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Pharmacological characterization of metabotropic glutamate receptors coupled to phospholipase D in the rat hippocampus

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1 Phospholipase D (PLD) is the key enzyme in a signal transduction pathway leading to the formation of the second messengers phosphatidic acid and diacylglycerol. In order to define the pharmacological profile of PLD-coupled metabotropic glutamate receptors (mGluRs), PLD activity was measured in slices of adult rat brain in the presence of mGluR agonists or antagonists. Activation of the phospholipase C (PLC) pathway by the same agents was also examined.

2 The mGluR-selective agonist (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3**R**)-ACPD] induced a concentration-dependent (10-300 μM) activation of PLD in the hippocampus, neocortex, and striatum, but not in the cerebellum. The effect was particularly evident in hippocampal slices, which were thus used for all subsequent experiments.

3 The rank order of potencies for agonists stimulating the PLD response was: quisqualate > ibotenate > (2S,3S,4S)-α-(carboxycyclopropyl)-glycine > (1S,3**R**)-ACPD > L-cysteine sulphinic acid > L-aspartate > L-glutamate. L-(+)-2-Amino-4-phosphonobutyric acid and the ionotropic glutamate receptor agonists N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainate failed to activate PLD. (**RS**)-3,5-dihydroxyphenylglycine (100-300 μM), an agonist of mGluRs of the first group, stimulated PLC but inhibited the PLD response elicited by 100 μM (1S,3**R**)-ACPD.

4 (+)-α-Methyl-4-carboxyphenylglycine (0.1-1 mM), a competitive antagonist of mGluRs of the first and second group, elicited a significant PLD response. L-(+)-2-Amino-3-phosphonopropionic acid (1 mM), an antagonist of mGluRs of the first group, inhibited the 100 μM (1S,3**R**)-ACPD-induced PLC response but produced a robust stimulation of PLD.

5 12-*O*-Tetradecanoylphorbol 13-acetic acid and phorbol 12,13-dibutyrate (PDBu), activators of protein kinase C, at 1 μM had a stimulatory effect on mGluRs linked to PLD but depressed (1S,3**R**)-ACPD-induced phosphoinositide hydrolysis. The protein kinase C inhibitor, staurosporine (1 and 10 μM) reduced PLD activation induced by 1 μM PDBu but not by 100 μM (1S,3**R**)-ACPD.

6 Our results suggest that PLD-linked mGluRs in rat hippocampus may be distinct from any known mGluR subtype coupled to PLC or adenylyl cyclase. Moreover, they indicate that independent mGluRs coupled to the PLC and PLD pathways exist and that mGluR agonists can stimulate PLD through a PKC-independent mechanism.

Keywords: (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3**R**)-ACPD]; (**RS**)-3,5-dihydroxyphenylglycine (DHPG); L-(+)-amino-3-phosphonopropionic acid (L-AP3); metabotropic glutamate receptors (mGluRs); (+)-α-methyl-4-carboxyphenylglycine [(+)-MCPG]; phosphatidylethanol; phospholipase C; phospholipase D; protein kinase C

Introduction

Metabotropic glutamate receptors (mGluRs) are coupled to a variety of second-messenger systems via GTP-binding proteins (G-proteins) (Schoepp *et al.*, 1990; Conn *et al.*, 1994). Originally, and for several years, the term mGluR has been synonymous with the glutamate-mediated stimulation of phospholipase C (PLC), which leads to the formation of inositol phosphates (IP) and diacylglycerol (Sladeczek *et al.*, 1985; Nicoletti *et al.*, 1986). It is now clear that mGluRs have much greater molecular and functional diversity. For example, activation of mGluRs in mammalian brain slices or primary neuronal cultures has been shown to (i) inhibit forskolin-induced adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation (Prezeau *et al.*, 1992; Schoepp *et al.*, 1992), (ii) potentiate cyclic AMP responses to agonists of other receptors that are positively coupled to adenylyl cyclase (Alexander *et al.*, 1992; Winder & Conn, 1992), and (iii) increase arachidonic acid release when α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are coactivated (Dumuis *et al.*, 1990). Recently, it has also been demonstrated that the mGluR agonist (1S,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3**R**)-ACPD] is able to stimulate phospholipase D (PLD) activity in adult (Boss & Conn, 1992) and neonatal (Holler *et al.*, 1993) hippocampal slices. The excitatory amino acid L-cysteine sulphinic acid (L-CSA) has been proposed as an endogenous agonist of mGluRs linked to PLD (Boss *et al.*, 1994).

Several neurotransmitter and hormone receptors are coupled via G-proteins to activation of PLD, a key enzyme in a transduction pathway which has recently been implicated in the generation of second messengers (Billah & Anthes, 1990; Thompson *et al.*, 1991; Klein *et al.*, 1995). PLD catalyses the hydrolysis of phosphatidylcholine into choline and phosphatidic acid; the latter may directly affect a number of cellular events, or, alternatively, can be converted by a phosphatidic acid phosphoydrolase into diacylglycerol, which activates protein kinase C (PKC). Diacylglycerol can also be formed by phosphoinositide hydrolysis catalysed by PLC and many receptors, including mGluRs, have been shown to be coupled to both PLC and PLD. However, since phosphatidylcholine is the major phospholipid component of the membrane, PLD is expected to yield much higher levels of diacylglycerol than PLC.

To date, eight different mGluR clones have been isolated

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from rat cDNA libraries (termed mGluR1–mGluR8) and have been classified into three groups based upon similarities in their sequence homology, effector coupling in functional expression systems, and agonist selectivity (Nakanishi, 1992; Schoepp & Conn, 1993; Pin & Duvoisin, 1995). The first group comprises mGluR1 and mGluR5, which are coupled to phosphoinositide hydrolysis and display the following rank order for agonist potency: quisqualate > L-glutamate ⩾ ibotenate > (2S,3S,4S)-α-(carboxycyclopropyl)-glycine (L-CCG-I) > (1S,3R)-ACPD. Recently, (RS)-3,5-dihydroxyphenylglycine (DHPG) has been proposed as a rather selective agonist for mGluRs of this group (Schoepp *et al.*, 1994). In addition, mGluR1 is capable of stimulating the formation of cyclic AMP and arachidonic acid release in Chinese hamster ovary cells (Aramori & Nakanishi, 1992). The members of the second group (mGluR2 and mGluR3), as well as those of the third group (mGluR4, mGluR6, mGluR7, and mGluR8), inhibit the accumulation of cyclic AMP but, whereas the agonist rank order of potencies for mGluR2 and mGluR3 is 2-2,3-dicarboxycyclopropylglycine (DCG-IV) = L-CCG-I > L-glutamate ⩾ (1S,3R)-ACPD > ibotenate > quisqualate, the order for mGluRs of the third group is: L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) > L-serine-*O*-phosphate > L-glutamate > (1S,3R)-ACPD > quisqualate > ibotenate.

The aim of our study was to define whether the pharmacology of mGluRs coupled to PLD coincides with the profile of any known mGluR subtype linked to PLC or adenylyl cyclase. In addition, we investigated whether agonist-induced PLD activity is independent or dependent upon stimulation of other transduction pathways such as phosphoinositide hydrolysis or PKC activation. Part of this work has been reported in preliminary form (Pellegrini-Giampietro *et al.*, 1994).

Methods

Preparation of rat brain slices

Adult male Wistar rats (Nossan strain, Milan) weighing 180–200 g were used. After decapitation, brains were rapidly removed and the fronto-parietal cortices, striata, hippocampi or cerebella dissected and placed into chilled Krebs-bicarbonate buffer (in mM: NaCl 122, KCl 3.1, $MgSO_4$ 1.2, KH_2PO_4 0.4, $NaHCO_3$ 25, $CaCl_2$ 1.3 and glucose 10) gassed with 95% O_2/5% CO_2. Slices (350 μm thick) from different brain regions were prepared with a McIlwain tissue chopper and then placed in gassed Krebs-bicarbonate solution for 1 h at 37°C before use.

Determination of PLD activity

The experimental approach was based on the ability of PLD to catalyse transphosphatidylation between phosphatidylcholine and primary alcohols. Thus, in the presence of exogenously added ethanol, PLD preferentially transfers the alcohol rather than water to the phosphatidyl moiety of phosphatidylcholine, producing phosphatidylethanol (PEt) in place of phosphatidic acid. The formation of agonist-induced PEt is generally accepted as a useful marker for PLD activity in intact cells (Pai *et al.*, 1988; Llahi & Fain, 1992).

Membrane phospholipids were labelled by incubating the slices with [^{3}H]-glycerol (final concentration: 60 μCi ml^{-1}) for 2 h at 37°C. Slices were then rinsed thoroughly in freshly oxygenated buffer and transferred to test-tubes (two slices each) containing 500 μl of drug-containing buffer gently stirred at 37°C by bubbling 95% O_2/5% CO_2. Staurosporine was applied for 30 min and D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (+)-α-methyl-4-carboxyphenylglycine [(+)-MCPG] and DHPG were applied for 5 min before adding the agonists together with 170 mM ethanol; the reaction was then carried out for one additional hour. Preliminary time course experiments revealed that, in our conditions, a steady-state level of PEt formation was reached within 30 min and was stable for at least 2 h (see also Holler *et al.*, 1993). All experiments were run in triplicate; two control sets of triplicate samples were always included in which (i) only Krebs-bicarbonate buffer (background) and (ii) only buffer plus 170 mM ethanol (basal PLD activity) were present.

The reaction was stopped by the addition of 2 ml of ice cold chloroform/methanol/HCl (100:200:2). The organic and inorganic phases were separated by addition of 0.65 ml chloroform and 0.65 ml water and, after sonication (30 min), by low-speed centrifugation. Aliquots (1 ml) of the lower organic phase were dried under a stream of N_2, resuspended in 70 μl of chloroform, and spotted on heat-activated (3 h at 55°C) precoated silica gel 60A plates. Standard solutions (at 10 μg μl^{-1}) of PEt, phosphatidic acid, diacylglycerol, and phosphatidylcholine were also spotted onto the plates. [^{3}H]-PEt was separated from other phospholipids by thin layer chromatography using the upper phase of the solvent system, ethyl acetate/2,2,4-trimethyl pentane/acetic acid/water (12:5:1:10). Spots were visualized with iodine vapour and [^{3}H]-PEt was identified by comparison with the PEt standard. The region corresponding to [^{3}H]-PEt was scraped and counted by liquid scintillation spectrometry.

The formation of [^{3}H]-PEt for each individual sample was expressed as the percentage of radioactivity incorporated into the total lipids present in the organic phase. Since PEt can be formed only in the presence of ethanol (see below), the amount of label co-migrating with [^{3}H]-PEt in ethanol-free controls was considered as background and subtracted from the mean of each ethanol-containing triplet. Radioactivity present in ethanol-free (background) samples never exceeded 10% of the radioactivity present in ethanol-containing samples. After subtracting the background, basal [^{3}H]-PEt formation, expressed as [^{3}H]-PEt/[^{3}H]-total lipids × 10^4, was consistently about 12.0 ± 0.8 (see, for example, Table 1). Data are expressed as percentages of incorporation of label into [^{3}H]-PEt occurring under agonist-free (basal) conditions.

Synthesis of PEt

Phosphatidylcholine (final concentration: 500 μg ml^{-1}) was incubated for 30 min at 37°C in 0.1 M sodium acetate buffer, pH 5.6, containing 140 mM $CaCl_2$ and 10 unit ml^{-1} of PLD in the presence or the absence of 170 mM ethanol. Lipids were extracted and separated by thin layer chromatography as described above.

Determination of PLC activity

Agonist-induced phosphoinositide hydrolysis was assayed essentially as described (Pellegrini-Giampietro *et al.*, 1988). Briefly, hippocampal slices incubated for 2 h at 37°C with [^{3}H]-inositol (final concentration: 20 μCi ml^{-1}) were rinsed in freshly oxygenated buffer and then transferred to test-tubes (two slices each) with 500 μl of drug-containing buffer gently stirred at 37°C in the presence of 10 mM LiCl by bubbling 95% O_2/ 5% CO_2. L-(+)-2-Amino-3-phosphonopropionic acid (L-AP3) and the phorbol esters 12-*O*-tetradecanoylphorbol 13-acetic acid (TPA) or phorbol 12,13-dibutyrate (PDBu) were applied for 40 min before adding the agonists, which were then allowed to react for an additional 15 min. All experiments were run in triplicate. The reaction was stopped by adding 1.88 ml of ice cold chloroform/methanol (1:2). The phases were separated by adding 0.65 ml of chloroform and 0.65 ml of water and, after brief sonication, by low-speed centrifugation. The upper phase, containing the water soluble [^{3}H]-IPs (inositol monophosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate), was transferred to Dowex AG 1-X 8 (formate form, 100–200 mesh) anion exchange resin columns. After washing with water and eluting [^{3}H]-glycerophosphorylinositol with 16 ml of 5 mM sodium tetraborate, 60 mM ammonium formate, the three [^{3}H]-IPs were eluted together with 16 ml of 1 M ammonium formate, 0.1 M formic

acid. The d.p.m. of the corresponding fractions was determined by liquid scintillation spectrometry and the radioactivity present in [^{3}H]-IPs was normalized to radioactivity incorporated into [^{3}H]-glycero-phosphorylinositol. Data are expressed as percentages of incorporation of label into [^{3}H]-IPs occurring under agonist-free (basal) conditions.

Materials

(1S,3**R**)-ACPD, AMPA, L-AP3, L-AP4, D-AP5, L-CCG-I, CNQX, DHPG, ibotenate, kainate, (+)-MCPG, N-methyl-D-aspartate (NMDA), and quisqualate were purchased from Tocris Cookson (Bristol, UK). L-Aspartate, L-CSA, L-glutamate, PDBu, L-α-phosphatidylcholine (type III-B from bovine brain), PLD (type I from cabbage), staurosporine and TPA were from Sigma Chimica (Milan, Italy). The PEt standard was from Avanti Polar Lipids (Pelham, AL, U.S.A.), and the phosphatidic acid and diacylglycerol standards were from Sigma Chimica (Milan, Italy). [1,2,3-^{3}H]-glycerol (30–60 Ci mmol^{-1}) and *myo*-[2-^{3}H(N)]-inositol (10–25 Ci mmol^{-1}) were purchased from Du Pont/NEN (Milan, Italy). Dowex AG-1-X 8 anion exchange resin (100–200 mesh) was from Sigma Chimica (Milan, Italy) and precoated silica gel 60A (LK6D) plates were from Whatman.

Statistical analysis

Statistical significance of differences between results was evaluated by performing the analysis of variance (ANOVA) followed by Tukey's *w* test for multiple comparisons.

Results

Identification of PEt

As a measure of PLD activity, we investigated the accumulation of labelled PEt in the presence of ethanol in rat brain slices preloaded with [^{3}H]-glycerol. Thin layer chromatography of phospholipid standards demonstrated that PEt could be clearly separated in our solvent system from phosphatidic acid, diacylglycerol and major phospholipids such as phosphatidylcholine. Application of mGluR agonists to prelabelled rat brain slices or incubation of phosphatidylcholine with PLD in the presence of Ca^{2+} caused the appearance of a lipid (which was tritiated in the former case) that co-migrated with standard PEt. The formation of the non-radioactive lipid was totally dependent on the presence of ethanol in the medium, further confirming that it was indeed PEt.

Effects of mGluR agonists on PLD activity

The mGluR-selective agonist (1S,3**R**)-ACPD induced concentration-dependent increases in [^{3}H]-PEt formation in slices from various brain regions (Figure 1). The effect was particularly evident in the hippocampus, where the maximal response was twice the basal value, with an approximate EC_{50} of 30 μM. The response was also present in neocortical and striatal slices but not in the cerebellum (Figure 1). Thus, hippocampal slices were used in subsequent experiments to define the pharmacological profile of mGluRs coupled to PLD.

Other mGluR agonists (see Schoepp *et al.*, 1990), as well as the putative endogenous agonist L-CSA (Boss *et al.*, 1994), were tested on PLD activity in adult rat hippocampal slices (Figure 2). The formation of [^{3}H]-PEt was enhanced by a variety of mGluR agonists, and the rank order of potencies was: quisqualate > ibotenate > L-CCG-I > (1S,3**R**)-ACPD > L-CSA > L-aspartate > L-glutamate. L-AP4, the preferential agonist for mGluRs of the third group, was inactive up to 1 mM, and so were the ionotropic glutamate receptor agonists NMDA, AMPA and kainate (all at 100 μM) (data not shown). In addition, the PLD responses of L-CSA, L-aspartate or L-

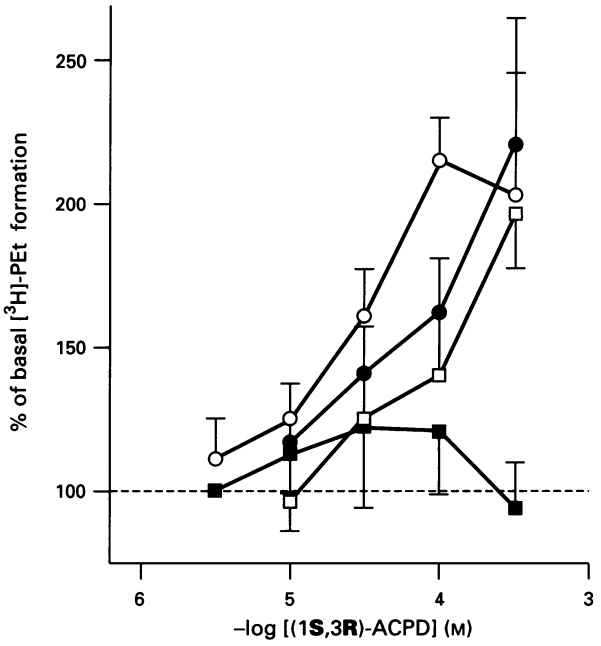

Figure 1 (1S,3**R**)-ACPD increases in a concentration-dependent manner the PLD-catalyzed formation of [^{3}H]-PEt in the hippocampus (○), neocortex (●) and striatum (□), but not in the cerebellum (■). Slices from various rat brain areas were labelled with [^{3}H]-glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of increasing concentrations of (1S,3**R**)-ACPD. PLD activity is expressed as the percentage of incorporation of label into [^{3}H]-PEt under (1S,3**R**)-ACPD-free (basal) conditions. Each point represents the mean ± s.e. mean of at least four experiments run in triplicate.

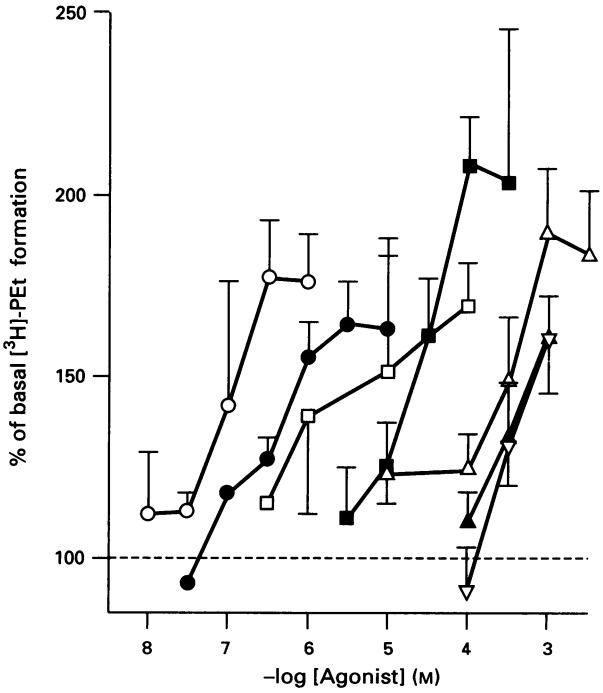

Figure 2 mGluR agonists increase the PLD-catalyzed formation of [^{3}H]-PEt in hippocampal slices. Slices were labelled with [^{3}H]-glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of increasing concentrations of agonists. PLD activity is expressed as indicated in Figure 1. Each point represents the mean ± s.e. mean of at least four experiments run in triplicate. (○) Quisqualate; (●) ibotenate; (□) L-CCG-I; (■) (1S,3**R**)-ACPD; (△) L-CSA; (▲) L-aspartate; (▽) L-glutamate.

glutamate were not affected when tested in the presence of the ionotropic glutamate receptor antagonists D-AP5 (100 μM) and CNQX (50 μM) (data not shown).

We then examined the effects of DHPG, a recently characterized selective agonist of mGluRs of the first group coupled to phosphoinositide hydrolysis, on both PLC and PLD activity. As described (Schoepp *et al.*, 1994), 100 μM DHPG promoted the formation of [^{3}H]-IPs from hippocampal slices, although it produced only about half the maximal response of 100 μM (1S,3R)-ACPD. The application of 100 μM (1S,3R)-ACPD plus 100 μM DHPG did not result in an additive effect (Figure 3). When tested on PLD activity, DHPG displayed totally different properties: it was virtually inactive as an agonist (up to 1 mM) but, at 100 and 300 μM, it inhibited the formation of [^{3}H]-PEt induced by 100 μM (1S,3R)-ACPD in a concentration-dependent manner (Figure 3).

Effects of mGluR antagonists on agonist-stimulated PLD activity

Among the mGluR antagonists developed to date, (+)-MCPG has proved to be a competitive antagonist acting at a number of subtypes (Watkins & Collingridge, 1994; Roberts, 1995). In hippocampal slices, (+)-MCPG (0.1 – 1 mM) stimulated the formation of [^{3}H]-PEt (Figure 4), while it displayed no agonist effect on phosphoinositide hydrolysis (data not shown). The increase in PLD activity induced by (+)-MCPG was rather weak as compared to 100 μM (1S,3R)-ACPD, but was significant at the same concentration (1 mM) that antagonizes the agonist-induced formation of [^{3}H]-IPs (Eaton *et al.*, 1993). (+)-MCPG at 1 mM was also able to inhibit significantly the PLD response elicited by 100 μM (1S,3R)-ACPD (Figure 4).

L-AP3 has been shown to act as a weak partial agonist or antagonist on mGluRs linked to phosphoinositide hydrolysis or accumulation of cyclic AMP (Schoepp & Conn, 1993). In our experimental conditions, when hippocampal slices were pre-incubated with 1 mM L-AP3 for 40 min, the formation of [^{3}H]-IPs induced by 100 μM (1S,3R)-ACPD was significantly reduced (Figure 5). L-AP3 alone had no effect on phosphoinositide hydrolysis. On the contrary, the same concentration of L-AP3 produced a robust stimulation of PLD; the response was as great as the maximal response obtained with (1S,3R)-ACPD (Figure 5). The effects of (1S,3R)-ACPD and L-AP3 on the formation of [^{3}H]-PEt were only partially additive (not significant vs. (1S,3R)-ACPD or L-AP3 alone).

Effects of PKC activators and inhibitors on PLD activity

Since PKC has been demonstrated to play a regulatory role in the function of mGluRs (Canonico *et al.*, 1988; Schoepp & Johnson, 1988; Aramori & Nakanishi, 1992), we compared the effects of the phorbol esters TPA and PDBu, activators of PKC, on the PLC and PLD cascades in hippocampal slices. Whereas TPA or PDBu alone (1 μM) did not alter the basal formation of [^{3}H]-IPs, pretreatment of slices with phorbol esters inhibited the stimulation of PLC in response to (1S,3R)-ACPD: the reduction induced by TPA was modest (by 15%, non-significant) but the inhibition induced by PDBu was much larger (by 64%, $P<0.05$) (Figure 6). In contrast, the formation of [^{3}H]-PEt was enhanced by phorbol esters. Again, the effect of PDBu (about seven fold the basal levels) was much more pronounced than that observed with TPA, which was comparable to the PLD response induced by 100 μM (1S,3R)-ACPD (Figure 6). In addition, the stimulatory effects of TPA and (1S,3R)-ACPD on PLD activity were additive when assayed together (Figure 6).

In order to determine whether activation of PLD may be a consequence of PKC stimulation, we also investigated the effect of 1 and 10 μM staurosporine, an inhibitor of PKC, on the formation of [^{3}H]-PEt induced by (1S,3R)-ACPD and PDBu in hippocampal slices. Although staurosporine by itself was able to stimulate PLD activity at the higher concentration, preincubation with the PKC inhibitor strongly depressed the effect induced by 1 μM PDBu but failed to affect (1S,3R)-ACPD-stimulated PLD activity (Table 1).

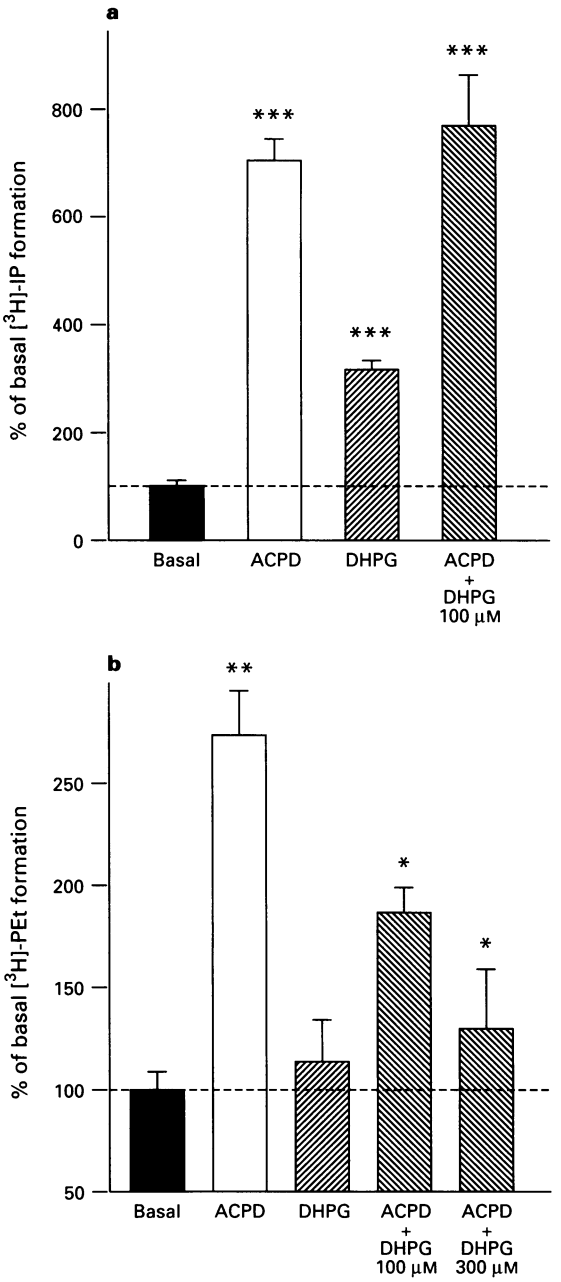

Figure 3 DHPG stimulates the formation of [^{3}H]-IPs but reduces the (1S,3R)-ACPD-induced formation of [^{3}H]-PEt in hippocampal slices. (a) Slices were labelled with [^{3}H]-inositol, washed, and incubated for 15 min at 37°C in buffer containing 100 μM (1S,3R)-ACPD and/or 100 μM DHPG. PLC activity is expressed as percentage of incorporation of label into [^{3}H]-IPs under agonist-free (basal) conditions. Each column represents the mean ± s.e. mean of four experiments run in triplicate. ***$P<0.001$ vs. basal. (b) Slices were labelled with [^{3}H]-glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of 100 μM (1S,3R)-ACPD or 100 μM DHPG. When assayed together, 100 or 300 μM DHPG was applied 5 min before adding 100 μM (1S,3R)-ACPD and ethanol. PLD activity is expressed as indicated in Figure 1. Each column represents the mean ± s.e.mean of at least four experiments run in triplicate. **$P<0.01$ vs. basal, *$P<0.05$ vs. (1S,3R)-ACPD alone.

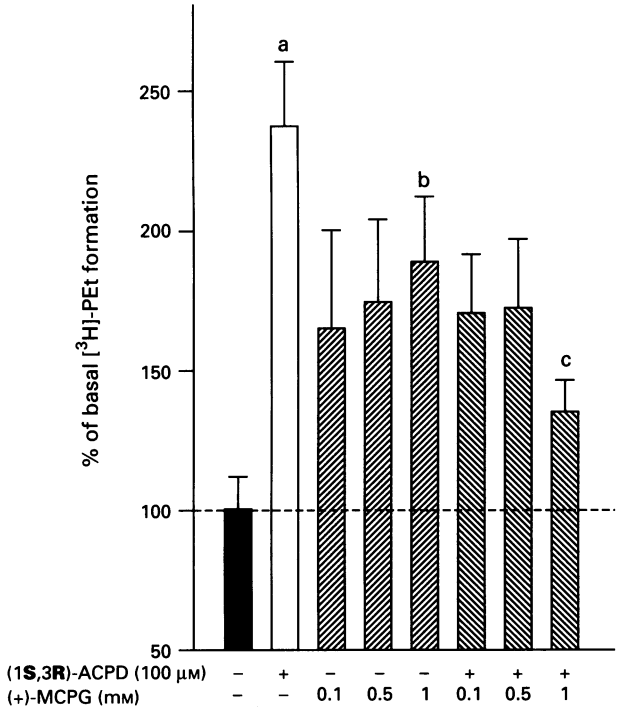

Figure 4 (+)-MCPG stimulates the formation of [^{3}H]-PEt and reduces the PLD response induced by (1S,3**R**)-ACPD in hippocampal slices. Slices were labelled with [^{3}H]-glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of 100 μM (1S,3**R**)-ACPD or increasing concentrations of (+)-MCPG. When assayed together, (+)-MCPG was applied 5 min before adding (1S,3**R**)-ACPD and ethanol. PLD activity is expressed as indicated in Figure 1. Each column represents the mean ± s.e.mean of at least four experiments run in triplicate. $^aP<0.01$ vs. basal, $^bP<0.05$ vs. basal, $^cP<0.05$ vs. (1S,3**R**)-ACPD alone.

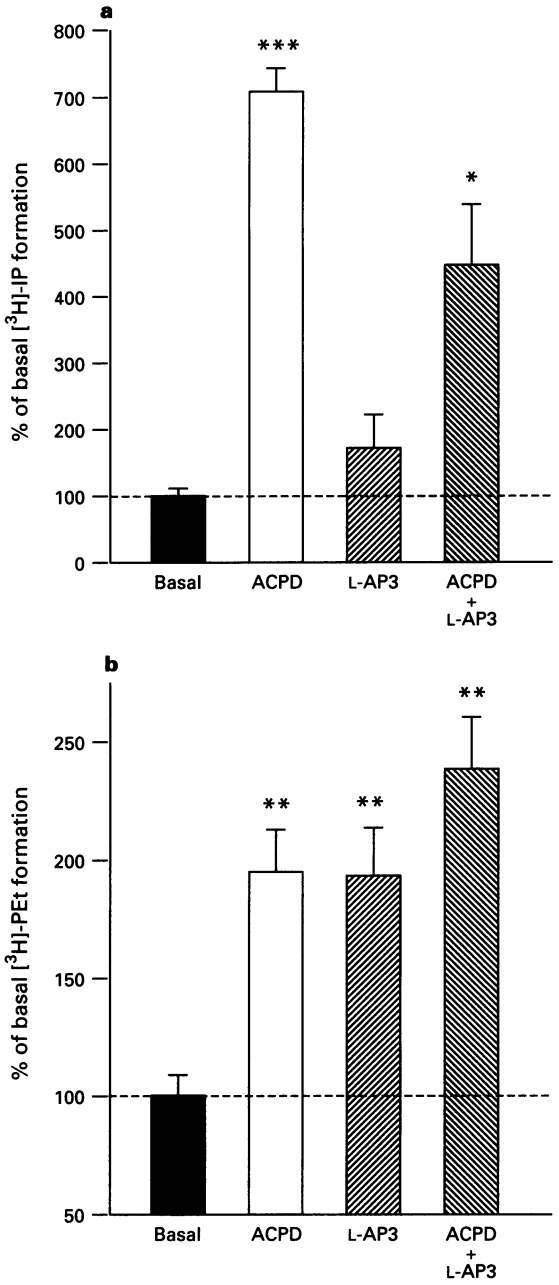

Figure 5 L-AP3 reduces the (1S,3**R**)-ACPD-induced formation of [^{3}H]-IPs but stimulates PLD activity in hippocampal slices. (a) Slices were labelled with [^{3}H]-inositol, washed, and incubated for 15 min at 37°C in buffer containing 100 μM (1S,3**R**)-ACPD or 1 mM L-AP3. When assayed together, 1 mM L-AP3 was applied 40 min before adding 100 μM (1S,3**R**)-ACPD. PLC activity is expressed as indicated in Figure 3. Each column represents the mean ± s.e.mean of four experiments run in triplicate. $^{***}P<0.001$ vs. basal, $^*P<0.05$ vs. (1S,3**R**)-ACPD alone. (b) Slices were labelled with [^{3}H]-glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of 100 μM (1S,3**R**)-ACPD and/or 1 mM L-AP3. PLD activity is expressed as indicated in Figure 1. Each column represents the mean ± s.e.mean of at least four experiments run in triplicate. $^{**}P<0.01$ vs. basal.

Discussion

Our results show that (1S,3**R**)-ACPD, a mGluR agonist acting on multiple subtypes, stimulates PLD activity in adult rat hippocampal, neocortical and striatal slices at concentrations having no effect on ionotropic glutamate receptors (Schoepp & Conn, 1993) or glutamate uptake systems (Robinson *et al.*, 1993). A previous study in hippocampal slices (Boss & Conn, 1992) revealed that this effect is stereoselective, since (1S,3**R**)-ACPD and (1S,3S)-ACPD but not (1**R**,3S)-ACPD are able to generate a PLD response. Other typical mGluR agonists, such as quisqualate, ibotenate, and L-CCG-I were also able effectively to induce the formation of [^{3}H]-PEt in the hippocampus, and so was L-AP3, which is a weak partial agonist or antagonist of mGluRs linked to phosphoinositide hydrolysis (Schoepp & Conn, 1993) but an agonist of mGluRs negatively coupled to adenylyl cyclase (Schoepp & Johnson, 1993). In addition, ionotropic glutamate receptor agonists or antagonists failed to affect PLD activity (see also Boss & Conn, 1992; Holler *et al.*, 1993;), thus indicating that the PLD-coupled receptors activated by (1S,3**R**)-ACPD should be regarded as mGluRs. (1S,3**R**)-ACPD was unable to activate PLD in cerebellar slices and similar results were obtained in cultured cerebellar granule cells (unpublished results), suggesting that PLD-coupled mGluRs are present in the hippocampus, neocortex and striatum but not in the cerebellum.

The issue of whether synaptically released L-glutamate, L-aspartate, or other putative neurotransmitters activate these receptors is controversial. Holler *et al.* (1993) reported that 1 mM L-glutamate stimulates PLD in hippocampal slices from adult rats, but only in the presence of a glutamate uptake blocker (L-aspartate-β-hydroxamate). Moreover, Boss *et al.* (1994) observed that, whereas L-CSA at 1 mM increases PLD activity in the same preparation, neither L-aspartate at 1 mM nor L-glutamate at 10 mM and in the presence of another uptake blocker (*trans*-pyrrolidine-2,4-dicarboxylate) have any effect; hence, they proposed that L-CSA is more likely to be the primary endogenous agonist of the hippocampal PLD-coupled receptor than L-glutamate or L-aspartate. However, in both studies 1 mM L-glutamate alone induced a robust PLD response in hippocampal slices from newborn rats, suggesting

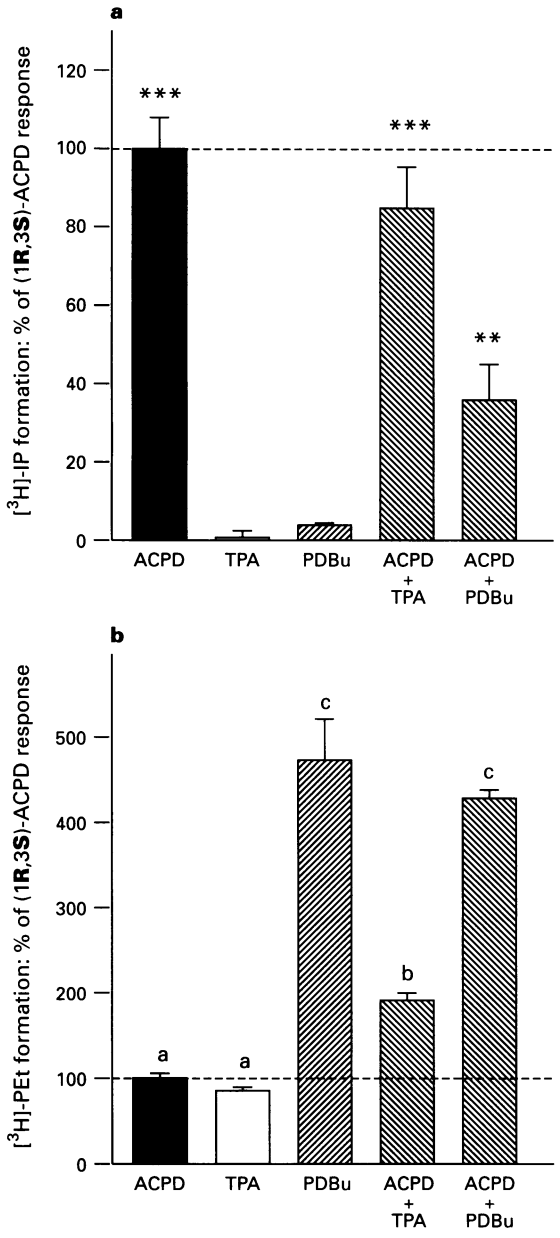

Figure 6 Differential effects of the phorbol esters TPA and PDBu on PLC and PLD activity in hippocampal slices. (a) Slices were labelled with [^{3}H]-inositol, washed, and incubated for 15 min at 37°C in buffer containing 100 μM (1S,3**R**)-ACPD, 1 μM TPA or 1 μM PDBu. When assayed together, 1 μM TPA or 1 μM PDBu were applied 40 min before adding 100 μM (1S,3**R**)-ACPD. PLC activity is expressed as percentage of 100 μM (1S,3**R**)-ACPD-induced [^{3}H]-IP formation. For experiments using TPA, (1S,3**R**)-ACPD-induced [^{3}H]-IP formation was 710±36% of basal formation; for those using PDBu, (1S,3**R**)-ACPD-induced [^{3}H]-IP formation was 650±75% of basal formation. Each column represents the mean±s.e.mean of four experiments run in triplicate. ***$P<0.001$ vs. basal; **$P<0.01$ vs. (1S,3**R**)-ACPD alone. (b) Slices were labelled with [^{3}H]-glycerol, washed, and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of 100 μM (1S,3**R**)-ACPD and/or TPA or PDBu (both at 1 μM). PLD activity is expressed as percentage of 100 μM (1S,3**R**)-ACPD-induced [^{3}H]-PEt formation. For experiments using TPA, (1S,3**R**)-ACPD-induced [^{3}H]-PEt formation was 154±8% of basal formation; for those using PDBu, (1S,3**R**)-ACPD-induced [^{3}H]-PEt formation was 229±21% of basal formation. Each column represents the mean±s.e.mean of at least four experiments run in triplicate. [a]$P<0.05$ vs. basal, [b]$P<0.01$ vs. basal and $P<0.05$ vs. (1S,3**R**)-ACPD or TPA alone, [c]$P<0.001$ vs. basal.

that maturation of uptake systems might conceal PLD activation by L-glutamate in adult tissue or that the expression of PLD-coupled receptors might be reduced during postnatal development. Our results demonstrate that not only L-CSA, but also L-aspartate and L-glutamate, induce a concentration-dependent accumulation of [^{3}H]-PEt in adult hippocampal slices in the absence of any uptake blocker. The lack of effects of added ionotropic glutamate receptor antagonists, as well as the fact that NMDA, AMPA and kainate failed to stimulate PLD activity, should rule out the possibility that L-CSA, L-aspartate or L-glutamate activate PLD indirectly via ionotropic glutamate receptors. The PLD response of L-CSA, L-aspartate or L-glutamate was seen at 300 μM and 1 mM and was approximately 75% of the maximal response observed with (1S,3**R**)-ACPD, which is consistent with the presence of uptake systems in neurones and glial cells in slices reducing the effective concentration of exogenously added amino acids (see Holler *et al.*, 1993). Taken together, these data suggest that L-CSA, L-aspartate and L-glutamate may act as endogenous agonists of mGluRs linked to stimulation of PLD activity. It is reasonable to propose that multiple PLD-coupled mGluRs may exist, perhaps at different developmental stages, bearing different affinities for their agonists. At present, however, it appears that additional studies are required before the hypothesis of Boss *et al.* (1994) can be fully supported, claiming that these receptors belong to a specific, L-glutamate-insensitive, receptor family.

One of the aims of our study was to determine whether the PLD-coupled mGluR(s) can be pharmacologically identified as any of the known mGluR subtypes, or can be at least classified into any of the three mGluR groups characterized to date. The rank order of potencies for agonists stimulating the PLD response in hippocampal slices was: quisqualate > ibotenate > L-CCG-I > (1S,3**R**)-ACPD > L-CSA > L-aspartate > L-glutamate. This pattern is similar to that reported by Boss *et al.* (1994); except for the fact that ibotenate is much weaker and, as discussed above, L-glutamate and L-aspartate are inactive in their system. In evaluating the potencies of PLD agonists it should be taken into account that some of these agents (such as quisqualate, L-CCG-I or L-AP3) have been demonstrated to interact with sodium-dependent L-[^{3}H]-glutamate uptake into synaptosomes (Robinson *et al.*, 1993), and thus may be substrates for carriers reducing their concentration at the receptor level. In any case, these rank orders do not appear to correspond to that of any specific mGluR subtype and, in particular, the insensitivity to L-AP4 clearly rules out the possibility that PLD-coupled receptors might be members of the third group. Nonetheless, some of our data could be interpreted as if mGluRs of the first and second group could promote PLD activity in hippocampal slices. Similarities to mGluRs of the first group, coupled to phosphoinositide hydrolysis, can be recognized by the facts that quisqualate is the most potent agonist and that PLD activity is evoked in the same regions (hippocampus, neocortex, striatum but not cerebellum) that have been demonstrated to express the mGluR5 subtype (Abe *et al.*, 1992; Shigemoto *et al.*, 1993). On the other hand, L-CCG-I and L-AP3 are effective agonists of PLD-coupled receptors, suggesting the involvement of mGluRs of the second group negatively coupled to adenylyl cyclase.

However, there are a number of observations suggesting that PLD-coupled mGluRs are not likely to be members of the first or the second group: (i) L-AP3 is an antagonist of responses mediated by mGluR1 (Aramori & Nakanishi, 1992; Ito *et al.*, 1992) and has no effect on those mediated by mGluR5 (Abe *et al.*, 1992) in functional expression systems. Conversely, L-AP3 is a relatively efficaceous agonist of mGluRs linked to PLD in hippocampal slices (see also Boss *et al.*, 1994). Although L-AP3 has been reported as an agonist of mGluRs mediating an inhibition of forskolin-stimulated cyclic AMP accumulation in adult rat hippocampus (Schoepp & Johnson, 1993), no effect of L-AP3 has been observed on functionally expressed mGluR2 or mGluR3 subtypes (see Suzdak *et al.*, 1994) (ii) L-CSA stimulates PLD activity but has no effect on phosphoinositide hydrolysis (Boss *et al.*, 1994). (iii) Phorbol esters attenuate agonist-stimulated phosphoinositide hydrolysis in mammalian cells expressing mGluR1

Table 1 Staurosporine reduces the stimulation of PLD activity induced by PDBu but does not affect the PLD response elicited by (1S,3R)-ACPD in hippocampal slices

	Control	*Staurosporine* (1 μM)	*Staurosporine* (10 μM)
Basal	12.2 ± 1.4	14.9 ± 0.7	25.1 ± 3.2[b]
(1S,3R)-ACPD (100 μM)	28.0 ± 3.1[b]	28.0 ± 4.8[a]	29.6 ± 6.1[a]
PDBu (1 μM)	86.6 ± 17.1[c]	58.4 ± 7.1[b]	35.3 ± 6.9[d]

Slices were labelled with [^{3}H]-glycerol, washed and incubated for 1 h at 37°C in buffer with 170 mM ethanol in the presence of 100 μM (1S,3R)-ACPD or 1 μM PDBu. Staurosporine (1 or 10 μM) was applied 30 min before adding (1S,3R)-ACPD or PDBu plus ethanol. PLD activity is expressed as [^{3}H]-PEt/[^{3}H]-total lipids × 10^4. Values are mean ± s.e.mean of at least four experiments run in triplicate. [a]$P<0.05$ vs. basal control; [b]$P<0.01$ vs. basal control; [c]$P<0.001$ vs. basal control; [d]$P<0.05$ vs. basal control and vs. PDBu control.

(Aramori & Nakanishi, 1992; Thomsen *et al.*, 1993) but display a stimulatory effect on [^{3}H]-PEt formation in our study. It should be noted, however, that the release of arachidonic acid is potentiated by phorbol esters in cells expressing mGluR1 (Aramori & Nakanishi, 1992), indicating that the possibility exists for a specific subtype to be linked to multiple transduction pathways that are differentially regulated by PKC. (iv) The antagonist pharmacology of PLD-linked mGluRs appears to be different from that of mGluR1/mGluR5 or mGluR2/mGluR3. (+)-MCPG has been demonstrated to be a competitive antagonist in mammalian cells expressing mGluR1 or mGluR2 (Hayashi *et al.*, 1994; Thomsen *et al.*, 1994), clearly devoid of any obvious agonist activity (Hayashi *et al.*, 1994), whereas it exhibits agonist as well as antagonist effects on PLD-coupled mGluRs. Finally, particularly striking and conclusive is the finding that, whereas DHPG is a potent agonist in functional systems expressing mGluR1 and mGluR5 (Ito *et al.*, 1992; Brabet *et al.*, 1995), it is an effective antagonist of mGluRs linked to PLD in hippocampal slices.

(1S,3R)-ACPD and other mGluR agonists may activate both PLC and PLD. In addition, activation of PKC by phorbol esters in our study produced stimulation of PLD in hippocampal slices (see also Holler *et al.*, 1991; Llahi & Fain, 1992). Thus, it is possible that mGluR agonist-induced activation of PLD may actually be secondary to phosphoinositide hydrolysis: activation of PLC may lead to diacylglycerol formation and PKC activation which, in turn, stimulates PLD. Our results, however, suggest that independent mGluRs coupled to the PLC and PLD pathways exist in hippocampal slices. In parallel experiments, L-AP3 was able to stimulate PLD but not PLC, whereas DHPG stimulated PLC but not PLD. In particular, the experiments with DHPG clearly show that activation of mGluRs coupled to phosphoinositide hydrolysis does not necessarily lead to the formation of [^{3}H]-PEt. Recently, a similar activation of PLD independent of phosphoinositide hydrolysis has been demonstrated in hippocampal slices (Holler *et al.*, 1994). This conclusion is further supported by the finding that the phorbol esters TPA and PDBu stimulate PLD but not PLC. In our study, PDBu inhibited mGluR-mediated phosphoinositide hydrolysis, in confirmation of previous studies (Canonico *et al.*, 1988; Schoepp & Johnson, 1988; Aramori & Nakanishi, 1992); thus, PKC activation may lead to desensitization (Catania *et al.*, 1991) and feedback inhibition (Ryu *et al.*, 1990) of PLC but to stimulation of PLD. The positive feedback loop between PKC and PLD may be important in generating increasing amounts of diacylglycerol in response to receptor stimulation (Löffelholz, 1989).

It has been demonstrated that noradrenaline and a phorbol ester activate PLD in rat cortical slices by two different mechanisms (Llahi & Fain, 1992). Similarly, two different mechanisms of activation of PLD are suggested by the additive effects on the formation of [^{3}H]-PEt induced by (1S,3R)-ACPD and TPA in hippocampal slices. PDBu did not display an additive effect when assayed together with (1S,3R)-ACPD presumably because, being more water-soluble than TPA, it attained a higher, close to maximal, degree of PLD activation, as well as a more effective inhibition of agonist-induced [^{3}H]-IP formation. Moreover, the PKC inhibitor, staurosporine, was able to inhibit the formation of [^{3}H]-PEt induced by PDBu but not by (1S,3R)-ACPD, indicating that the former mechanism is dependent and the latter independent of PKC activation. Emerging evidence indicates that receptor-mediated stimulation of PLD via the PKC-independent pathway might involve direct G-protein coupling between the receptor and PLD in the brain. Activation of monomeric and heterotrimeric G-proteins results in receptor-mediated stimulation of PLD in several neuronal cell lines (see Klein *et al.*, 1995), canine cortical synaptosomes (Qian & Drewes, 1989), and rat hippocampal slices (Holler *et al.*, 1994). In granulocytes, pre-incubation with pertussis toxin inhibits PLD activity induced by agonists (Pai *et al.*, 1988; Kanaho *et al.*, 1991) but not that induced by phorbol esters (Billah *et al.*, 1989). Similarly, PKC-independent activation of PLD by α_1-adrenoceptors in neocortex (Llahi & Fain, 1992) or by mGluRs in hippocampus (this study) could be, in part, a G-protein-mediated process.

In conclusion, we have demonstrated that the mGluR agonist (1S,3R)-ACPD evokes a PLD response in hippocampal, neocortical, and striatal, but not in cerebellar slices from adult rats. The receptors coupled to PLD in hippocampal slices possess many of the attributes of mGluRs but appear to be distinct from any known mGluR subtype linked to PLC or adenylyl cyclase. In addition, our results indicate that the PLC and PLD pathways are independently linked to distinct mGluRs and that (1S,3R)-ACPD may stimulate PLD through a PKC-independent mechanism. Elucidation of the physiological function of these mGluRs in the central nervous system awaits the development of selective antagonists or the molecular cloning of the PLD-coupled mGluR subtype(s). Nonetheless, it may already be predicted that mGluRs linked to PLD activation are likely to play a role in developmental plasticity, synaptogenesis, and in mediating long-term formation of diacylglycerol and activation of PKC.

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