly via a MK-801-sensitive mechanism. This conclusion is supported by experiments where slices were stimulated in the nominal absence of extracellular Ca^{2+} ($[Ca^{2+}]_e$ = 2–5 μM). Under these conditions, the [^{3}H]-InsP$_x$ response to 1**R**, 3**R**-ACPD (300 μM) was markedly reduced (by 89 ± 8%), whereas only a modest reduction (by 23 ± 5%) in the response to 1S, 3**R**-ACPD (300 μM) was observed (data not shown).

The ability of NMDA receptor activation to stimulate phosphoinositide turnover in cerebral cortex slices was also assessed. Both NMDA and 1**R**, 3**R**-ACPD stimulated Ins(1,4,5)P$_3$ accumulation in a concentration-dependent manner (Figure 3). However, while the NMDA-evoked response was completely antagonized by MK-801, a small component of the 1**R**, 3**R**-ACPD stimulated response remained in the pre-

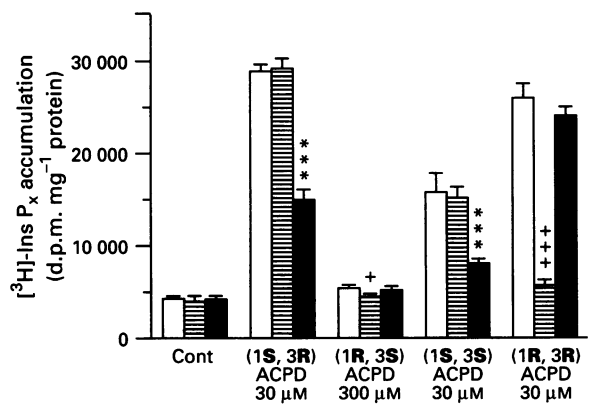

Figure 2 Effects of MK-801 and (±)-MCPG on the [^{3}H]-InsP$_x$ accumulation stimulated by the four diastereoisomers of ACPD. Neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60 min. After this labelling period, LiCl (final concentration 5 mM) and MK-801 (1 μM; hatched column), (±)-MCPG (1 mM; solid column) or vehicle (open column) were added. After 30 min, slices were stimulated by addition of 30 μM 1S, 3**R**-, 1S, 3S-, 1**R**, 3**R**-ACPD, or 300 μM 1**R**, 3S-ACPD. Incubations were terminated after 15 min. Samples were processed for [^{3}H]-InsP$_x$ assay as described in the Methods section. Values are presented as means ± s.e.mean for three separate experiments each performed in triplicate. Statistically significant decreases in [^{3}H]-InsP$_x$ accumulations are indicated as $^+P<0.05$; $^{+++}P<0.001$ for the effect of MK-801, and as $^{***}P<0.001$ for (±)-MCPG.

sence of NMDA receptor blockade suggesting that this agent may stimulate phosphoinositide turnover by more than one mechanism in neonatal rat cerebral cortex.

Antagonism of metabotropic glutamate receptor-stimulated phosphoinositide turnover by (±)-MCPG

The inhibitory effect of (±)-MCPG on phosphoinositide turnover stimulated by the metabotropic glutamate receptor agonist 1S, 3R-ACPD was investigated further. Considering the observed discrepancy between EC_{50} values for 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 mass and [^{3}H]-InsP_x responses (see Figure 1), the effect of 1 mM (±)-MCPG on both these indices of phosphoinositide turnover was studied (Figure 4). In agreement with the experiments shown in Figure 1, a 4–5 fold higher concentration of 1S, 3R-ACPD was required to half-maximally activate Ins(1,4,5)P_3 accumulation compared to the [^{3}H]-InsP_x response (respective −log EC_{50} values: -4.11 ± 0.04, and -4.76 ± 0.02). The presence of (±)-MCPG (1 mM) shifted both curves to the right in an apparently parallel fashion (Figure 4a,b). However, the dose-ratios yielded by comparisons of EC_{50}s for each response to 1S, 3R-ACPD in the absence and presence of (±)-MCPG were significantly different (log EC_{50} for Ins(1,4,5)P_3 response in the presence of (±)-MCPG -3.36 ± 0.03; dose-ratio, 5.59 ± 0.24 ($n=4$); log EC_{50} for [^{3}H]-InsP_x response in the presence of (±)-MCPG -4.28 ± 0.05; dose-ratio, 3.04 ± 0.34; $P<0.01$; $n=4$) giving rise to respective apparent K_d values of 218 and 490 μM for (±)-MCPG antagonism of Ins(1,4,5)P_3 mass and [^{3}H]-InsP_x responses stimulated by 1S, 3R-ACPD.

Time-course studies revealed that 1S, 3R-ACPD (100 μM) stimulated a linear accumulation of [^{3}H]-InsP_x over at least a 20 min period (Figure 5). Pre-addition of (±)-MCPG (1 mM) appeared to have a more dramatic inhibitory effect over the initial 0–2 min period of 1S, 3R-ACPD challenge; thus, at 1 min after agonist addition the [^{3}H]-InsP_x response was inhibited by $67.7 \pm 3.4\%$, whereas after 15 min the inhibition had decreased to $27.1 \pm 3.4\%$ (Figure 5).

The ability of the (+)- and (−)-enantiomers of MCPG to inhibit the initial accumulation of Ins(1,4,5)P_3 stimulated by 100 μM 1S, 3R-ACPD is shown in Figure 6 together with the effect of the (±)-MCPG racemate. These data confirm that the metabotropic glutamate receptor antagonistic property of MCPG resides principally with the (+)-enantiomer, with this enantiomer causing almost 100% inhibition of agonist-stimulated Ins(1,4,5)P_3 accumulation at 1 mM whilst a similar concentration of the (−)-enantiomer caused only a 28% inhibitory effect. Analysis of the MCPG inhibition curves using the method of Lazareno & Birdsall (1993) yielded antagonist dissociation constants (K_d) of 55 and 120 μM for the (+)- and (±)-enantiomers respectively. The latter value is in fair agreement with the K_d value obtained from (±)-MCPG-induced shift in the concentration-response curve for 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 accumulation stated above.

What underlies the potency differences for metabotropic glutamate receptor agonist-stimulated Ins(1,4,5)P_3 mass and [^{3}H]-InsP_x responses?

The possible involvement of Ca^{2+}-influx pathways in the ability of 1S, 3R-ACPD (and 1S, 3S-ACPD; see Figure 1) to stimulate [^{3}H]-InsP_x accumulation some 4–5 fold more po-

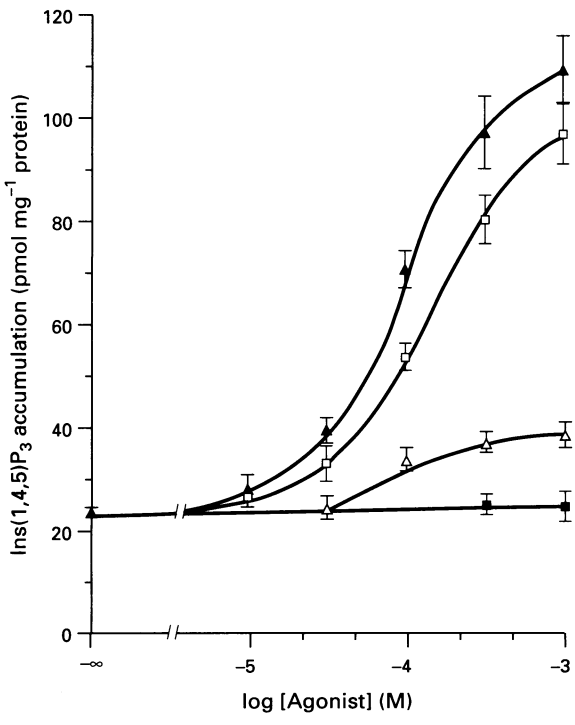

Figure 3 Effects of 1R, 3R-ACPD and NMDA on Ins(1,4,5)P_3 mass accumulation and susceptibility to antagonism by MK-801. Neonatal cerebral cortex slices were incubated in the absence (▲, □) or presence (△, ■) of 1 μM MK-801 for 30 min prior to addition of the indicated concentrations of either 1R, 3R-ACPD (▲, △) or NMDA (□, ■) for 15 s. Samples were processed for Ins(1,4,5)P_3 mass assay as described in the Methods section. Values are presented as means ± s.e.mean for three separate experiments each performed in triplicate.

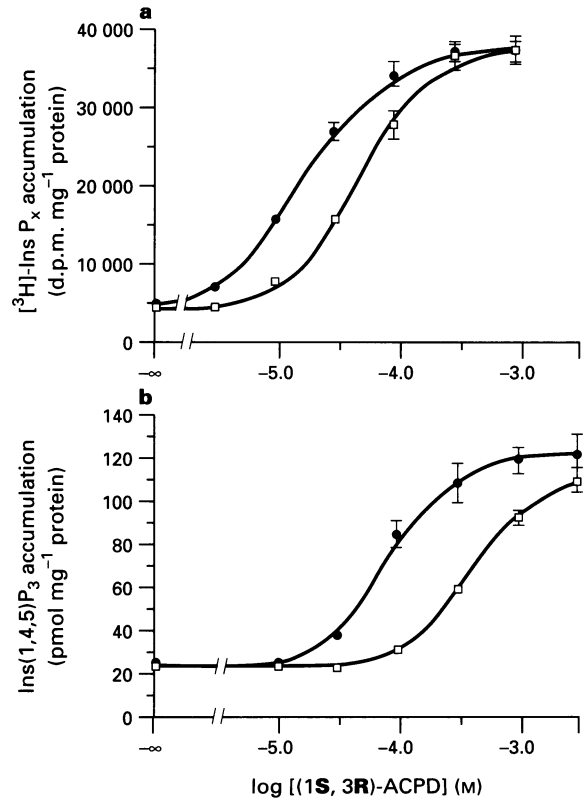

Figure 4 Antagonism of 1S, 3R-ACPD-stimulated [^{3}H]-InsP_x and Ins(1,4,5)P_3 mass accumulations by (±)-MCPG. In (a), neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60 min. LiCl was added (final concentration 5 mM) and slices were incubated for 30 min in the absence (●) or presence (□) of 1 mM (±)-MCPG. Slices were stimulated by addition of the indicated concentrations of 1S, 3R-ACPD. Incubations were terminated after 15 min. In (b), a similar experimental design was followed except that 1S, 3R-ACPD additions were made for 15 s to unlabelled neonatal cerebral cortex slices in the absence (●) or presence (□) of 1 mM (±)-MCPG and in the absence of LiCl. Samples were processed for [^{3}H]-InsP_x and Ins(1,4,5)P_3 mass assay respectively as described in the Methods section. Values are presented as means ± s.e.mean for four separate experiments each performed in triplicate.

tently than Ins(1,4,5)P_3 mass accumulation was investigated. Figure 7 shows the effect of removal of extracellular Ca^{2+} (reducing the $[Ca^{2+}]_e$ from 1.3 mM to 2–5 μM) on the initial time-course of Ins(1,4,5)P_3 mass accumulation in cerebral cortex slices. Removal of $[Ca^{2+}]_e$ decreased basal Ins(1,4,5)P_3 levels by $\geqslant$50% and had a similar effect on the initial peak increase at 15 s. The fact that basal and stimulated values were both reduced meant that the initial peak increase was unaffected if expressed as a fold increase over basal (Figure 7). Concentration-response curves to 1S, 3R-ACPD under normal, nominally Ca^{2+}-free and very low Ca^{2+} ($\leqslant$100 nM; achieved by omission of Ca^{2+} and addition of EGTA) are shown in Figure 8. For 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 mass accumulation (Figure 8b), the basal and maximum values were progressively suppressed by decreasing $[Ca^{2+}]_e$; however, EC_{50} values were largely unaffected (log EC_{50} values: +Ca, -4.04 ± 0.05; −Ca, -4.02 ± 0.11; −Ca+EGTA -4.01 ± 0.04). In contrast, nominally Ca^{2+}-free conditions suppressed the 1S, 3R-ACPD-stimulated [^{3}H]-InsP_x response by only 14%, but caused a 3–4 fold rightward shift in the EC_{50} value (log EC_{50}+Ca, -4.80 ± 0.04; −Ca, -4.26 ± 0.07); further decreasing $[Ca^{2+}]_e$ dramatically attenuated the [^{3}H]-InsP_x response and further right-shifted the EC_{50} value (−Ca+EGTA, -4.13 ± 0.08) to a value not significantly different from that observed for the ACPD-stimulated Ins(1,4,5)P_3 response (Figure 8a).

Furthermore, (±)-MCPG (1 mM) was a more effective antagonist against the 1S, 3R-ACPD-stimulated [^{3}H]-InsP_x response when $[Ca^{2+}]_e$ was reduced to $\leqslant$100 nM by EGTA addition (Figure 9). Thus, the antagonist dissociation constant derived from 1S, 3R-ACPD-stimulated [^{3}H]-InsP_x concentration-response curves generated in the presence compared to the absence of (±)-MCPG was 278 μM (dose-ratio, 4.59 ± 0.30; log EC_{50} values: −MCPG, -4.24 ± 0.13; +MCPG, -3.58 ± 0.05; $n=3$), compared to a value of 459 μM (dose-ratio, 3.18 ± 0.17; $P<0.02$; $n=3$) obtained from a parallel series of experiments performed in normal KHB ($[Ca^{2+}]_e$ = 1.3 mM; data not shown).

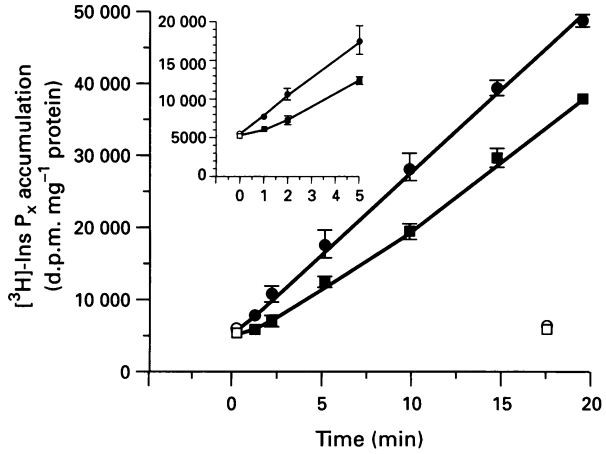

Figure 5 Time-course of 1S, 3R-ACPD-stimulated [^{3}H]-InsP_x accumulation in the absence and presence of (±)-MCPG. Neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60 min. LiCl was added (final concentration 5 mM) and slices were incubated for 30 min in the absence (○, ●) or presence (□, ■) of 1 mM (±)-MCPG. Slices were stimulated by addition of 100 μM 1S, 3R-ACPD (●, ■) for the times indicated. The inset shows changes in [^{3}H]-InsP_x accumulation over the initial 5 min stimulation period. Samples were processed for [^{3}H]-InsP_x assay as described in the Methods section. Values are presented as means±s.e.mean for a single experiment performed in triplicate (similar data were obtained in two further experiments).

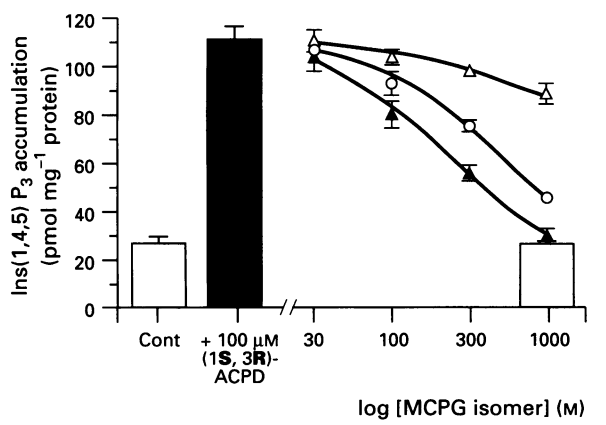

Figure 6 Inhibition of 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 mass accumulation by (±)-, (+)- or (−)-MCPG. Neonatal cerebral cortex slices were pre-incubated with the indicated concentrations of (±)-MCPG (○), (+)-MCPG (▲) or (−)-MCPG (△) for 30 min prior to addition of 100 μM 1S, 3R-ACPD for 15 s. The columns show basal Ins(1,4,5)P_3 mass accumulation in the absence or presence of 1 mM (+)-MCPG (open column) or following addition of 1S, 3R-ACPD for 15 s (solid column). Values are presented as means±s.e.mean for three separate experiments each performed in triplicate.

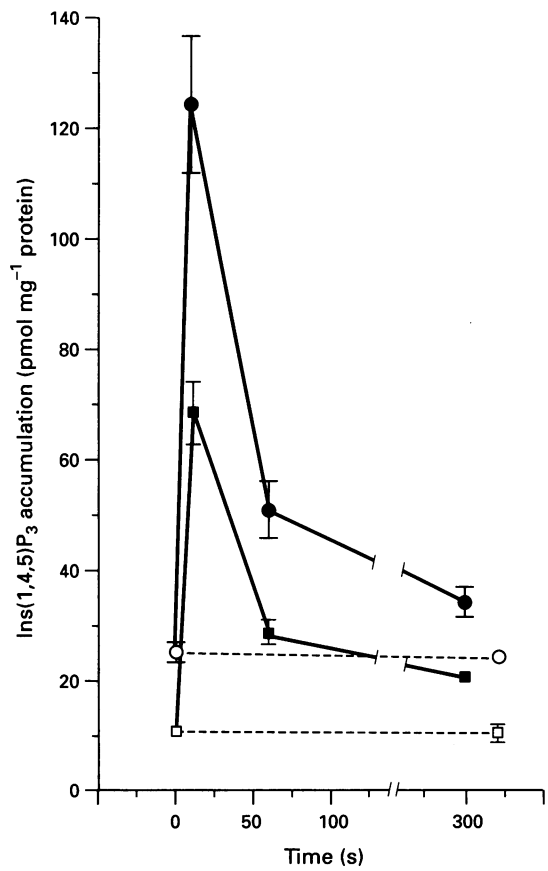

Figure 7 Effect of extracellular [Ca^{2+}] on 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 mass accumulation. Neonatal cerebral cortex slices were incubated for 60 min in KHB containing normal [Ca^{2+}] (=1.3 mM). After this time slices were either maintained in normal KHB or washed extensively with nominally calcium-free KHB ([Ca^{2+}] =2–5 μM). Slices were transferred to incubation vials containing either normal KHB (○, ●) or nominally calcium-free KHB (□, ■) and challenged with 300 μM 1S, 3R-ACPD (●, ■) for the times indicated. Values are presented as means±s.e.mean for five separate experiments each performed in duplicate or triplicate.

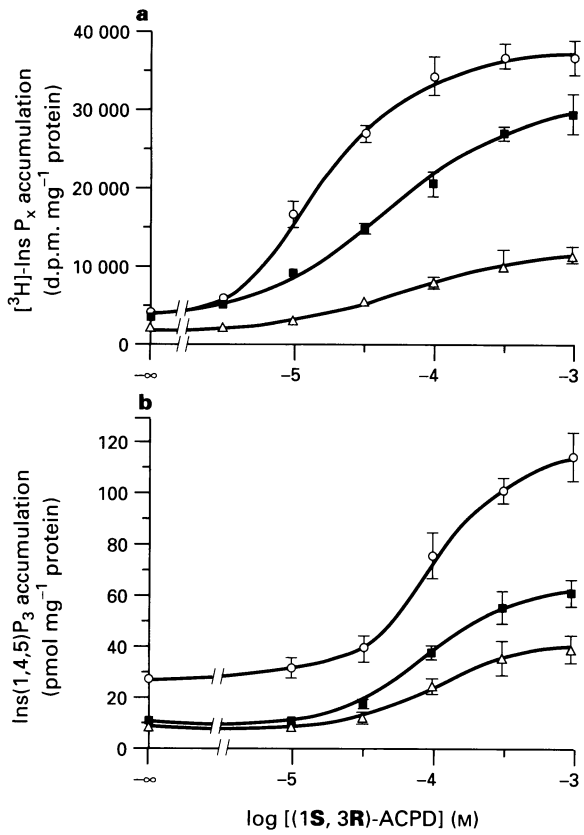

Figure 8 Effect of extracellular $[Ca^{2+}]$ on the concentration-dependencies of $[^3H]$-$InsP_x$ and $Ins(1,4,5)P_3$ mass accumulations stimulated by 1S, 3R-ACPD. In (a), neonatal cerebral cortex slices were incubated with $[^3H]$-inositol (0.5 μCi/vial) for 60 min in normal KHB ($[Ca^{2+}]$ = 1.3 mM). Slices were either transferred to vials containing normal KHB (○), or extensively washed in nominally calcium-free KHB before addition to vials containing nominally calcium-free KHB (■) or calcium-free KHB + 100 μM EGTA (△). In all cases LiCl was added (final concentration 5 mM) and slices were stimulated by addition of the indicated concentrations of 1S, 3R-ACPD for 15 min. In (b), similar 1S, 3R-ACPD additions were made to unlabelled cerebral cortex slices maintained in normal KHB (○), nominally calcium-free KHB (■) or calcium-free KHB + 100 μM EGTA (△) in the absence of LiCl. In this case incubations were terminated after 15 s. Samples were processed for $[^3H]$-$InsP_x$ and $Ins(1,4,5)P_3$ mass assay respectively as described in the Methods section. Values are presented as means ± s.e.mean for three separate experiments each performed in triplicate.

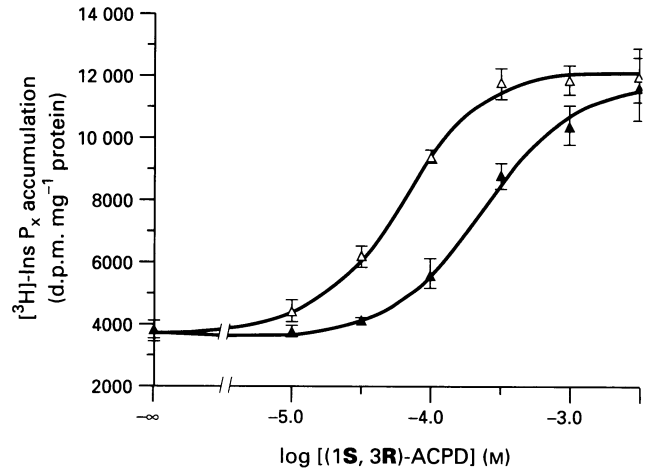

Figure 9 Antagonism of 1S, 3R-ACPD-stimulated $[^3H]$-$InsP_x$ accumulation by (±)-MCPG in the presence of very low (≤ 100 nM) extracellular $[Ca^{2+}]$. Neonatal cerebral cortex slices were incubated with $[^3H]$-inositol (0.5 μCi/vial) for 60 min in normal KHB ($[Ca^{2+}]$ = 1.3 mM). Slices were either transferred to vials containing normal KHB (data not shown), or extensively washed in nominally calcium-free KHB before addition to vials containing nominally calcium-free KHB + 100 μM EGTA. In all cases LiCl was added (final concentration 5 mM) and slices were incubated in the absence (△) or presence (▲) of 1 mM (±)-MCPG for 30 min prior to addition of the indicated concentrations of 1S, 3R-ACPD for 15 min. Samples were processed for $[^3H]$-$InsP_x$ assay as described in the Methods section. Values are presented as means ± s.e.mean for three separate experiments each performed in triplicate.

Discussion

In parallel with the cloning and functional characterization of the eight mGluRs which presently constitute the mGluR family (Nakanishi, 1994; Pin & Duvoisin, 1995) has been the synthesis of an increasing array of molecules which exhibit selective agonist or antagonist properties at this class of receptor (Hayashi *et al.*, 1992; 1994; Schoepp & Conn, 1993; Birse *et al.*, 1994; Pin & Duvoisin, 1995). Although heterologous expression of specific mGluR subtypes can in theory provide almost unlimited supplies of material for pharmacological characterization of putative mGluR agonists/antagonists using radioligand binding methods, few studies have adopted this approach (Thomsen *et al.*, 1993; Laurie *et al.*, 1995), probably because radiolabelled L-$[^3H]$-glutamate is the only ligand available at present. Thus, the vast majority of pharmacological studies have used the activation or inhibition of particular signal transduction pathways as an index of receptor activation or blockade.

In the case of studies of mGluRs which preferentially link to phosphoinositide turnover, the assay of choice has involved incubation of cells/tissues with $[^3H]$-inositol to label the inositol phospholipid pool, followed by stimulation of the pathway (usually for 30–45 min) in the presence of sufficient lithium (5–10 mM) to prevent inositol monophosphate dephosphorylation. In the presence of lithium all $[^3H]$-$InsP_x$ generated by PIC activation are trapped, and can easily be separated from inositol phospholipids and free *myo*-inositol. The increase in $[^3H]$-$InsP_x$ measured in such assays is generally interpreted as reflecting the extent of PIC activation. However, such simple assays have a number of limitations (see Nahorski & Challiss, 1991); in particular they provide at best limited information on the changes which may occur at the level of the pathway second messenger.

In the present study we have systematically compared the actions of the diastereoisomers of ACPD and the mGluR-selective antagonist MCPG on total $[^3H]$-$InsP_x$ (at 15 min, in the presence of lithium), and $Ins(1,4,5)P_3$ mass responses measured immediately following exposure to agonist (at 15 s). In close agreement with a number of previous studies carried out with a variety of brain slice preparations, $[^3H]$-$InsP_x$ accumulation was half-maximally stimulated by 1S, 3R-ACPD at a concentration of 10–20 μM (Schoepp *et al.*, 1991; Manzoni *et al.*, 1992; Jones & Roberts, 1993; Challiss *et al.*, 1994b). In contrast, 1R, 3S-ACPD was essentially without effect at concentrations up to 300 μM. Although this finding is in agreement with previous reports by Irving *et al.* (1990) and Schoepp *et al.* (1991), others have found that this isomer does stimulate PIC activity (Manzoni *et al.*, 1992; Jones & Roberts, 1993) and is a weak but full agonist at mGluRs mediating adenylyl cyclase inhibitory effects (Cartmell *et al.*, 1992). In our hands not only did 300 μM 1R, 3S-ACPD fail to evoke a $[^3H]$-$InsP_x$ response, but it had no inhibitory or facilitatory effect on the 1S, 3R-ACPD-stimulated phosphoinositide response, apparently ruling out any significant interaction with mGluR(s) mediating this effect in neonatal rat cerebral cortex. 1S, 3S-ACPD evoked a similar maximal $[^3H]$-$InsP_x$ response to that caused by 1S, 3R-ACPD, but this isomer was approximately 5 fold less potent (EC_{50} 62 μM). This is in agreement with another study on mGluR-mediated phosphoinositide re-

sponses (Jones & Roberts, 1993), and contrasts with the relative actions of these isomers to inhibit forskolin-stimulated cyclic AMP accumulation, where a potency of 1S, 3S-ACPD similar to, or greater than that of 1S, 3R-ACPD has been reported (Kemp *et al.*, 1994).

The relative effects of 1S, 3R-ACPD, 1S, 3S-ACPD and 1R, 3S-ACPD in causing an increase in Ins(1,4,5)P_3 mass accumulation were essentially similar; however comparison with the effects on the [^{3}H]-InsP_x responses revealed differences in their absolute potencies. Thus, EC_{50} values for the Ins(1,4,5)P_3 response were 6–9 fold greater for both 1S, 3R-ACPD and 1S, 3S-ACPD (82 and 550 μM respectively). Whilst the stimulatory effects of 1S, 3R-ACPD and 1S, 3S-ACPD on phosphoinositide turnover were unaffected by the uncompetitive NMDA-receptor antagonist MK-801, they were substantially antagonized by the mGluR-selective antagonist (±)-MCPG. At present there is some disagreement as to whether (±)-MCPG exhibits mGluR1/5 subtype selectivity. Whilst it is generally agreed that this antagonist is effective at mGluR1 (Hayashi *et al.*, 1994; Joly *et al.*, 1995; Saugstad *et al.*, 1995), contradictory findings have been reported with respect to antagonism at mGluR5, with Saugstad *et al.* (1995) reporting that (±)-MCPG is an antagonist at this subtype, whilst Joly *et al.* (1995) found that (±)-MCPG fails to block L-glutamate-stimulated [^{3}H]-InsP_x accumulation in LLC-PK1 cells transiently expressing either mGluR5a or mGluR5b splice variants. If the latter situation pertains in neonatal rat cerebral cortex, then the responses to 1S, 3R-ACPD and 1S, 3S-ACPD observed in these studies are likely to be mediated by mGluR1 rather than mGluR5.

In contrast, the stimulatory effect of 1R, 3R-ACPD on phosphoinositide turnover showed a reciprocal sensitivity to MK-801 and (±)-MCPG, consistent with this isomer being a selective NMDA-receptor agonist (Curry *et al.*, 1988). That NMDA-receptor activation can stimulate [^{3}H]-InsP_x accumulation in neonatal rat cerebral cortex has been demonstrated previously (Challiss *et al.*, 1994a, b); here we have shown that both NMDA and 1R, 3R-ACPD can also stimulate a rapid and substantial increase in Ins(1,4,5)P_3 accumulation, similar in magnitude to that caused by mGluR activation. In the case of NMDA this response is completely abolished by MK-801, whereas a small component of the response to 1R, 3R-ACPD is still apparent in the presence of this antagonist suggesting that 1R, 3R-ACPD might be a partial agonist at mGluR or cause a small Ins(1,4,5)P_3 response via another undefined mechanism. That 1R, 3R-ACPD exerts its predominant action via NMDA-receptor activation provides a possible explanation for the decreasing ability of this agent to stimulate [^{3}H]-InsP_x accumulation at higher concentrations (see Figure 1a). We have previously shown that substantial activation of NMDA-receptors in this tissue can cause a rapid Ca^{2+}-dependent neurotoxic effect which compromises subsequent or on-going phosphoinositide cycle activation (Challiss *et al.*, 1994b). Indeed, preliminary experiments have shown that, in contrast to the time-course of [^{3}H]-InsP_x accumulation stimulated by 1S, 3R-ACPD (see Figure 5), 300 μM 1R, 3R-ACPD causes a linear [^{3}H]-InsP_x for only the first 1–2 min after agonist addition with the rate decreasing progressively with increasing exposure time, reminiscent of the response seen previously following NMDA challenge (Challiss *et al.*, 1994b).

Antagonism of the 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 and [^{3}H]-InsP_x responses by (±)-MCPG also revealed differences in the calculated apparent dissociation constant for this agent (K_d values of 218 and 490 μM respectively). Use of an alternative experimental protocol to investigate further the effects of (±)-MCPG, and the constituent (+)- and (−)-enantiomers of MCPG, on the Ins(1,4,5)P_3 response to 1S, 3R-ACPD yielded slightly lower K_d values of 120 and 55 μM for (±)-MCPG and (+)-MCPG respectively. The latter value is in very close agreement to that reported by Hayashi *et al.* (1994) for the action of (+)-MCPG in CHO cells expressing mGluR1. Support for the proposition that the differences in K_d values for (±)-MCPG on 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 and [^{3}H]-InsP_x responses are of significance was provided by a detailed time-course of agonist-stimulated [^{3}H]-InsP_x accumulation in the absence and presence of the antagonist. Whilst the rate of [^{3}H]-InsP_x accumulation was clearly linear over the 15 min time-course in the presence of agonist only, it was evident that in the presence of (±)-MCPG the inhibitory effect of this agent was more pronounced during the initial 1–2 min following 1S, 3R-ACPD addition (see Figure 5). These data are consistent with the finding that (±)-MCPG causes a greater inhibition of the Ins(1,4,5)P_3 response (at 15 s) than the [^{3}H]-InsP_x response (at 15 min).

Many studies, particularly those in neuronal preparations, have demonstrated that agonist-stimulated PIC activation is facilitated by increases in $[Ca^{2+}]_i$ (Eberhard & Holz, 1988; Challiss & Nahorski, 1991; del Rio *et al.*, 1994; Wojcikiewicz *et al.*, 1994; Willars & Nahorski, 1995). Facilitation of agonist-stimulated PIC activity may be caused by Ca^{2+} mobilization from intracellular stores or Ca^{2+}-entry via a multitude of influx pathways (Simpson *et al.*, 1995).

Reducing $[Ca^{2+}]_e$ from 1.3 mM to 2–5 μM (by use of a nominally Ca^{2+}-free buffer) decreased both basal and 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 levels, whilst reducing $[Ca^{2+}]_e$ to $\leqslant$100 nM by EGTA addition further decreased the relative and absolute magnitude of the 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 response. Why such a diminution in the initial Ins(1,4,5)P_3 response to agonist challenge occurs under conditions of reduced $[Ca^{2+}]_e$ is unclear, but a number of explanations can be suggested. For example, it is possible that mGluR-stimulated Ins(1,4,5)P_3 generation is usually facilitated by a receptor-operated Ca^{2+}-influx pathway which is rapidly activated upon mGluR activation. Thus, it has been reported that histamine activates a nifedipine-insensitive Ca^{2+}-influx pathway in chromaffin cells within 20 ms of receptor occupation (Cheek *et al.*, 1994). In addition, the possibility that the attenuated mGluR-stimulated Ins(1,4,5)P_3 response may be an artifact of the preparation employed cannot be discounted; thus, it is possible that even short periods of exposure of neonatal rat cerebral cortex slices to low $[Ca^{2+}]_e$ can bring about a depletion of intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores, resulting in an attenuation of the Ca^{2+} positive feedback component of the initial Ins(1,4,5)P_3 response.

Irrespective of the explanation for the decreased Ins(1,4,5)P_3 response under conditions of reduced $[Ca^{2+}]_e$, a critical finding of the present study was that this manipulation affected neither the time-course of Ins(1,4,5)P_3 accumulation nor the EC_{50} value for this response to 1S, 3R-ACPD. In contrast, reducing $[Ca^{2+}]_e$ not only decreased the magnitude of the [^{3}H]-InsP_x response to 1S, 3R-ACPD, but also progressively rightward-shifted the EC_{50} value, such that in the presence of EGTA the EC_{50} values for the 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 and [^{3}H]-InsP_x responses were not significantly different (95 and 75 μM respectively). Similarly, the discrepancy between antagonist dissociation constants (K_d) derived for (±)-MCPG inhibition of 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 and [^{3}H]-InsP_x responses was no longer observed under conditions of very low $[Ca^{2+}]_e$.

One interpretation consistent with these data is that Ca^{2+} facilitation of the mGluR-stimulated PIC activity alters the potency of agonists to stimulate, and antagonists to inhibit, the [^{3}H]-InsP_x response: in contrast although the magnitude of the initial Ins(1,4,5)P_3 response is clearly influenced by decreasing $[Ca^{2+}]_e$, such manipulations do not appear to alter experimentally-derived EC_{50} and K_d values obtained at different $[Ca^{2+}]_e$. Thus, facilitation of the [^{3}H]-InsP_x response must involve an increase in the maximal response and a sensitization to mGluR-mediated agonist stimulation. The mechanism by which the mGluR(s) mediates such an effect can be speculated upon. It is known that mGluRs exert diverse effects on a variety of ion channels (Pin & Duvoisin, 1995). Recent evidence suggests that mGluR1/5 activation can affect both K^+- and Ca^{2+}-channels, and these effects may not necessarily be downstream of second messenger generation (Fang & Gallagher, 1992; Greene *et al.*, 1994; Guérineau *et al.*, 1994; Chavis

et al., 1995; Ikeda *et al.*, 1995). Thus, either by inhibiting membrane K^+-conductance (leading to membrane depolarization and increased open probability of voltage-gated Ca^{2+}-channels) or direct activation of voltage-gated Ca^{2+}-channels, mGluR1/5 activation could increase Ca^{2+}-influx, providing a source of Ca^{2+} to facilitate agonist-stimulated PIC activity. Alternatively, it is possible that Ca^{2+}-influx regulated by mechanisms downstream of PIC (e.g. capacitative Ca^{2+}-entry caused by intracellular Ca^{2+}-store depletion) may contribute, although the importance of such mechanisms in excitable tissues is uncertain (see Simpson *et al.*, 1995), and such mechanisms may not fully explain the contrasting effects on agonist and antagonist mGluR interactions.

Further support for the concept that mGluR-stimulated [^{3}H]-$InsP_x$ accumulation may represent a composite response resulting from direct agonist-mediated PIC activation and facilitation of Ca^{2+}-sensitive PIC activity by activation of a Ca^{2+}-influx mechanism has been provided in our previous work. Thus, in studies investigating the interactions between ionotropic and metabotropic glutamate receptor signalling mechanisms we demonstrated that modest concentrations of NMDA could not only enhance the maximal [^{3}H]-$InsP_x$ response to 1**S**, 3**R**-ACPD, but also significantly leftward-shift the EC_{50} value (from 16.6 to 4.9 μM; Challiss *et al.*, 1994b).

The increasing potency of 1**S**, 3**R**-ACPD with increasing $[Ca^{2+}]_i$ suggests that the direct and indirect components of the PIC response may have different sensitivities to this agonist. Such a difference, i.e. the Ca^{2+}-influx component is activated at lower concentrations of 1**S**, 3**R**-ACPD than is the direct mGluR-stimulated PIC, may result from the activation of a single mGluR (exhibiting different efficacies with respect to the two components of the composite [^{3}H]-$InsP_x$ response) or the activation of distinct mGluRs. Furthermore, one possible explanation of the data presented here on the difference in antagonist actions on 1**S**, 3**R**-ACPD-evoked Ins(1,4,5)P_3 and [^{3}H]-$InsP_x$ responses would be that the Ca^{2+}-mediated component is less susceptible (or is insensitive) to (±)-MCPG. Such a possibility takes on a new significance in the light of the reported differential (±)-MCPG effect on mGluR1/5-mediated responses (Joly *et al.*, 1995).

In summary, the present study has shown that in neonatal cerebral cortex, mGluR agonists and antagonists exhibit different EC_{50} and K_d values depending on which index of phosphoinositide turnover is measured. Further experiments in well-defined homogeneous cell systems (e.g. cerebellar granule cells, or neurones transfected to express single mGluR subtypes; Ikeda *et al.*, 1995) which express appropriate complements of (mGluR-sensitive) ion channels will be necessary to resolve unambiguously the factors which contribute to mGluR agonist and antagonist activities in neurones.

We thank Professor S.R. Nahorski and Dr G.B. Willars (Cell Physiology & Pharmacology, University of Leicester) for their criticisms and helpful comments on this manuscript.

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(Received November 30, 1995
Revised December 18, 1995
Accepted January 3, 1996)