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Stimulatory effects of the putative metabotropic glutamate receptor antagonist L-AP3 on phosphoinositide turnover in neonatal rat cerebral cortex

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1 The effects of the metabotropic glutamate receptor (mGluR) antagonist, L-2-amino-3-phosphonopropionate (L-AP3) on phosphoinositide turnover in neonatal rat cerebral cortex slices has been investigated.

2 At concentrations of $\leqslant 300\ \mu M$, L-AP3 inhibited total [^{3}H]-inositol phosphate ([^{3}H]-InsP_X) and Ins(1,4,5)P_3 mass responses stimulated by the selective mGluR agonist, 1-amino-cyclopentane-1S, 3R-dicarboxylic acid (1S, 3R-ACPD). Comparison with the competitive mGluR antagonist (±)-α-methyl-4-carboxyphenylglycine ((±)-MCPG) clearly demonstrated that L-AP3 caused inhibition by a mechanism that was not competitive, as L-AP3 decreased the maximal response to 1S, 3R-ACPD (by ~40% at 300 μM L-AP3) without significantly affecting the concentration of 1S, 3R-ACPD required to cause half-maximal stimulation of the [^{3}H]-InsP_X response.

3 In contrast, at a higher concentration L-AP3 (1 mM) caused a large increase in [^{3}H]-InsP_X accumulation which was similar in magnitude in both the absence and presence of 1S, 3R-ACPD (300 μM). D-AP3 (1 mM) had no stimulatory effect alone and did not affect the response evoked by 1S, 3R-ACPD. L-AP3 (1 mM) also caused a large increase in Ins(1,4,5)P_3 accumulation. The magnitude of the response (4–5 fold increase over basal) approached that evoked by a maximally effective concentration of 1S, 3R-ACPD, but differed substantially in the time-course of the response. The stimulatory effects of 1S, 3R-ACPD and L-AP3 on Ins(1,4,5)P_3 accumulation were also similarly affected by decreases in extracellular calcium concentration.

4 Detailed analysis of the inositol phospholipid labelling pattern and the inositol (poly)phosphate isomeric species generated following addition of L-AP3 was also performed. In the continued presence of *myo*-[^{3}H]-inositol, L-AP3 (1 mM) stimulated a significant increase in phosphatidylinositol labelling, but not that of the polyphosphoinositides, and the inositol (poly)phosphate profile suggested that substantial Ins(1,4,5)P_3 metabolism occurs via both 5-phosphatase and 3-kinase routes.

5 A significant stimulatory effect of L-AP3 (1 mM) on [^{3}H]-InsP_X accumulation was also observed in neonatal rat hippocampus, and cerebral cortex and hippocampus slices prepared from adult rat brain.

6 These data demonstrate that whilst L-AP3 antagonizes mGluR-mediated phosphoinositide responses at concentrations of $\leqslant 300\ \mu M$, higher concentrations substantially stimulate this response. The ability of (±)-MCPG (1 mM) to attenuate significantly L-AP3-stimulated [^{3}H]-InsP_X accumulation, suggests that both the inhibitory and stimulatory effects of L-AP3 may be mediated by mGluRs.

Keywords: Metabotropic glutamate receptors; phosphoinositide turnover; inositol 1,4,5-trisphosphate; L-2-amino-3-phosphonopropionate; 1-amino-cyclopentane-1S, 3R-dicarboxylic acid; (±)-MCPG, (±)-α-methyl-4-carboxyphenylglycine; cerebral cortex (neonatal rat)

Introduction

Metabotropic glutamate receptors (mGluRs) possess a seven-transmembrane topology and thus belong to the G protein-coupled receptor superfamily (Nakanishi, 1992). To date, eight mGluRs have been cloned and sub-divided into 3 groups based on sequence homologies, the second messenger systems to which they preferentially couple, and pharmacological agonist potency-ranking profiles (Nakanishi, 1992; Pin & Duvoisin, 1995). Although recent developments have provided clear indications that selective mGluR antagonists are in prospect (Birse *et al.*, 1994; Hayashi *et al.*, 1994; Jane *et al.*, 1994; Thomsen *et al.*, 1994; Watkins & Collingridge, 1994), the prototypic mGluR antagonist, L-2-amino-3-phosphonopropionate (L-AP3) is still widely used to characterize and categorize mGluR-mediated physiological actions (e.g. Hu & Storm, 1992; Zheng & Gallagher, 1992; Collins, 1993; Sahara & Westbrook, 1993; Herrero *et al.*, 1994; Stefani *et al.*, 1994; Young *et al.*, 1994; Zirpel *et al.*, 1995; and see Schoepp & Conn, 1993 for review).

Initial studies which characterized the effects of L- (or DL-) AP3 on phosphoinositide responses stimulated by a variety of agonists acting at metabotropic glutamate receptors suggested that this agent was a selective, though non-competitive, mGluR antagonist (Schoepp & Johnson, 1989; Irving *et al.*, 1990; Schoepp *et al.*, 1990). In contrast, at mGluRs which preferentially couple to inhibition of adenylyl cyclase, L-AP3 has been reported either to have no effect (Cartmell *et al.*, 1992) or to exert an agonist action (Casabona *et al.*, 1992; Schoepp & Johnson, 1993). Although studies in model cell systems expressing mGluR1 (Aramori & Nakanishi, 1992; Pickering *et al.*, 1993; Thomsen *et al.*, 1993) or mGluR5 (Abe *et al.*, 1992; Saugstad *et al.*, 1995) have confirmed that L-AP3 is a mGluR antagonist, a number of questions remain regarding the mechanism of action of this agent. For example, Saugstad and coworkers (1995) have recently shown that L-AP3 appears to act competitively at mGluR1α and mGluR5 expressed in *Xenopus* oocytes, whilst a number of groups have reported

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stimulatory actions of L-AP3 on phosphoinositide responses in a variety of preparations (Schoepp *et al.*, 1990; Lonart *et al.*, 1992; Birrell & Marcoux, 1993; Littman & Robinson, 1994), and some studies have suggested that L-AP3 may exert a non-receptor-dependent inhibitory effect (Ikeda, 1993).

In the present study we have investigated the complex inhibitory *and* stimulatory actions exerted by L-AP3 in neonatal rat cerebral cortex slices. The possibility that these effects are independent of interactions with mGluRs is also addressed. A preliminary account of this work has been communicated to the British Pharmacological Society (Mistry *et al.*, 1994).

Methods

Incubation methods

Cerebral cortex or hippocampus from 7–8 day old neonatal rats (Wistar strain, either sex), or adult male Wistar rats (200–250 g) was cross-cut (350 × 350 μm) with a McIlwain 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 (or 50 μl for adult brain slice preparation) aliquots transferred to flat-bottomed polypropylene tubes containing 250 μl KHB. Where inositol phospholipid radiolabelling was required, *myo*-[^{3}H]-inositol was introduced at this stage (0.5 μCi [^{3}H]-inositol per vial (*i.e.* 1.67 μCi [^{3}H]-inositol ml^{-1}) for experiments involving subsequent total [^{3}H]-inositol phosphate ([^{3}H]-InsP_X) determinations, or 2.5 μCi [^{3}H]-inositol per vial for experiments where h.p.l.c. separation of [^{3}H]-inositol (poly)phosphate isomers was undertaken and incubations continued for 60 min at 37°C with regular purging with O_2/CO_2. In radiolabelling experiments only, LiCl (5 mM final concentration) was added at the end of the labelling period. 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, radiolabelled 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, or nominally Ca^{2+}-free KHB 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, 5 mM LiCl and 0.5 μCi [^{3}H]-inositol.

Where used as antagonists, L- (or D-) AP3, and (±)-α-methyl-4-carboxyphenylglycine (MCPG) 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 [^{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 h.p.l.c. separations, the sample preparation was similar except that triplicate samples were pooled for subsequent analysis. For Ins(1,4,5)P_3 mass determinations, 50 μl 60 mM $NaHCO_3$ and 50 μl 30 mM 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).

The slice pellet following TCA addition and centrifugation was processed in one of two ways. Routinely, the pellet was digested in NaOH overnight for subsequent protein determination (Lowry *et al.*, 1951) allowing [^{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. Alternatively the slice pellet was sequentially washed with TCA/1 mM EDTA and water before addition of 0.94 ml acidified chloroform/methanol (80:40:1 methanol/chloroform/conc HCl) and vortex-mixing for 15–20 min. Chloroform (0.31 ml) and 0.1 M HCl (0.56 ml) were then added and phases resolved by centrifugation (1000 *g*, 10 min). Following aspiration of the upper phase and interfacial material, an aliquot of the lower phase was taken for deacylation and resolution of the glycerophosphoinositol (phosphate) products of phosphatidylinositol ([^{3}H]-GroPIns), phosphatidylinositol 4-phosphate ([^{3}H]-GroPInsP) and phosphatidylinositol 4,5-bisphosphate ([^{3}H]-GroPInsP_2) by use of the methods described in detail by Simpson *et al.* (1987).

Materials

L- and D-2-amino-3-phosphonopropionic acid (L- and D-AP3), 1-amino-cyclopentane-1S, 3R-dicarboxylic acid (1S, 3R-ACPD) and (±)-α-methyl-4-carboxyphenylglycine (MCPG) were purchased from Tocris Cookson Ltd. (Langford, U.K.). All other agents were of the highest quality available and 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 by a commercially available programme (InPlot, GraphPad Software, San Diego CA, U.S.A.) and used to generate EC_{50}/IC_{50} values. Statistical comparisons were performed by Student's *t* test for unpaired observations.

Results

L-AP3 as a metabotropic glutamate receptor antagonist

Addition of 1S, 3R-ACPD to neonatal rat cerebral cortex slices for 15 min, in the presence of 5 mM LiCl, caused a 5–6 fold increase in [^{3}H]-InsP_X accumulation. Increasing concentrations of L-AP3 inhibited the 1S, 3R-ACPD-stimulated response with a maximal inhibitory effect of 43.0±2.8% being observed at 300 μM L-AP3 (Figure 1). At a concentration of 300 μM, L-AP3 had a small, but significant (18±6%; $P<0.01$) stimulatory effect on [^{3}H]-InsP_X accumulation (Figure 1). Higher concentrations of L-AP3 caused no further inhibition of the 1S, 3R-ACPD-stimulated response, but instead had a marked stimulatory effect on phosphoinositide turnover *per se* (see below). The time-course of the inhibitory action of L-AP3 (300 μM) on 1S, 3R-ACPD-stimulated [^{3}H]-InsP_X accumulation is shown in Figure 2. Pre-addition of L-AP3 appears to cause an essentially similar inhibition whether measured at 5 or 30 min after addition of 1S, 3R-ACPD.

The mechanism of antagonism by L-AP3 at the mGluR has been reported not to be competitive (Schoepp *et al.*, 1990). Comparison of the inhibitory effects of L-AP3 and the competitive mGluR antagonist (±)-MCPG on the concentration-dependency of 1S, 3R-ACPD-stimulated [^{3}H]-InsP_X accumulation lends support to this finding (Figure 3). Whilst (±)-MCPG (1 mM) causes a parallel rightward shift in the 1S, 3R-ACPD concentration-response curve without affecting basal or maximal values ($logEC_{50}$ (M): −MCPG, −4.76±0.06;

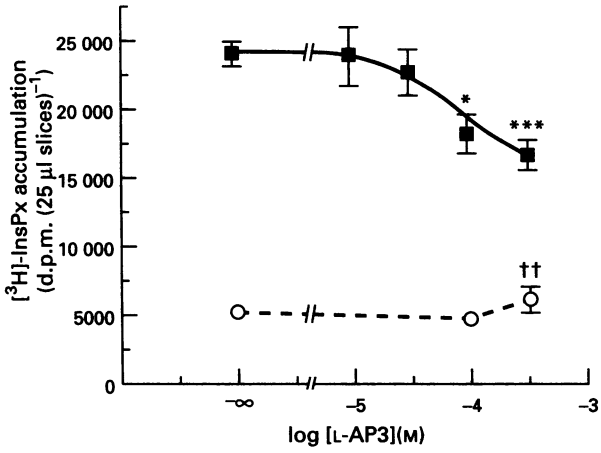

Figure 1 Concentration-dependent inhibitory effect of L-AP3 on [^{3}H]-InsP$_X$ accumulation stimulated by 1S, 3R-ACPD: neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. After this period, LiCl (5 mM, final concentration) and the indicated concentration of L-AP3 were added. After 20 min slices were stimulated by addition of 300 μM 1S, 3R-ACPD (■) or vehicle (○). Incubations were terminated after 15 min and 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 performed in triplicate. Statistically significant inhibitory effects of L-AP3 pre-addition on the subsequent 1S, 3R-ACPD-evoked response are indicated as $*P<0.05$; $***P<0.01$; in addition, 300 μM L-AP3 significantly increased basal [^{3}H]-InsP$_X$ accumulation ($††P<0.01$).

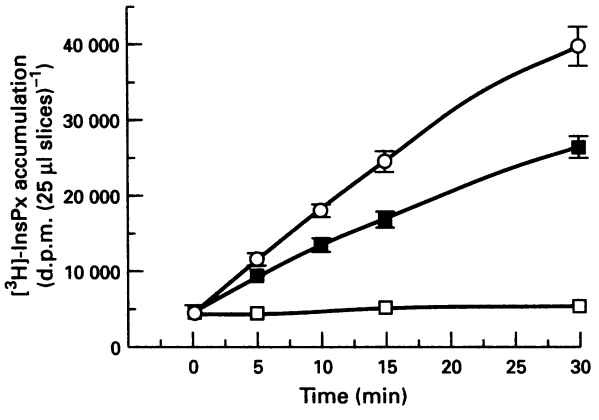

Figure 2 Time-course of [^{3}H]-InsP$_X$ accumulation stimulated by 1S, 3R-ACPD in the absence and presence of L-AP3: neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. After this period, LiCl (5 mM), and either L-AP3 (300 μM; ■) or vehicle (□) were added. After 20 min, slices were stimulated by addition of 300 μM 1S, 3R-ACPD (○, ■) for the periods indicated. Incubations 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.

+MCPG, −4.40 ± 0.05), the major effect of L-AP3 was to suppress the response to high concentrations of 1S, 3R-ACPD (at 300 μM inhibition by ~37%). As seen previously, 300 μM L-AP3 increased basal [^{3}H]-InsP$_X$ accumulation and did not significantly alter the concentration of 1S, 3R-ACPD required to stimulate half-maximally [^{3}H]-InsP$_X$ accumulation ($-\log EC_{50}$: −4.90 ± 0.11).

It has been suggested that L-AP3 may inhibit phosphoinositide turnover, not by direct mGluR antagonism, but through an indirect mechanism by interfering with substrate supply for the agonist-stimulated phosphoinositidase C (Ikeda, 1993). To address this possibility, we have studied the

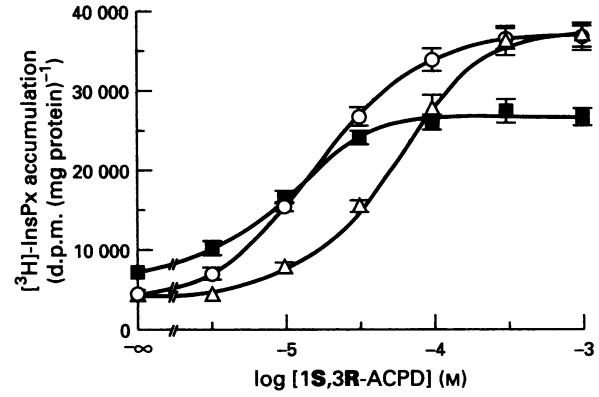

Figure 3 Effects of L-AP3 and (±)-MCPG on the concentration-dependency of [^{3}H]-InsP$_X$ accumulation stimulated by 1S, 3R-ACPD: neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. After this period, LiCl (5 mM, final concentration) and L-AP3 (300 μM; ■), (±)-MCPG (1 mM; △) or vehicle (○) were added. After 20 min slices were stimulated by addition of the indicated concentration of 1S, 3R-ACPD. Incubations were terminated after 15 min and processed for [^{3}H]-InsP$_X$ assay as described in the Methods section. Values are presented as means ± s.e.mean for either three (+MCPG) or four (control; +L-AP3) separate experiments performed in triplicate.

changes in radiolabelling of PtdIns, PtdIns(4)P and PtdIns(4,5)P$_2$ following addition of L-AP3 and/or 1S, 3R-ACPD (Figure 4). Neonatal cerebral cortex slices were labelled with *myo*-[^{3}H]-inositol (at 1.67 μCi ml^{-1}) for 60 min, incubated with 5 mM LiCl in the absence and presence of 300 μM L-AP3 for a further 20 min, and then challenged with 1S, 3R-ACPD (300 μM) for 15 min. L-AP3 had no effect on basal labelling patterns and had no significant effect on the magnitude of the increase in phosphoinositide labelling associated with stimulation by 1S, 3R-ACPD (Figure 4). Similarly, L-AP3 (300 μM) had no significant effect on changes in phosphoinositide levels stimulated by the muscarinic cholinoceptor agonist, carbachol, or the [^{3}H]-InsP$_X$ response evoked by this agent (data not shown). Thus, at least under these conditions, the inhibitory effects of L-AP3 exhibit a selectivity for mGluR-specific responses and are very unlikely to result from changes in phosphoinositide availability for agonist-stimulated phosphoinositidase C.

Experiments were also performed to assess the effects of L-AP3 on the immediate second messenger product of phosphoinositidase C activation. Addition of 1S, 3R-ACPD (300 μM) caused a characteristic rapid 4–5 fold increase in Ins(1,4,5)P$_3$ mass accumulation which was maximal by 15 s and declined throughout the subsequent 10 min time-course (Figure 5). L-AP3 (300 μM) alone caused a small, but significant increase in basal Ins(1,4,5)P$_3$ levels (−L-AP3, 23.8 ± 0.7; +L-AP3, 35.4 ± 2.1 pmol mg^{-1} protein; $n=4$; $P<0.01$). Pre-addition of L-AP3 did not affect the initital peak response to 1S, 3R-ACPD (causing only an 11% decrease in the absolute increase evoked by 1S, 3R-ACPD), but it did have significant inhibitory effects at 30, 60 and 300 s after agonist addition (Figure 5).

L-AP3 as a stimulus of phosphoinositide turnover

At higher concentrations, L-AP3 had a profound stimulatory effect on phosphoinositide turnover (Figure 6). Thus, addition of 1 mM L-AP3 caused a large [^{3}H]-InsP$_X$ accumulation, which was 140–150% of the response evoked by 1S, 3R-ACPD after a similar exposure time (15 min). The [^{3}H]-InsP$_X$ accumulation evoked by co-addition of L-AP3 and 1S, 3R-ACPD was not significantly greater than that evoked by L-AP3 alone. In contrast, 1 mM D-AP3 had no effect on either basal or 1S, 3R-ACPD-stimulated [^{3}H]-InsP$_X$ accumulations (Figure 6). Time-course studies showed that whilst [^{3}H]-InsP$_X$ accumulation

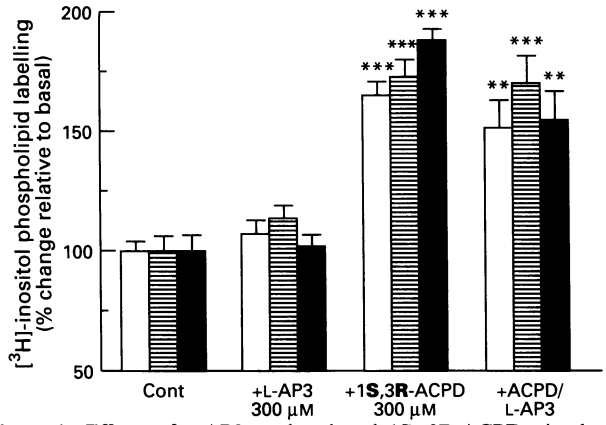

Figure 4 Effects of L-AP3 on basal and 1S, 3R-ACPD-stimulated changes in [^{3}H]-inositol phospholipid labelling patterns: neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. After this period, LiCl (5 mM, final concentration) was added. Slices were incubated in the absence (Cont) or presence of 300 μM L-AP3 for 20 min, before addition of 1S, 3R-ACPD (300 μM) or vehicle and incubations continued for 15 min before termination and processing by inositol phospholipid extraction and separation of glycerophosphoinositol(phosphate)s as described in the Methods section. Values are presented as means ± s.e.mean for 4–8 separate experiments performed in triplicate. Respective basal values (expressed as d.p.m. (25 μl slices)$^{-1}$ and set to 100%) were: PtdIns (34979 ± 1281; open columns); PtdIns(4)P (4010 ± 243; lined columns) and PtdIns(4,5)P_2 (3959 ± 255; solid columns). Statistically significant changes are indicated as $^{**}P<0.01$ or $^{***}P<0.001$ for increases relative to respective control values.

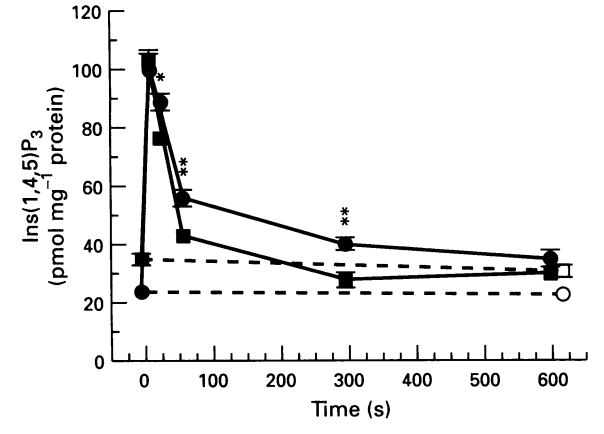

Figure 5 Time-course of 1S, 3R-ACPD-stimulated Ins(1,4,5)P_3 mass accumulation in the absence and presence of L-AP3: neonatal cerebral cortex slices were incubated for 60 min prior to addition of L-AP3 (300 μM □, ■) or vehicle (○, ●). After 20 min 1S, 3R-ACPD (300 μM; ●, ■) was added and incubations terminated at the times indicated and 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 four separate experiments performed in triplicate. The presence of 300 μM L-AP3 significantly increased ($P<0.01$) the basal Ins(1,4,5)P_3 level throughout the time-course. Statistically significant effects of L-AP3 on the 1S, 3R-ACPD-stimulated response are indicated as $^{*}P<0.05$; $^{**}P<0.01$.

stimulated by 1S, 3R-ACPD was essentially linear for 30 min, a maximal rate of increase in L-AP3-stimulated [^{3}H]-InsP_X accumulation was observed between 5 and 15 min (Figure 7), with a slower initial rate of increase and little increase in accumulation between 15 and 30 min after L-AP3 addition.

Like 1S, 3R-ACPD, L-AP3 caused a large increase in Ins(1,4,5)P_3 mass accumulation, however, the time-courses of Ins(1,4,5)P_3 accumulation stimulated by each agent differed considerably (Figure 8). In contrast to the rapid peak and

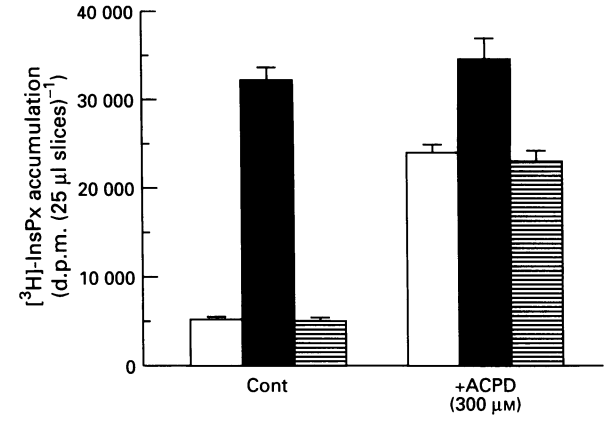

Figure 6 Effects of L- and D-AP3 (1 mM) on basal and 1S, 3R-ACPD-stimulated [^{3}H]-InsP_X accumulation: neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. After this period, LiCl (5 mM, final concentration) and 1 mM L-AP3 (solid columns), D-AP3 (lined columns) or vehicle (open columns) were added. After 20 min, 1S, 3R-ACPD (ACPD; 300 μM) or vehicle (Cont) was added and incubations terminated 15 min later and processed for [^{3}H]-InsP_X assay as described in the Methods section. Values are presented as means ± s.e.mean for four separate experiments performed in triplicate.

decaying plateau response characteristic of 1S, 3R-ACPD (300 μM), L-AP3 (1 mM) stimulated a slowly developing increase in Ins(1,4,5)P_3 which approached a maximum only 5–10 min after challenge (Figure 8). D-AP3 (1 mM) had no effect on Ins(1,4,5)P_3 accumulation *per se* and did not significantly affect the response evoked by 1S, 3R-ACPD (data not shown). The effect of omitting Ca^{2+} from the incubation medium on the Ins(1,4,5)P_3 responses stimulated by 1 mM-ACPD (300 μM) or L-AP3 (1 mM) was also investigated. Reduction of $[Ca^{2+}]_e$ from 1.3 mM to 2–5 μM decreased the basal level of Ins(1,4,5)P_3 and reduced the absolute maximal increase (measured at 15 s for 1S, 3R-ACPD and at 300 s for L-AP3) by ~50% for both agents. However, this manipulation had no effect on the time-course of changes in Ins(1,4,5)P_3 accumulation, and no effect on the respective 4 and 6 fold-over-basal increases evoked by L-AP3 and 1S, 3R-ACPD (Figure 9).

Whether similar stimulatory effects of L-AP3 on phosphoinositide turnover occur in other brain preparations was investigated in neonatal hippocampus, and in the adult brain. The effects of L-AP3 (and for comparison, 1S, 3R-ACPD and carbachol) were assessed in slices prepared from cerebral cortex and hippocampus of neonatal or adult rats (Table 1). Although, the largest stimulatory effect of L-AP3 (1 mM) was observed in neonatal cerebral cortex, a marked increase in [^{3}H]-InsP_X accumulation was also observed in neonatal hippocampus. It is noteworthy that whilst the response to L-AP3 was slightly greater than that to 1S, 3R-ACPD in neonatal cerebral cortex, this was not the case in neonatal hippocampus where the response to 1S, 3R-ACPD was 2–3 fold greater than that evoked by L-AP3 (Table 1). In adult cerebral cortex and hippocampus L-AP3 again caused significant increases in [^{3}H]-InsP_X accumulation which were 55% and 35% of the respective responses evoked by 1S, 3R-ACPD (Table 1). Thus, in two brain regions at different developmental stages, L-AP3 appears to exert significant stimulatory effects on phosphoinositide turnover.

Effects of L-AP3 on inositol (poly)phosphate intermediates and phosphoinositide labelling

H.p.l.c. analysis of inositol (poly)phosphate accumulations stimulated by L-AP3 (1 mM), 1S, 3R-ACPD (300 μM) and carbachol (100 μM) in neonatal cerebral cortex are shown in

Table 2. It is clear that L-AP3 caused dramatic increases in all inositol (poly)phosphate isomers resolved by this analysis. In agreement with the previously shown $Ins(1,4,5)P_3$ mass data, [^{3}H]-$Ins(1,4,5)P_3$ was increased ~8 fold 10 min after L-AP3 addition, whilst 1S, 3R-ACPD elicited only ~ 3 fold increase at this time-point (Table 2). There was evidence of significant accumulations of the products of both $Ins(1,4,5)P_3$ 5-phosphatase and $Ins(1,4,5)P_3$ 3-kinase routes of $Ins(1,4,5)P_3$ metabolism in L-AP3-stimulated slices; in the case of the latter it was interesting to note that [^{3}H]-$Ins(1,3,4,5)P_4$ was dramatically elevated (basal, 632±87; +L-AP3, 18867±2264 d.p.m. (75 μl slices)$^{-1}$) at this time-point. Estimates of the relative contribution of the 3-kinase route of $Ins(1,4,5)P_3$ metabolism (calculated as the percentage that $Ins(1,3,4,5)P_4$ + $Ins(1,3,4)P_3$ + $Ins(1,3,)P_2$ + $Ins(3,4)P_2$ + Ins(1/3)P contribute to the total increase in $InsP_X$) indicate a 37, 31 and 42% contribution for the L-AP3-, 1S, 3R-ACPD- and carbachol-stimulated responses respectively.

Phosphoinositide labelling by [^{3}H]-inositol was also assessed in the samples processed for h.p.l.c. analysis. Challenge with L-AP3, 1S,3R-ACPD or carbachol caused marked 50–90% increases in [^{3}H]-PtdIns labelling (Table 2). However, different labelling patterns were observed with respect to [^{3}H]-PtdIns(4)P and [^{3}H]-$PtdIns(4,5)P_2$. Thus, whilst 1S,3R-APCD and carbachol also caused 70–90% increases in the labelling of these inositol phospholipids, L-AP3 did not enhance the labelling of [^{3}H]-PtdIns(4)P or [^{3}H]-$PtdIns(4,5)P_2$ (Table 2).

Is the stimulatory effect of L-AP3 on phosphoinositide turnover mediated via mGluRs?

Although this and other studies (Schoepp *et al.*, 1990; Lonart *et al.*, 1992; Birrell & Marcoux, 1993; Littman & Robinson, 1994) have demonstrated that L-AP3 can elicit a robust phosphoinositide response in a variety of brain slice preparations, it is unclear whether this occurs via an agonist action at mGluRs. Pre-incubation of cerebral cortex slices with the mGluR antagonist (±)-MCPG (1 mM) significantly attenuated (by 43%; $P<0.001$) the subsequent [^{3}H]-$InsP_X$ accumulation stimulated by L-AP3 (1 mM) addition (Figure 10), and completely suppressed the small stimulatory effect of 300 μM L-AP3 (data not shown), implicating an action at mGluR(s) linked to phosphoinositide turnover in the stimulatory action of L-AP3.

Discussion

L-AP3 was the first, and for sometime the only, mGluR antagonist available for interrogating the occurrence and physiological roles of this important class of receptors for the excitatory neurotransmitter glutamate (Schoepp & Conn, 1993). Initial studies of L-AP3 demonstrated that this agent could markedly suppress phosphoinositide responses stimulated by the mGluR agonists, quisqualate or ibotenate, in rat

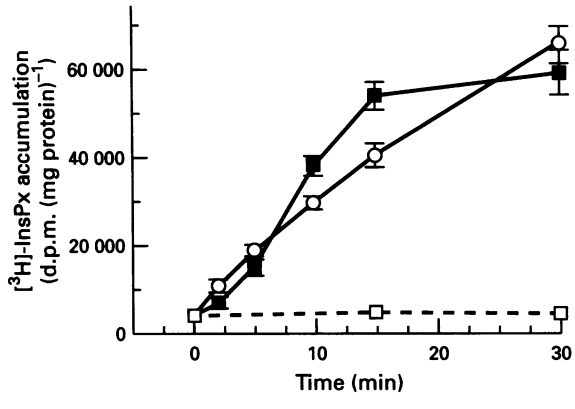

Figure 7 Time-course of [^{3}H]-$InsP_X$ accumulation stimulated by 1S, 3R-ACPD or L-AP3: neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. After this period, LiCl (5 mM, final concentration) was added. After 20 min, 1S, 3R-ACPD (300 μM; ○), 1 mM L-AP3 (1 mM; ■) or vehicle (□) was added and incubations terminated at the times indicated and processed for [^{3}H]-$InsP_X$ assay as described in the Methods section. Values are presented as means±s.e.mean for one experiment performed in triplicate and representative of three other experiments.

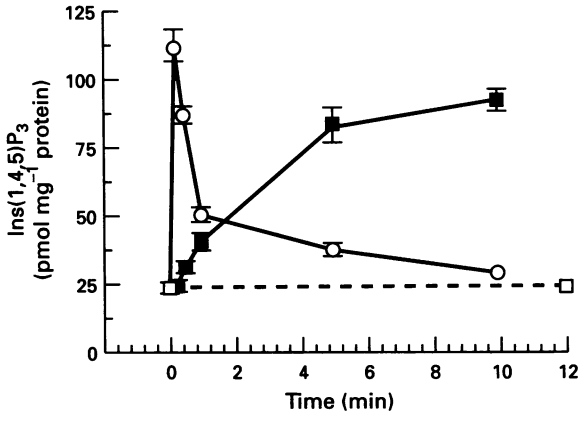

Figure 8 Time-course of $Ins(1,4,5)P_3$ mass accumulation stimulated by 1S, 3R-ACPD or L-AP3: neonatal cerebral cortex slices were incubated for 60 min prior to addition of either 1S, 3R-ACPD (300 μM; ○), L-AP3 (1 mM; ■) or vehicle (□) for the indicated times. Incubations were terminated and processed for $Ins(1,4,5)P_3$ mass assay as described in the Methods section. Values are presented as means±s.e.mean for four separate experiments performed in triplicate.

Table 1 Comparative effects of L-AP3, 1S,3R-ACPD and carbachol on [^{3}H]-$InsP_x$ accumulations in neonatal and adult cerebral cortex and hippocampus

	Control	*L-AP3* (1 mM)	*1S,3R-ACPD* (300 μM)	*Carbachol* (100 μM)
Neonatal				
Cerebral cortex	4365±185	47904±1818	36180±1237	76287±1471
Hippocampus	7220±772	43392±4693	105341±6365	110731±8176
Adult				
Cerebral cortex	3307±216	7058±439	10093±681	13660±902
Hippocampus	3255±115	6982±418	13812±750	16812±1272

Cerebral cortex and hippocampal slices were prepared from neonatal (7–8 day old, either sex) or adult (weight 250–300 g, male) rats. Aliquots of gravity-packed slices (neonate, 25 μl; adult 50 μl) were incubated with [^{3}H]-inositol (0.5 μCi) in a final volume of 300 μl for 60 min. LiCl was added to a final concentration of 5 mM and slices were challenged with L-AP3 (1 mM), 1S,3R-ACPD (300 μM) or carbachol (100 μM) for 15 min. [^{3}H]-$InsP_x$ were recovered and separated as described in the Methods section. Values (expressed as d.p.m. (25 or 50 μl of slices)$^{-1}$), are presented as means±s.e.mean for four separate experiments, each performed in triplicate. In all cases, addition of L-AP3 causes a highly statistically significant ($P<0.001$) increase in [^{3}H]-$InsP_x$ accumulation compared to respective basal values.

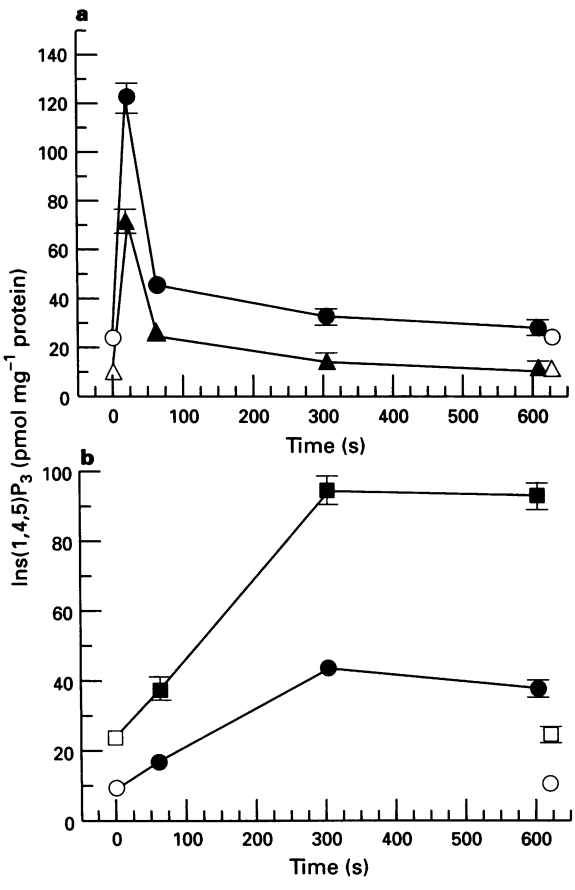

Figure 9 Extracellular Ca^{2+} dependence of Ins(1,4,5)P_3 accumulation stimulated by 1S, 3R-ACPD and L-AP3: neonatal cerebral cortex slices were incubated for 60 min in normal KHB (1.3 mM Ca^{2+}). Slices were then washed either in normal KHB or nominally Ca^{2+}-free KHB. ([Ca^{2+}] = 2.5 μM) before dispensing into incubation vials containing the appropriate buffer. In (a), slices in normal KHB (○, ●) or Ca^{2+}-free KHB (△, ▲) were challenged with either 1S, 3R-ACPD (300 μM; filled symbols), or vehicle (open symbols); in (b), slices in normal KHB (□, ■) or Ca^{2+}-free KHB (○, ●) were challenged with either L-AP3 (1 mM; filled symbols), or vehicle (open symbols). Incubations were terminated at the times indicated and 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 performed in triplicate.

cerebral cortex and hippocampus slices, with the predominant effect being a suppression of the maximal agonist-stimulated response (Schoepp & Johnson, 1989; Schoepp *et al.*, 1990). Furthermore the latter study demonstrated a degree of stereoselectivity, with L-AP3 being more effective than the D-enantiomer as an antagonist. These observations have been confirmed by others with respect to mGluR-mediated phosphoinositide turnover (Manzoni *et al.*, 1991; Jones & Roberts, 1993; Littman & Robinson, 1994) and Ca^{2+} mobilization (Irving *et al.*, 1990). However, antagonism of mGluR-stimulated phosphoinositide turnover by L-AP3 has not been found in all studies. For example, tissue-specific (Vecil *et al.*, 1992; Littman & Robinson, 1994) and agonist-specific (Chung *et al.*, 1994) differences to antagonism by L-AP3 have been reported. In addition, the tendency for higher concentrations of L-AP3 to have an apparent partial agonist action (Schoepp *et al.*, 1990; Manzoni *et al.*, 1991; Lonart *et al.*, 1992; Vecil *et al.*, 1992; Birrell & Marcoux, 1993; Littman & Robinson, 1994) may explain the reduced or lacking inhibitory effects of this agent, at least in some brain preparations.

Heterologous expression cloning of specific mGluR subtypes has provided some clarification of the activity and selectivity of L-AP3 for phosphoinositide-linked mGluRs. Thus, it has been shown that L-AP3 is a weak antagonist of glutamate-stimulated phosphoinositide turnover in model cell systems expressing mGluR1α (Aramori & Nakanishi, 1992; Thomsen *et al.*, 1993), and GluR1β (Pickering *et al.*, 1993), but not mGluR5 (Abe *et al.*, 1992). These data suggest a mechanistic basis for the L-AP3-sensitive and insensitive responses seen in different brain preparations. However, the possibility that L-AP3 may discriminate between mGluR1- and mGluR5-mediated responses has been thrown into doubt by a recent paper which showed that L-AP3 antagonizes a mGluR-activated calcium-dependent chloride current in *Xenopus* oocytes injected with RNA transcripts for either mGluR1α or mGluR5 (Saugstad *et al.*, 1995). Furthermore, in this system, L-AP3 appeared to antagonize competitively the glutamate-induced current (Saugstad *et al.*, 1995). It is noteworthy that one earlier report also concluded that antagonism by DL-AP3 occurred by a competitive mechanism, on the basis of effects of this agent on quisqualate-stimulated phosphoinositide turnover in cultured striatal neurones (Manzoni *et al.*, 1991).

The present study has clearly shown that in neonatal cerebral cortex, L-AP3 causes inhibition of 1S, 3R-ACPD-stimulated [^{3}H]-InsP_x accumulation by an apparently non-competitive mechanism. Thus, L-AP3 significantly decreases the maximal response, but has no effect on the EC_{50} value; in

Table 2 Effects of L-AP3, 1S,3R-ACPD and carbachol on changes in inositol phospholipid levels and inositol (poly)phosphate accumulations in neonatal cerebral cortex slices

	No addition	*+ L-AP3* (1000 μM)	*+ 1S,3R-ACPD* (300 μM)	*+ Carbachol* (100 μM)
(i) [^{3}H]-PtdIns(P_x) isomers				
PtdIns	222668 ± 10581	360521 ± 14600	334221 ± 11342	415278 ± 21794
PtdIns(4)P	22319 ± 1616	25238 ± 331	37106 ± 851	41132 ± 4245
PtdIns(4,5)P_2	21066 ± 960	19659 ± 873	34889 ± 1050	39302 ± 4524
(ii) [^{3}H]-InsP_x isomers				
Ins(1/3)P	12836 ± 669	77907 ± 7355	49995 ± 4270	231790 ± 25635
Ins(4)P	2907 ± 783	60129 ± 4977	52502 ± 1920	180775 ± 25038
Ins(1,3)P_2	119 ± 40	3381 ± 366	1942 ± 257	16400 ± 1581
Ins(1,4)P_2	2174 ± 891	98110 ± 9111	49509 ± 1239	206585 ± 19980
Ins(3,4)P_2	137 ± 40	985 ± 239	367 ± 51	2460 ± 210
Ins(1,3,4)P_3	1920 ± 256	2816 ± 274	4395 ± 303	6641 ± 785
Ins(1,4,5)P_3	1076 ± 181	9243 ± 813	3646 ± 62	14742 ± 957
Ins(1,3,4,5)P_4	632 ± 87	18867 ± 2264	2542 ± 418	34699 ± 2767

Neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (2.5 μCi.(300 μl)$^{-1}$) for 60 min. LiCl was added to a final concentration of 5 mM and slices were challenged with L-AP3 (1 mM), 1S,3R-ACPD (300 μM) or carbachol (100 μM) for 10 min. [^{3}H]-InsP_x and [^{3}H]-inositol phospholipids were recovered and separated as described in the Methods section. Values are presented as means ± s.e.mean for three separate experiments. In each experiment triplicate incubations were pooled for [^{3}H]-InsP_x and [^{3}H]-inositol phospholipid recovery and separation; therefore all values are expressed as d.p.m. (75 μl slices)$^{-1}$.

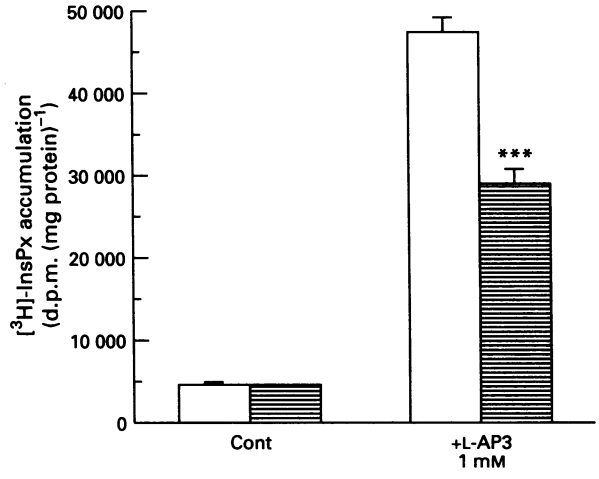

Figure 10 Inhibition of L-AP3-stimulated [^{3}H]-InsP$_X$ accumulation by (±)-MCPG: neonatal cerebral cortex slices were incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. After this period, LiCl (5 mM, final concentration) and (±)-MCPG (1 mM lined columns) or vehicle (open columns) were added. After 20 min slices were stimulated by addition of buffer (Cont) or L-AP3. Incubations were terminated after 15 min and 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 performed in triplicate. A statistically significant decrease in the [^{3}H]-InsP$_X$ response evoked by L-AP3 in the presence compared to the absence of (±)-MCPG is indicated as ***$P<0.001$.

contrast the competitive mGluR antagonist, (±)-MCPG, has no effect on [^{3}H]-InsP$_X$ accumulation stimulated by maximally effective concentrations of 1S, 3R-ACPD, but significantly increases the concentration of the mGluR agonist required to evoke half-maximal response. In addition, significant inhibitory effects of L-AP3 were observed to occur at the level of 1S, 3R-ACPD-stimulated Ins(1,4,5)P$_3$ mass accumulation.

The unusual nature of the mGluR antagonistic effect of L-AP3, and the fact that a number of studies have reported that the L-AP3 effect cannot be reversed by extensive washing of slice preparations (Schoepp *et al.*, 1990; Littman *et al.*, 1992; Lonart *et al.*, 1992; Ikeda, 1993) has led some workers to propose alternative explanations for the inhibitory effects of L-AP3. For example, results presented by Ikeda (1993) led this author to suggest that L-AP3 may exert an indirect (and non-receptor selective) effect through inhibition of the phosphoinositide kinases responsible for PtdIns(4)P and PtdIns(4,5)P$_2$ synthesis in rat hippocampus slices. Indeed, Ikeda (1993) demonstrated that L-AP3 was not only effective in substantially inhibiting phosphoinositide hydrolysis stimulated by 1S, 3R-ACPD, but also that evoked by the muscarinic cholinoceptor agonist, carbachol. Data presented in the present study provide no support for L-AP3 acting via this indirect mechanism in cerebral cortex. Thus, pre-incubation with 300 μM L-AP3 had no effect on the labelling of any phosphoinositide, and had little effect on the increase in PtdIns, PtdIns(4)P and PtdIns(4,5)P$_2$ labelling seen after addition of 1S, 3R-ACPD. Furthermore, the modest inhibitory effect of L-AP3 on the initial increase in 1S, 3R-ACPD-stimulated Ins(1,4,5)P$_3$ mass accumulation argues against a disruption of PtdIns(4,5)P$_2$ supply for agonist-stimulated phosphoinositidase C activity.

It should be noted also that in studies where L-[^{3}H]-glutamate binding has been assessed with membranes prepared from clonal cells expressing specific mGluRs, L-AP3 has been shown to displace specfic [^{3}H]-glutamate binding with K_i values of 298 and 125 μM for mGluR1α (Thomsen *et al.*, 1993) and mGluR3 (Laurie *et al.*, 1995) respectively, suggesting that L-AP3 interacts directly with the mGluR to affect agonist binding.

Another important aspect of the present study has been the characterization of the stimulatory effects of L-AP3 on phosphoinositide turnover in neonatal rat cerebral cortex. Although a small stimulatory effect of L-AP3 was observed at 300 μM, 1 mM L-AP3 caused a dramatic increase in [^{3}H]-InsP$_X$ accumulation which, when assessed at 10 or 15 min after challenge, was 25–50% greater than the response evoked by a maximally effective concentration of 1S, 3R-ACPD. Phosphoinositide responses evoked L-AP3 or 1S, 3R-ACPD were non-additive; indeed almost identical responses were observed following addition of either L-AP3 or L-AP3-plus-1S, 3R-ACPD. These data contrast with a previous report by Birrell & Marcoux (1993) who found essentially additive effects of L-AP3 with either 1S, 3R-ACPD or quisqualate on [^{3}H]-InsP$_X$ accumulation in cultures of cerebrocortical neurones. Although our data are consistent with L-AP3 and 1S, 3R-ACPD acting via a common or convergent mechanism, there are substantial differences between the agents with respect to the patterns of changes in phosphoinositide cycle intermediates each evokes. For example, although both 1S, 3R-ACPD and L-AP3 caused substantial increases in Ins(1,4,5)P$_3$ accumulation, the former evoked a rapid ($\leqslant$15 s) peak increase which declines towards basal values by 10 min after addition, whilst L-AP3 caused a much more slowly developing increase in Ins(1,4,5)P$_3$ which was sustained for $\geqslant$10 min. Furthermore, these differences were maintained in the nominal absence of extracellular Ca^{2+}.

These differences were confirmed and extended by a detailed analysis of isomeric changes in inositol (poly)phosphates and phosphoinositides seen after 1S, 3R-ACPD and L-AP3 addition. Although both 1S, 3R-ACPD and L-AP3 stimulated Ins(1,4,5)P$_3$ generation and subsequent metabolism by both 5-phosphatase and 3-kinase routes, the labelling of the pathway second messengers Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ at 10 min was substantially (3 and 7 fold respectively) greater in the case of L-AP3 stimulation, consistent with the Ins(1,4,5)P$_3$ mass data also reported here. With respect to changes in phosphoinositide labelling, both mGluR and muscarinic cholinoceptor activation, by 1S, 3R-ACPD and carbachol respectively, increased the radioactivity associated with all phosphoinositide fractions, whilst L-AP3 increased only that in PtdIns. Thus for PtdIns(4,5)P$_2$, 1S, 3R-ACPD and carbachol increased labelling by 66 and 87% respectively: in contrast L-AP3 caused a small decrease (−7%) in the radioactivity associated with this phospholipid. Although these data suggest that L-AP3 may exert an additional direct or indirect effect at the level of PtdIns 4-kinase and/or PtdIns(4)P 5-kinase which are responsible for sequential phosphorylation of PtdIns to PtdIns(4,5)P$_2$, such an action is clearly insufficient to affect significantly the ability of L-AP3 to stimulate Ins(1,4,5)P$_3$ generation.

The marked differences in phosphoinositide cycle intermediate levels stimulated by L-AP3 and 1S, 3R-ACPD suggest that these agents may not be working by a common mechanism (*i.e.* via interaction with mGluR(s)), however, other evidence presented here does argue for this possibility. Thus, L-AP3-stimulated [^{3}H]-InsP$_X$ accumulation was blocked (by 43%) by (±)-MCPG. Although this effect was only partial, it should be noted that (±)-MCPG is likely to antagonize the effect of L-AP3 via a competitive mechanism. Thus, at a high concentration of 1S, 3R-ACPD ($\geqslant$100 μM), which causes a smaller increase in [^{3}H]-InsP$_X$ accumulation than L-AP3, (±)-MCPG has no significant inhibitory effect.

The sensitivity of the L-AP3 response to (±)-MCPG, and the previously discussed lack of additivity between L-AP3 and 1S, 3R-APCD, suggest that the stimulatory effect of this agent is mediated via mGluR(s). Despite such evidence, it is difficult to reconcile the apparent antagonist and agonist effects of L-AP3 being mediated at a single site of action. Further studies will be needed to establish whether this is indeed the case. In this context it may be informative to establish whether, and if so with what concentration-dependency, L-AP3 causes, or affects the ability of other mGluR agonists to cause, other events associated with mGluR activation. For example, investigations

of mGluR phosphorylation (Alaluf *et al.*, 1995) or electrophysiological correlates of mGluR1/mGluR5 activation (Gereua & Conn, 1995) may provide further support for a bimodal activity of L-AP3.

In conclusion, although the present study has presented data to suggest that L-AP3 exerts complex actions on mGluR-linked phosphoinositide responses in cerebral cortex, this agent may still prove a useful tool for investigating the (patho)physiological roles of mGluRs in the brain. Indeed, providing studies are carefully controlled to avoid use of concentrations of this agent which might exert a stimulatory action, the non-competitive nature of mGluR antagonism by L-AP3 may be highly advantageous. Thus L-AP3, unlike the more recently developed competitive mGluR antagonists, will inhibit the actions of endogenous or exogenously applied agonists at mGluRs, irrespective of their concentration. The disadvantage imposed by the fact that L-AP3 is a relatively weak antagonist, and therefore must be applied at high concentrations, may be overcome by the availability of the essentially inactive D-isomer allowing adequate control experiments to be performed.

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