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Regulation of phosphoinositide turnover in neonatal rat cerebral cortex by group I- and II- selective metabotropic glutamate receptor agonists

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1 The interactive effects of different metabotropic glutamate (mGlu) receptor subtypes to regulate phosphoinositide turnover have been studied in neonatal rat cerebral cortex and hippocampus by use of agonists and antagonists selective between group I and II mGlu receptors.

2 The group II-selective agonist 2**R**,4**R**-4-aminopyrrolidine-2,4-dicarboxylate (2**R**,4**R**-APDC; 100 μM) had no effect on basal total inositol phosphate ([^{3}H]-InsP_x) accumulation (in the presence of Li^+) in *myo*-[^{3}H]-inositol pre-labelled slices, but enhanced the maximal [^{3}H]-InsP_x response to the group I-selective agonist (**S**)-3,5-dihydroxyphenylglycine (DHPG) by about 100% in both hippocampus and cerebral cortex. In cerebral cortex the enhancing effect of 2**R**,4**R**-APDC occurred with respect to the maximal responsiveness and had no effect on EC_{50} values for DHPG (-log EC_{50} (M): control, 5.56 ± 0.05; + 2**R**,4**R**-APDC, 5.51 ± 0.08). 2**R**,4**R**-APDC also caused a significant enhancement of the DHPG-stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) mass response over an initial 0–300 s time-course.

3 The enhancing effects of 2**R**,4**R**-APDC on DHPG-stimulated [^{3}H]-InsP_x accumulation were observed in both the presence and nominal absence of extracellular Ca^{2+}, and irrespective of whether 2**R**,4**R**-APDC was added before, simultaneous with, or subsequent to DHPG. Furthermore, increasing the tissue cyclic AMP concentration up to 100 fold had no effect on DHPG-stimulated Ins(1,4,5)P_3 accumulation in the absence or presence of 2**R**,4**R**-APDC.

4 2**R**,4**R**-APDC and (2**S**, 1′**R**, 2′**R**, 3′**R**)-2-(2,3-dicarboxylcyclopropyl)glycine (DCG-IV), the latter agent in the presence of MK-801 to prevent activation of NMDA-receptors, each inhibited forskolin-stimulated cyclic AMP accumulation by about 50%, with respective EC_{50} values of 1.3 and 0.04 μM (-log EC_{50} (M): 2**R**,4**R**-APDC, 5.87 ± 0.09; DCG-IV, 7.38 ± 0.05). In the presence of DHPG (30 μM), 2**R**,4**R**-APDC and DCG-IV also concentration-dependently increased [^{3}H]-InsP_x accumulation with respective EC_{50} values of 4.7 and 0.28 μM (-log EC_{50} (M): 2**R**,4**R**-APDC, 5.33 ± 0.04; DCG-IV, 6.55 ± 0.09) which were 3–7 fold rightward-shifted relative to the adenylyl cyclase inhibitory responses.

5 The group II-selective mGlu receptor antagonist LY307452 (30 μM) caused parallel rightward shifts in the concentration-effect curves for inhibition of forskolin-stimulated adenylyl cyclase, and enhancement of DHPG-stimulated [^{3}H]-InsP_x accumulation, by 2**R**,4**R**-APDC yielding similar equilibrium dissociation constants (K_ds, 3.7 ± 1.1 and 4.1 ± 0.4 μM respectively) for each response.

6 The ability of 2**R**,4**R**-APDC to enhance receptor-mediated [^{3}H]-InsP_x accumulation appeared to be agonist-specific; thus although DHPG (100 μM) and the muscarinic cholinoceptor agonist carbachol (10 μM) stimulated similar [^{3}H]-InsP_x accumulations, only the response to the former agonist was enhanced by co-activation of group II mGlu receptors.

7 These data demonstrate that second messenger-generating phosphoinositide responses stimulated by group I mGlu receptors are positively modulated by co-activation of group II mGlu receptors in cerebral cortex and hippocampus. The data presented here are discussed with respect to the possible mechanisms which might mediate the modulatory activity, and the physiological and pathophysiological significance of such crosstalk between mGlu receptors.

Keywords: Metabotropic glutamate receptors; phosphoinositide turnover; inositol 1,4,5-trisphosphate; cyclic AMP; 3,5-dihydroxyphenylglycine; 2**R**,4**R**-APDC; LY307452; cerebral cortex (neonatal rat)

Introduction

The discovery of receptors for the neurotransmitter glutamate which are not ligand-gated cation channels, but instead belong to the G protein-coupled receptor superfamily has led to the cloning and characterization of at least eight metabotropic glutamate (mGlu) receptors encoded by distinct genes in the mammalian genome (Nakanishi, 1994). The mGlu receptor family can be subdivided into three subgroups on the basis of sequence homology, preferential coupling to different signal transduction pathways, and pharmacological criteria (Conn & Pin, 1997). Group I mGlu receptors (types 1 and 5 mGlu receptors and splice variants) stimulate phosphoinositide turnover and therefore can influence neuronal function through Ca^{2+}-mobilization from inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3)-sensitive stores, and activation of various isozymes of protein kinase C (Nahorski, 1988; Conn & Pinn, 1997). Group I mGlu receptors can be selectively activated by (**S**)-3,5-dihydroxyphenylglycine (Ito *et al*., 1992; Schoepp *et al*., 1994). In contrast, group II mGlu receptors (types 2 and 3) link to the inhibition of adenylyl cyclase and also modulate a variety of ion channel activities (Guérineau *et al*., 1994; Ikeda *et al*., 1995; Holmes *et al*., 1996; Conn & Pin, 1997), and can be selectively activated by (2**S**, 1′**R**, 2′**R**, 3′**R**)-2-(2,3-dicarboxylcy-

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clopropyl)glycine (Ishida *et al*., 1993) and 2**R**,4**R**-4-aminopyrrolidine-2,4-dicarboxylate (Schoepp *et al*., 1995).

Recent studies have indicated an unexpected interaction between group I and II mGlu receptors. In hippocampal slice preparations the phosphoinositide hydrolysis stimulated by agonists selective for group I mGlu receptor activation is enhanced by co-activation of group II mGlu receptors (Nicoletti *et al*., 1993; Genazzani *et al*., 1994; Schoepp *et al*., 1996b). The enhancing effect of group II mGlu receptor activation has been suggested to be specific to hippocampal preparations (Genazzani *et al*., 1994) and to vary with development (Schoepp *et al*., 1996b); thus, it has been shown that in slices prepared from neonatal hippocampus co-activation of group II mGlu receptors shifts the group I mGlu receptor agonist concentration-effect curve to the left, whilst in adult preparations a marked enhancement of the maximal effect is seen (Schoepp *et al*., 1996b).

In the present study we have investigated whether similar group I/II interactions occur in rat cerebral cortex, whether co-activation of group II mGlu receptors also enhances the accumulation of the pathway second messenger Ins(1,4,5)P_3 stimulated by group I mGlu receptor agonists, and have compared the effects of the group II mGlu receptor agonists and antagonists to interfere with phosphoinositide and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) responses. A preliminary account of this work has been presented to the British Pharmacological Society (Mistry *et al*., 1997).

Methods

Incubation methods

Cerebral cortex or hippocampus from 7–8 day old neonatal rats (Wistar strain, either sex) 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 and 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 or cyclic AMP 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 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 ($[Ca^{2+}]$ = 1.3 mM) or nominally Ca^{2+}-free KHB ($[Ca^{2+}]$ = 2–5 mM) 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 plus 5 mM LiCl.

Where indicated antagonists (MK-801, LY307452) were added 20 min before the agonist challenge. Unless otherwise stated, group II mGlu receptor agonists were added 10 min before group I mGluR agonist addition for experiments where [^{3}H]-inositol (poly)phosphate ([^{3}H]-InsP_x) and Ins(1,4,5)P_3 mass levels were assessed. 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 and cyclic AMP

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]-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 before column separation being carried out within 48 h. For Ins(1,4,5)P_3 and/or cyclic AMP determinations 50 μl $NaHCO_3$ and 50 μl EDTA were added to 200 μl neutral extract and samples were stored at 4°C before 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). Cyclic AMP levels were determined with a radio-receptor assay by use of a crude preparation of the regulatory subunit of cyclic AMP-dependent protein kinase from bovine adrenal cortex as the binding protein (Brown *et al*., 1971). To allow [^{3}H]-InsP_x, Ins(1,4,5)P_3 and cyclic AMP values to be expressed as d.p.m. mg^{-1} protein and pmol mg^{-1} protein, respectively, the slice pellet was routinely digested with 1 M NaOH overnight for subsequent protein determination (Lowry *et al*., 1951).

Materials

(**S**)-3,5-dihydroxyphenylglycine (DHPG), (2**S**,1′**R**,2′**R**,3′**R**)-2-(2,3-dicarboxylcyclopropyl)glycine (DCG-IV) and 1-amino-cyclopentane-1**S**,3**R**-dicarboxylate (1**S**,3**R**-ACPD) were purchased from Tocris Cookson Ltd. (Langford, U.K.). 2**R**,4**R**-4-aminopyrrolidine-2,4-dicarboxylate (2**R**,4**R**-APDC) and LY307452 (2**S**,4**S**-2-amino-4-(4,4-diphenylbut-1-yl)pentan-1,5-dioic acid) were generous gifts from Dr D.D. Schoepp (Lilly Research Laboratories, Indianapolis, U.S.A.). 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 by use of 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 from 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. Statistical comparisons were performed by Student's *t* test for unpaired observations.

Results

Group I/II mGlu receptor interaction in cerebral cortex and hippocampus

In [^{3}H]-inositol-labelled slices the group II mGlu receptor agonist 2**R**,4**R**-APDC (100 μM) had no stimulant effect on [^{3}H]-InsP_x accumulation in the presence of LiCl, whilst the group I mGlu receptor agonist DHPG (100 μM) stimulated a robust [^{3}H]-InsP_x accumulation in both cerebral cortex and hippocampus (Table 1). Co-addition of 2**R**,4**R**-APDC with DHPG greatly enhanced the response seen in the presence of DHPG alone by 104% in cortex and 83% in hippocampus (Table 1). A marked enhancement of the DHPG-stimulated [^{3}H]-InsP_x response was observed in cerebral cortex slices irrespective of whether 2**R**,4**R**-APDC was added 10 min before, simultaneous with, or 5 min after addition of the group I mGlu receptor agonist (Figure 1).

Characterization of group I/II mGlu receptor interaction: [^{3}H]-InsP_x response

The positive interaction between group I and II agonist effects on phosphoinositide turnover was also observed if LiCl was omitted from the incubation, or in the nominal absence of extracellular Ca^{2+} (Table 2). Further analysis of the effect of 2**R**,4**R**-APDC on the phosphoinositide response to DHPG demonstrated that group II mGlu receptor activation enhanced the maximal response without affecting the EC_{50} for DHPG-stimulated [^{3}H]-InsP_x accumulation (-log EC_{50} (M): control, 5.56 ± 0.05; + 2**R**,4**R**-APDC (100 μM), 5.51 ± 0.08; $n = 4$; Figure 2).

*Comparison of effects of 2**R**,4**R**-APDC and DCG-IV: [^{3}H]-InsP_x and cyclic AMP responses*

DCG-IV was the first group II-selective mGlu receptor agonist to be described (Ishida *et al*., 1993; Ohfune *et al*., 1993) and we have therefore compared the actions of 2**R**,4**R**-APDC with this prototypic agent. Preliminary experiments revealed that unlike 2**R**,4**R**-APDC, DCG-IV stimulated [^{3}H]-InsP_x accumulation *per se*, and caused a biphasic enhancement of DHPG-stimulated [^{3}H]-InsP_x accumulation, which at high concentrations of DCG-IV (< 1 μM) was much greater than that observed in the presence of a maximally-effective concentration of 2**R**,4**R**-APDC (data not shown). However, these effects of DCG-IV were completely abolished in the presence of the uncompetitive NMDA-receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzyl[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801; 5 μM). Thus, in the presence of MK-801, DCG-IV had no stimulating effect on phosphoinositide hydrolysis alone and enhanced the DHPG-stimulated [^{3}H]-InsP_x response to the same extent as that seen in the presence of 2**R**,4**R**APDC (Figure 3). DCG-IV (-log EC_{50} (M), 6.55 ± 0.09; $n = 3$) was approximately 15 fold more potent than 2**R**,4**R**-APDC (-log EC_{50} (M), 5.33 ± 0.04; $n = 4$) in its potentiating effect (Figure 3).

These group II mGlu receptor agonists were also assessed for their respective actions on cyclic AMP levels in cerebral cortex slices. Neither 2**R**,4**R**-APDC (100 μM) nor DCG-IV (10 μM, in the presence of MK-801) significantly affected basal levels of cyclic AMP, but each concentration-dependently inhibited forskolin-stimulated cyclic AMP accumulation (Figure 4). Thus each agent decreased the cyclic AMP accumulation stimulated by 10 μM forskolin by approx. 50% at maximally-effective concentrations, with DCG-IV (-log EC_{50} (M), 7.38 ± 0.05; $n = 3$) being 30 fold more potent than 2**R**,4**R**-APDC (-log EC_{50} (M), 5.87 ± 0.09; $n = 5$) in this action. Comparison of the respective actions of 2**R**,4**R**-APDC and DCG-IV to inhibit cyclic AMP and enhance DHPG-stimulated [^{3}H]-InsP_x responses revealed that these agents were 3.5 and 7 fold more potent for the former compared to the latter second messenger response.

Table 1 Effect of 2**R**,4**R**-APDC on DHPG-stimulated [^{3}H]-InsP_x accumulation in cerebral cortex and hippocampal slices

	[^{3}H]-InsP_x accumulation (d.p.m. mg^{-1} protein)	
Agonist addition	*Cerebral cortex*	*Hippocampus*
No addition	4809 ± 521	7630 ± 885
2**R**,4**R**-APDC	4603 ± 799	7482 ± 1010
DHPG	26221 ± 903[a]	84102 ± 2786[a]
DHPG + 2**R**,4**R**-APDC	41582 ± 1344[a,b]	140821 ± 6607[a,b]

Neonatal rat slices were prepared from either cerebral cortex or hippocampus and incubated with [^{3}H]-inositol (0.5 μCi per vial) for 60 min. LiCl was added (final concentration 5 mM) and slices stimulated by addition of DHPG (100 μM) and 2**R**,4**R**-APDC (100 μM) alone or in combination. Incubations were terminated after 15 min. Samples were processed as described in the Methods section and values are presented as means ± s.e.mean for four separate experiments performed in triplicate (cerebral cortex) or duplicate (hippocampus). Statistically significant differences from respective control values are shown as [a] $P < 0.001$; and differences in the presence versus the absence of 2**R**,4**R**-APDC shown as [b] $P < 0.001$.

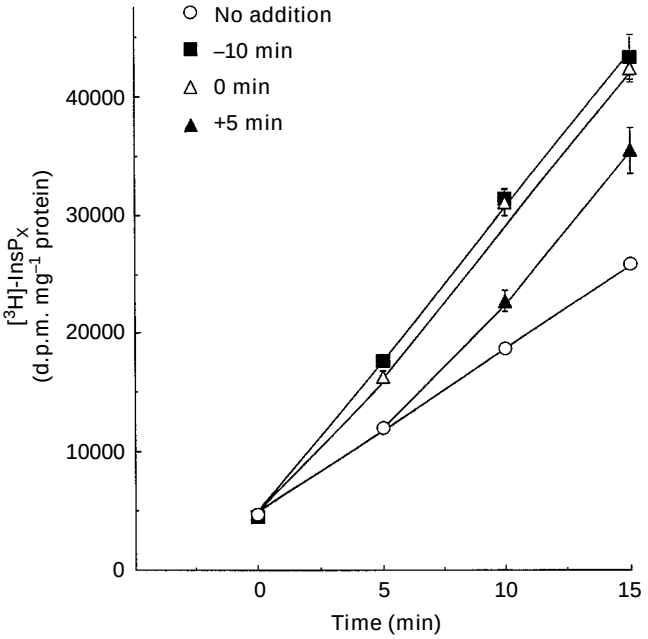

Figure 1 Time-course of DHPG-stimulated [^{3}H]-InsP_x accumulation in the absence or presence of 2**R**,4**R**-APDC. Neonatal rat cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60 min. LiCl was added (final concentration 5 mM) and slices stimulated by addition of DHPG (100 μM) for the times indicated. The effects of prior (−10 min), simultaneous (0 min) or subsequent (+5 min) addition of 2**R**,4**R**-APDC (100 μM) are shown. Samples were processed for analysis of [^{3}H]-InsP_x as described in the Methods section. Values are presented as means for a single experiment performed in triplicate (similar data were obtained in two further experiments); vertical lines show s.e.mean.

Table 2 Effects of LiCl or $[Ca^{2+}]_e$ omission on the enhancing effect of 2**R**, 4**R**-APDC on DHPG-stimulated [^{3}H]-InsP_x accumulation in cerebral cortex slices

Incubation condition	*No addition*	*[^{3}H]-InsP_x accumulation (d.p.m. mg^{-1} protein) + 2**R**,4**R**-APDC*	*+ DHPG*	*+ DHPG/2**R**,4**R**-APDC*
LiCl omission				
+ LiCl	4114 ± 343	4800 ± 519	24269 ± 1568[b]	44103 ± 1833[b,d]
− LiCl	3887 ± 373	3753 ± 339	5976 ± 530[a]	8166 ± 473[a,c]
$[Ca^{2+}]_e$ omission				
Normal KHB	4236 ± 290	4515 ± 481	27374 ± 1703[b]	46026 ± 2300[b,d]
KHB − Ca^{2+}	4058 ± 240	4189 ± 398	15178 ± 936[b]	31182 ± 1922[b,d]

Neonatal rat slices were prepared from cerebral cortex and incubated with [^{3}H]-inositol (1.67 μCi ml^{-1}) for 60 min. As indicated 25 µl gravity-packed slices were dispensed into KHB ± LiCl (final concentration 5 mM) or washed extensively in nominally Ca^{2+}-free KHB before dispensing into Ca^{2+}-free incubation medium. Slices were stimulated by addition of DHPG (100 μM) and 2**R**,4**R**-APDC (100 µM) alone or in combination. Incubations were terminated after 15 min. Samples were processed as described in the Methods section and values are presented as means ± s.e.mean for three (LiCl omission) or four ($[Ca^{2+}]_e$ omission) separate experiments performed in triplicate. Statistically significant differences from respective control values are shown as: [a]$P<0.05$; [b]$P<0.001$ and differences in the presence versus the absence of 2**R**,4**R**-APDC shown as [c]$P<0.05$; [d]$P<0.001$.

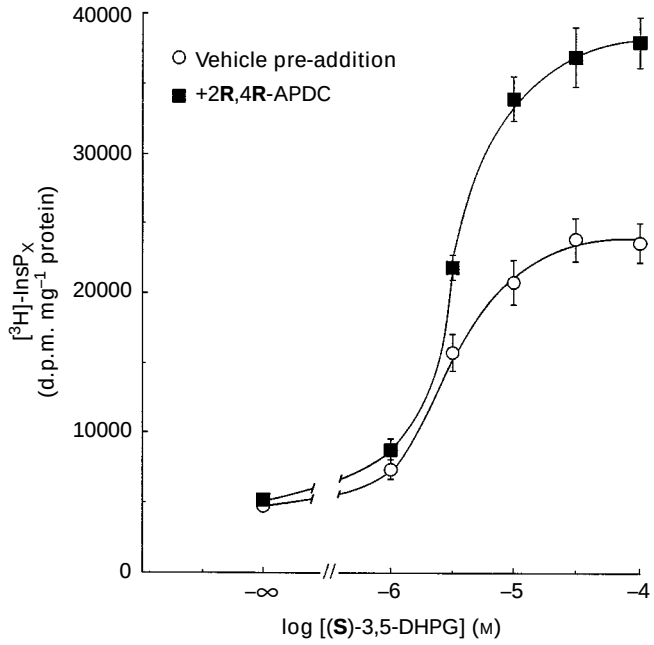

Figure 2 Concentration-dependent increases in DHPG-stimulated [^{3}H]-InsP_x accumulation in the absence and presence of 2**R**,4**R**-APDC. Neonatal rat cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60 min. LiCl was added (final concentration 5 mM) and slices stimulated by addition of the indicated concentrations of DHPG in the absence or presence of 2**R**,4**R**-APDC (100 μM) for 15 min. Samples were processed for analysis of [^{3}H]-InsP_x as described in the Methods section. Values are presented as means for 4 experiments performed in triplicate; vertical lines show s.e.mean.

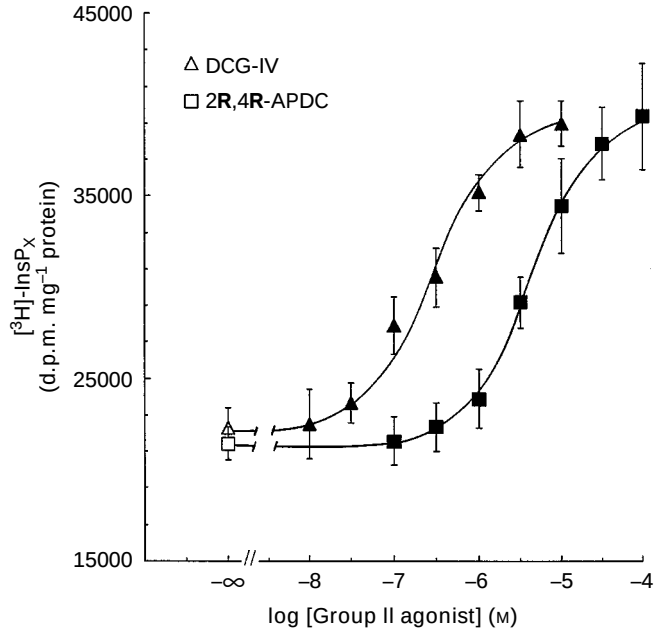

Figure 3 Concentration-dependent effects of DCG-IV and 2**R**,4**R**-APDC to enhance DHPG-stimulated [^{3}H]-InsP_x accumulation. Neonatal rat cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60 min. LiCl was added (final concentration 5 mM) and where DCG-IV was used MK-801 (5 μM) was also added (see text). Slices were stimulated by addition of DHPG (30 μM) in the absence or presence of the indicated concentrations of 2**R**,4**R**-APDC or DCG-IV for 15 min. Samples were processed for analysis of [^{3}H]-InsP_x as described in the Methods section. MK-801 had no effect *per se* on basal or DHPG-stimulated [^{3}H]-InsP_x responses. Values are presented as means for 4 (2**R**,4**R**-APDC) or 3 (DCG-IV) experiments performed in triplicate; vertical lines show s.e.mean.

Characterization of group I/II mGlu receptor interaction: Ins(1,4,5)P_3 mass response

We have also addressed whether the enhancement of DHPG-stimulated phosphoinositide hydrolysis by co-addition of a group II agonist is observed at the level of Ins(1,4,5)P_3 mass accumulation. Pre-addition of 2**R**,4**R**-APDC (100 μM) to cerebral cortex slices caused a small (19.8 ± 3.7%; $n=6$), but significant decrease in the basal Ins(1,4,5)P_3 level (basal, 41.5 ± 2.9; + 2**R**,4**R**-APDC, 33.2 ± 1.4 pmol mg^{-1} protein; $n=5$; $P<0.05$) which was evident within 5 min of 2**R**,4**R**-APDC addition and persisted for at least 25 min (see Figure 5). DHPG stimulated a rapid increase in Ins(1,4,5)P_3 accumulation which was approximately 4 fold greater than basal levels at 15 s (Figure 5). Despite the decrease in the basal Ins(1,4,5)P_3 level caused by 2**R**,4**R**-APDC, the accumulation of this second messenger was significantly increased by DHPG over the 5 min time-course studied (two-way ANOVA: $P=6.5\times10^{-5}$), in the presence, compared to the absence, of group II mGlu receptor co-activation (Figure 5). The peak Ins(1,4,5)P_3 accumulation caused by co-addition of DHPG and 2**R**,4**R**-APDC was not significantly different from that stimulated by a maximally-effective concentration (300 μM) of 1**S**,3**R**-ACPD (Ins(1,4,5)P_3 accumulation at 15 s: + DHPG/2**R**,4**R**-APDC, 180 ± 8; + 1**S**,3**R**-ACPD, 189 ± 18 pmol mg^{-1} protein; $n=3$). The concentration-dependency of the increase in Ins(1,4,5)P_3

(at 15 s) stimulated by DHPG (-log EC_{50} (M), 4.51 ± 0.04) was not affected by the co-addition of 2**R**,4**R**-APDC (100 μM) (-log EC_{50} (M), 4.57 ± 0.10; $n=3$). Thus, the group I/II mGlu receptor interaction appears to have similar characteristics whether assessed at the level of total phosphoinositide turnover or pathway second messenger generation, although the effects were more easily observed with respect to the former response.

Is the ability of group II mGlu receptor agonists to enhance group I mGlu receptor-stimulated phosphoinositide responses related to adenylyl cyclase inhibition?

It is possible that the inhibitory effect of group II mGlu receptor agonists on adenylyl cyclase activity (Figure 4) is linked in a causal manner to their ability to enhance phosphoinositide responses to group I mGlu receptor agonists (Figure 3). Evidence supporting an inhibitory effect of cyclic AMP elevation on receptor-mediated phosphoinositide turnover has been put forward for some neuronal systems (Akil & Fisher, 1989; Fowler & Tiger, 1991; Undie & Friedman, 1994). To address this possibility, the effect of manipulating cerebral cortex slice cyclic AMP concentration on the peak Ins(1,4,5)P_3 response to DHPG in the absence and presence of 2**R**,4**R**-APDC was studied. The peak Ins(1,4,5)P_3 response evoked by these agents was unchanged by either moderate or large increases in cyclic AMP levels stimulated by forskolin in the absence or presence of the PDE4 inhibitor rolipram (Table 3), strongly suggesting that the effects of 2**R**,4**R**-APDC on adenylyl cyclase activity are not causally linked to its modulatory effects on group I mGlu receptor-stimulated phosphoinositide turnover.

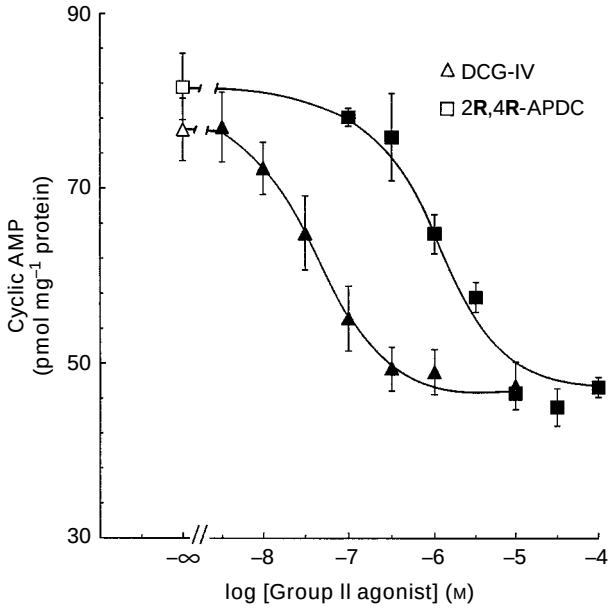

Figure 4 Concentration-dependent inhibitory effects of DCG-IV and 2**R**,4**R**-APDC on forskolin-stimulated cyclic AMP accumulation. Neonatal rat cerebral cortex slices were incubated for 60 min. For incubations involving DCG-IV, slices were pre-incubated with MK-801 (5 μM; see text) for 15 min before addition of the indicated concentrations of 2**R**,4**R**-APDC or DCG-IV. After a further 5 min forskolin (10 μM) was added and incubations terminated after 10 min. Samples were processed for analysis of cyclic AMP as described in the Methods section. MK-801 had no effect *per se* on basal or forskolin-stimulated cyclic AMP accumulation. Values are presented as means for 5 (2**R**,4**R**-APDC) or 3 (DCG-IV) experiments performed in triplicate; vertical lines show s.e.mean.

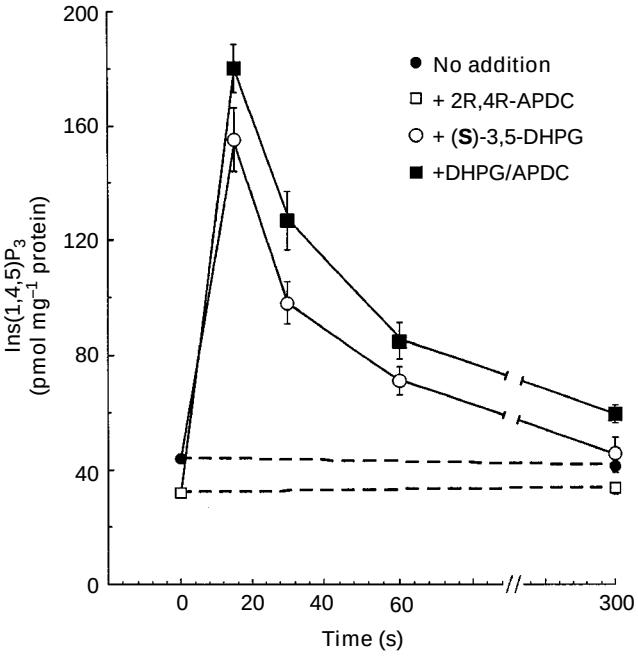

Figure 5 Time-course of DHPG-stimulated Ins(1,4,5)P_3 mass accumulation in the absence and presence of 2**R**,4**R**-APDC. Neonatal rat cerebral cortex slices were incubated for 60 min. Where indicated 2**R**,4**R**-APDC (100 μM) was added 10 min before challenge with DHPG (100 μM) or vehicle. Incubations were terminated at the indicated times and processed for analysis of Ins(1,4,5)P_3 as described in the Methods section. Values are presented as means for 4 experiments performed in triplicate; vertical lines show s.e.mean.

Table 3 Effect of varying cyclic AMP levels on DHPG-stimulated Ins(1,4,5)P_3 mass accumulation in the absence or presence of 2**R**,4**R**-APDC

±2**R**,4**R**-APDC (100 μM)	*Control*	*No addition*	*+forskolin*	*+forskolin/rolipram*
Cyclic AMP (pmol mg^{-1} protein)				
–	7.3±1.1	10.3±3.1	84.8±4.6	846±98
+	6.9±1.7	7.2±2.7	51.8±11.0	501±42
Ins(1,4,5)P_3 (pmol mg^{-1} protein)				
–	44.0±2.0	155.2±7.6	150.0±8.5	156.4±10.4
+	33.8±2.4	187.1±7.4	181.1±11.7	184.6±8.1

Neonatal rat cerebral cortex slices were prepared and incubated for 60 min. 2**R**,4**R**-APDC (100 μM) or vehicle was added 5 min before addition of forskolin (10 μM), forskolin + rolipram (10 μM), or vehicle. After a further 5 min DHPG (100 μM) was added for 15 s before the incubation was terminated with 1 M trichloroacetic acid. Samples were processed as described in the Methods section for measurement of cyclic AMP and Ins(1,4,5)P_3. Values are presented as means±s.e.mean for 3 separate experiments performed in triplicate.

*Effects of a selective group II mGlu receptor antagonist on the modulatory actions of 2**R**,4**R**-APDC*

The ability of the group II mGlu receptor-selective antagonist LY307452 (Wermuth *et al*., 1996; Schoepp *et al*., 1996b) to inhibit the modulatory effects of 2**R**,4**R**-APDC on adenylyl cyclase and phosphoinositide turnover was also investigated. In the presence of 30 μM LY307452 the concentration-effect curve for 2**R**,4**R**-APDC inhibition of forskolin-stimulated adenylyl cyclase was shifted in a parallel manner (Figure 6). At this antagonist concentration a 10.2 ± 2.1 fold ($n=3$) rightward shift was observed allowing a K_d for LY307452 of 3.7 ± 1.1 μM to be calculated. Similarly, the presence of LY307452 (30 μM) also caused a parallel rightward shift of the 2**R**,4**R**-APDC concentration-effect curve for enhancement of DHPG-stimulated [^{3}H]-InsP_x accumulation (Figure 7). In this case an 8.4 ± 0.9 fold ($n=3$) shift was observed generating a K_d for LY307452 of 4.1 ± 0.4 μM with respect to this response. Higher concentrations of LY307452 (300 μM) also had no effect on the DHPG-stimulated [^{3}H]-InsP_x response, demonstrating the selectivity of this antagonist for group II compared to group I mGlu receptors.

Receptor specificity of the modulatory action of group II mGlu receptor agonists

The ability of 2**R**,4**R**-APDC to enhance the [^{3}H]-InsP_x response stimulated by a different class of G protein-coupled receptor was assessed, to establish whether this modulatory action was a general phenomenon for all receptors which preferentially link to phosphoinositide turnover. To do this we compared the ability of 2**R**,4**R**-APDC to enhance the [^{3}H]-InsP_x response stimulated by either DHPG or carbachol. Muscarinic receptor activation evokes a robust phosphoinositide response in the cerebral cortex preparation (Challiss *et al*., 1994a) and a sub-maximally effective concentration of carbachol was chosen to equalize the [^{3}H]-InsP_x responses to these agonists *per se* (Figure 8). Although 2**R**,4**R**-APDC dramatically enhanced the [^{3}H]-InsP_x response to DHPG, group II mGlu receptor agonism had no effect on the response to carbachol (Figure 8).

Discussion

The initial objective of this study was to evaluate whether group II mGlu receptors can modulate the ability of group I mGlu receptor-selective agonists to stimulate phosphoinositide hydrolysis in neonatal rat cerebral cortex. Previous studies have provided evidence for a positive modulatory interaction in hippocampus, whereby group II mGlu receptor activation potentiates phosphoinositide turnover stimulated by group I-selective agonists (Nicoletti *et al*., 1993; Genazzani *et al*., 1994; Schoepp *et al*., 1996a). Here we showed that in cerebral cortex, as in hippocampus, co-activation of group II mGlu receptors by 2**R**,4**R**-APDC increases the maximal [^{3}H]-InsP_x accumulation (by $\sim 100\%$) evoked by the group I mGlu receptor agonist DHPG. Despite the increase in the maximal response, the concentration-dependence for stimulation of phosphoinositide hydrolysis by DHPG was similar in the absence and presence of 2**R**,4**R**-APDC.

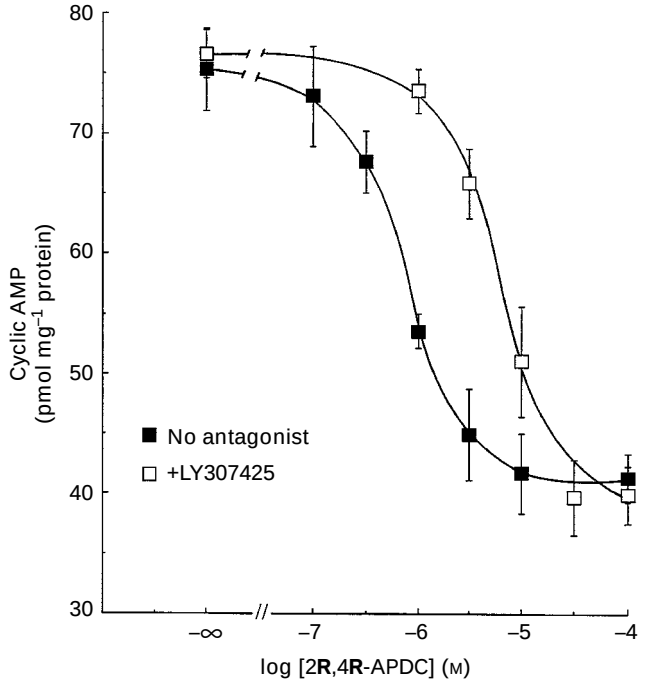

Figure 6 Effect of the group II mGlu receptor-selective antagonist LY307452 on the concentration-dependent inhibitory effect of 2**R**,4**R**-APDC on forskolin-stimulated cyclic AMP accumulation. Neonatal rat cerebral cortex slices were incubated for 60 min. Slices were then pre-incubated with LY307452 (30 μM) for 15 min before addition of the indicated concentrations of 2**R**,4**R**-APDC. After a further 5 min forskolin (10 μM) was added and incubations terminated after 10 min. Samples were processed for analysis of cyclic AMP as described in the Methods section. Due to inter-experimental variability in the forskolin-stimulated cyclic AMP levels, values are presented as means for a single representative experiment performed in triplicate; vertical lines show s.e.mean. Dose-ratio and K_d data presented in the text were obtained from this and 2 further experiments.

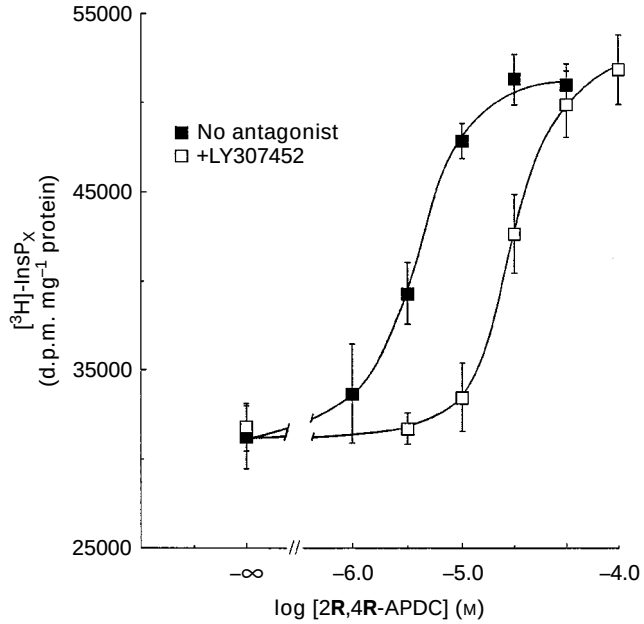

Figure 7 Effect of the group II mGlu receptor-selective antagonist LY307452 on the enhancement of DHPG-stimulated [^{3}H]-InsP_x accumulation by 2**R**,4**R**-APDC. Neonatal rat cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) for 60Dmin. LiCl was added (final concentration 5 mM) and slices were pre-incubated with LY307452 (30 μM) for 15 min before addition of DHPG (30 μM) in the absence or presence of the indicated concentrations of 2**R**,4**R**-APDC for 15 min. Samples were processed for analysis of [^{3}H]-InsP_x as described in the Methods section. Values are presented as means for 3 experiments performed in triplicate; vertical lines show s.e.mean.

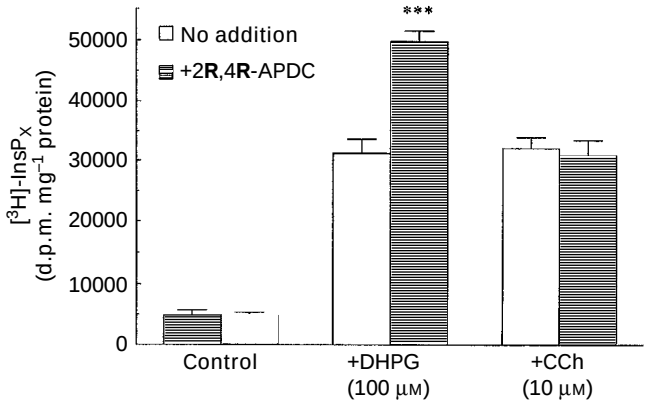

Figure 8 Contrasting effects of 2**R**,4**R**-APDC on the DHPG- and carbachol-stimulated [^{3}H]-InsP_x responses. Neonatal rat cerebral cortex slices were incubated with [^{3}H]-inositol (0.5 μCi/vial) and LiCl (final concentration 5 mM) added after 60 min. 2**R**,4**R**-APDC (100 μM) or vehicle was added to slices 10 min before challenge with DHPG (100 μM) or carbachol (CCh; 10 μM) for 15 min. Samples were processed for analysis of [^{3}H]-InsP_x as described in the Methods section. Values are presented as means s.e.mean for 3 experiments performed in triplicate. The statistically significant difference in the presence versus the absence of 2**R**,4**R**-APDC is shown as ***$P<0.001$.

We also demonstrated for the first time that group II mGlu receptor activation causes a small decrease in basal levels of Ins(1,4,5)P_3, whilst the time-course of the Ins(1,4,5)P_3 response to group I/II co-activation was significantly enhanced relative to that seen following addition of group I mGlu receptor agonist alone. Again the effect was observed as an increase in the maximal responsiveness with no change in the concentration-dependence for stimulation of Ins(1,4,5)P_3 accumulation by DHPG. The magnitude of the group II mGlu receptor-induced enhancement was more modest with respect to the Ins(1,4,5)P_3 mass compared to the total [^{3}H]-InsP_x response to DHPG. This difference is probably reflective of the fact that whilst in the presence of Li^+ [^{3}H]-InsP_x accumulates as a pseudo-endproduct, Ins(1,4,5)P_3 mass is a much more dynamic measurement with the level of the second messenger being determined by the relative rates of synthesis and breakdown of Ins(1,4,5)P_3.

The effect of another group II-selective mGlu receptor agonist upon DHPG-stimulated [^{3}H]-InsP_x accumulation was also evaluated. In the presence of the noncompetitive NMDA-receptor blocker MK-801 to eliminate non-group II mGlu receptor actions of this agent (Wilsch *et al*., 1994; Uyama *et al*., 1997), DCG-IV caused essentially identical effects to 2**R**,4**R**-APDC with respect to both enhancement of DHPG-stimulated [^{3}H]-InsP_x accumulation and inhibition of forskolin-stimulated cyclic AMP accumulation. In contrast, the action of 2**R**,4**R**-APDC on DHPG-stimulated [^{3}H]-InsP_x accumulation was unaffected by MK-801, suggesting that although this agonist is 15–30 fold less potent than DCG-IV with respect to group II mGlu receptors, it has the advantage of being devoid of NMDA-receptor agonist activity.

In addition to the use of group-selective mGlu receptor agonists, the putative group II-selective antagonist LY307452 (Wermuth *et al*., 1996) was also utilized. This agent, at a concentration of $\leqslant 300$ μM, did not inhibit forskolin-stimulated cyclic AMP accumulation, or have any effect on DHPG-stimulated [^{3}H]-InsP_x accumulation. However, it potently inhibited the actions of 2**R**,4**R**-APDC on each process yielding K_d values of 3.7 and 4.1 μM respectively. Thus, LY307452 is a useful tool for discriminating between group I and II mGlu receptor-mediated actions and as such joins a growing number of group-selective antagonists available for pharmacologically dissecting mGlu receptor activities (Hayashi *et al*., 1994; Bedingfield *et al*., 1996; Thomsen *et al*., 1996).

The enhancement of group I mGlu receptor-mediated phosphoinositide hydrolysis by 2**R**,4**R**-APDC occurred irrespective of the absence or presence of $[Ca^{2+}]_e$, and did not appear to be related to the ability of the group II mGlu receptor agonist to inhibit adenylyl cyclase activity. In contrast to the enhancement of group I-stimulated cyclic AMP accumulation by group II mGlu receptors (Schoepp *et al*., 1996a), which appears to be mediated indirectly via changes in endogenous adenosine and consequent activation of adenosine receptors linked positively to adenylyl cyclase, the enhancement of the phosphoinositide response mediated by co-activation of group I/II mGlu receptors was unaltered by addition of the adenosine metabolizing enzyme adenosine deaminase, or the adenosine receptor antagonist 8-phenyltheophylline (data not shown). Furthermore, the enhancement of receptor-mediated phosphoinositide turnover by group II mGlu receptor agonists appears to be dependent on the nature of the phospholipase C-linked receptor. Thus, whilst a robust enhancement of phosphoinositide hydrolysis is seen when group I mGlu receptors are activated, muscarinic acetylcholine receptor-mediated phosphoinositide hydrolysis is similar in the absence and presence of 2**R**,4**R**-APDC.

Much of the data presented here lend support to the proposition that group I/II mGlu receptor modulation of phosphoinositide responses occurs via a direct mechanism and, at least to some extent, the types 1/5, and 2/3 mGlu receptor populations must therefore be co-localized within cells in the cerebral cortical slice preparation. With respect to group I mGlu receptors, developmental studies suggest that the types 1β and 5a mGlu receptor splice variants are the major species, present in cerebral cortex during the postnatal period relevant to the present study (Minakami *et al*., 1995; Casabona *et al*., 1997), and the use of a putative mGlu1 receptor-selective antagonist, cyclopropan[b]chromen-1a-carboxylic acid ethylester, has provided evidence for the mGlu5 receptor subtype being responsible for phosphoinositide turnover in brain slice preparations from neonatal rats (Casabona *et al*., 1997). Less is known about the expression of group II mGlu receptors, although Northern blot analysis (Tanabe *et al*., 1992) and *in situ* hybridization (Tanabe *et al*., 1993; Testa *et al*., 1994) has provided evidence that both mGlu2 and mGlu3 receptors are expressed in rat cerebral cortex. Although group I and II mGlu receptors are co-expressed in cerebral cortex, evidence is lacking to demonstrate expression within the same cellular compartment. Immunocytochemical studies have shown predominant pre- and postsynaptic localizations of mGlu2 and mGlu3 (Ohishi *et al*., 1994), and mGlu1 and 5 (Martin *et al*., 1992; Shigemoto *et al*., 1993) respectively. However, this differential localization is likely to be far from absolute (Fotuhi *et al*., 1993; Prézeau *et al*., 1994; Neki *et al*., 1996b) except in one or two specific cases; for example, mGlu5 and mGlu2 receptors are expressed in non-overlapping populations of Golgi cells in the cerebellum (Neki *et al*., 1996a). Thus, it is likely that specific sub-populations of neurones (and/or glia) express groups I and II mGlu receptors within the same compartment allowing the observed crosstalk to occur.

The mechanism whereby mGlu2 and/or 3 receptors cause an enhancement of mGlu1- and/or mGlu5 receptor-mediated phosphoinositide signalling in neonatal rat cerebral cortex remains a matter of speculation. One possible mechanism which has been put forward (Schoepp *et al*., 1996b) involves the potential differential roles of Gα and G$\beta\gamma$ generated

following activation of $G_{i/o}$-linked receptors such as mGlu2/3 receptors (Clapham & Neer, 1997; Singer *et al*., 1997). Thus, $\beta\gamma$-subunits released by $G_{i/o}$ activation, as well as $G_{q/11}\alpha$-subunits generated by other receptor subtypes, can activate phospholipase C-β isoenzymes (Wu *et al*., 1993; Smrcka & Sternweis, 1993; Singer *et al*., 1997). Although this is an attractive hypothesis, the present data demonstrate that co-activation of group II mGluRs increases the efficacy, without affecting the potency, of DHPG. Such an effect is not entirely consistent with a $G\beta\gamma$ involvement in the group II mGlu receptor mechanism as $G_{q/11}\alpha$ and $G\beta\gamma$ interact with distinct sites on phospholipase C-β to exert independent and additive effects (Wu *et al*., 1993; Smrcka & Sternweis, 1993). If this is the case, then stimulation of group II mGlu receptors *per se* might be expected to cause activation of the phosphoinositide response, an effect clearly not seen here. Irrespective of the precise mechanism by which group I/II mGlu receptor interactions occur to regulate phosphoinositide hydrolysis, it is possible that such crosstalk will have an important bearing on our interpretation of how multiple mGlu receptor subtypes may interact to exert their actions on physiological and pathophysiological processes, where an mGlu receptor involvement has been implicated (Nakanishi, 1994; Conn & Pin, 1997).

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