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Activation of phospholipase D by metabotropic glutamate receptor agonists in rat cerebrocortical synaptosomes

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1 The pharmacological profile of metabotropic glutamate receptor (mGluR) activation of phospholipase D (PLD), and the associated signalling pathways, were examined in rat cerebrocortical synaptosomes. The assay was conducted using a transphosphatidylation reaction in synaptosomes which were pre-labelled with either [^{3}H]-arachidonic acid or [^{32}P]-orthophosphate.

2 The mGluR agonists (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) and (RS)-3,5-dihydroxyphenylglycine (DHPG), both activated PLD, while phorbol 12,13-dibutyrate (PDBu) treatment caused receptor-independent activation of PLD and had an additive effect on 1S,3R-ACPD induced PLD activity.

3 A protein kinase C (PKC) inhibitor, GF109203X, failed to antagonize mGluR receptor-coupled PLD activity. We could not detect any increase in the products of PI (phosphoinositide)-specific phospholipase C (PI-PLC), inositol(1,4,5)trisphosphate or diacylglycerol, by 1S, 3R-ACPD at 15 s. However, diacylglycerol increased monophasically in response to mGluR agonists and remained elevated for at least 15 min. Phosphatidic acid phosphohydrolase (PAP) activity, which converts PA to DAG, was present in the synaptosomes.

4 These data suggest that, in rat cerebrocortical synaptosomes, the 1S,3R-ACPD-sensitive mGluR is coupled to PLD through a mechanism that is independent of both PKC and PI-PLC.
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Abbreviations: 1S,3R-ACPD, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid; ADA, Adenosine Deaminase; BSA, bovine serum albumin; DAG, sn-1,2-diacylglycerol; DHPG, (RS)-3,5-Dihydroxyphenylglycine; IP_3, inositol (1,4,5) trisphosphate; L-AP3, L(+)-2-amino-3-phosphonopropionic acid; mGluRs, metabotropic glutamate receptors; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; PI, phosphoinositide; PIP_2, phosphatidylinositol (4,5) bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C; PLD, phospholipase D; TLC, thin layer chromatography; TPA, tetradecanoyl phorbol acetate

Introduction

The activation of PLD(s) in response to a variety of hormones, growth factors and neurotransmitters catalyzes the hydrolysis of phosphatidyl choline (PC), generating choline and phosphatidic acid (PA). PLD(s) are involved in a number of cellular functions which included protein trafficking and secretion (Jones *et al.*, 1999). The sequence and distribution of two PLD isoforms, PLD1 and PLD2, have been reported (Colley *et al.*, 1997; Exton, 1998) PLD1 has a low basal activity and can be regulated by PKC, PtdIns(4,5)P_2 and small GTP-binding proteins (Exton, 1998) and PLD2 has a high basal activity and can be activated by fatty acids (Kim *et al.*, 1999). The lipid product of PLD activity, PA, itself can induce a number of cellular functions and it can be converted by PA phosphohydrolase (PAP) into DAG, which is an activator of PKC (Chen *et al.*, 1997; Hodgkin *et al.*, 1998; Nishizuka, 1995). While it is possible that PLD activation requires a co-ordinated cross-talk between those signalling molecules, there is strong evidence that the activation of small G-proteins and PKC can occur independently of each other. Thus the specific mechanisms involved in the modulation of PLD activity remains unclear.

A number of neurotransmitters have now been described which activate PLD in preparations derived from distinct neuroanatomical locations. These include muscarinic stimulation of synaptosomes (Hattori & Kanfer, 1985; Qian & Drewes, 1989) and the activation of mGluRs in hippocampal and cortical slices (Boss & Conn, 1992; Holler *et al.*, 1993; Klein *et al.*, 1998; Pellegrini-Giampietro *et al.*, 1996). It has also been shown recently that elevated calcium levels and GTPγS application to permeabilized synaptosomes increased PLD activity (Sarri *et al.*, 1998) which suggests that calcium influx may be required for G-protein coupled receptors activation of PLD. The functional significance of this receptor-activated PLD remains unclear, although the developmental profile of the basal and receptor-activated PLD is consistent with a role in synaptogenesis (Klein *et al.*, 1997; 1998). Furthermore, the selective coupling between PLD and the stimulation of mGluR subtypes, together with a potential role for PLD in secretion (Williger *et al.*, 1999; Zheng *et al.*, 1997), may also indicate that PLD activation is involved in

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synaptic plasticity and may therefore play a role in the learning and memory process. Indeed it has been proposed that activation of PLD, which can occur secondary to PI-PLC activation, is a more sustainable signalling pathway when compared with PI turnover (Hodgkin *et al*., 1998; Klein *et al*., 1995; Nishizuka, 1995) and the distinct time scale of these signalling events suggests it may be involved in the process of long term potentiation. However, in order to ascribe the action of PLD to such a functional role in synaptic plasticity, the mechanisms controlling its activation, and the subtypes of mGluR involved, require clarification.

To date the pharmacological analysis of mGluR subtypes coupled to PLD has concluded that the receptor profiles do not conform to any of the characteristics of the known mGluRs, and, moreover, they were shown to function independently of PKC activation (Pellegrini-Giampietro *et al*., 1996). However, it is unknown whether these events occur at a pre- or post-synaptic location. Furthermore, the potential control of PLD at a presynaptic location would be of particular interest as it has recently been reported that synucleins (Jenco *et al*., 1998) and synaptojanin (Chung *et al*., 1997), proteins located abundantly at the presynaptic terminal, can act as endogenous PLD inhibitors, implying that PLD action at the presynaptic terminal is physiologically significant. The aim of our study was to examine the signal transduction pathway which links mGluRs to PLD in our presynaptic-rich rat cerebrocortical synaptosome preparation and to determine whether PKC activation, occurring secondary to PLC activation, is involved in the activation process.

Methods

Materials

[5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (specific radioactivity 209 Ci mol^{-1}), [γ-^{32}P]-orthophosphate were purchased from Amersham (Buckinghamshire, U.K.). [glycerol-^{14}C(U)]-L-alpha-dipalmitoyl phosphatidic acid (100–200 mCi mmol^{-1}), D-myo-[1,2-^{3}H] Ins(1,4,5)- P_3 (20–60 Ci mmol^{-1}) and En^{3}Hance were obtained from Dupont (Boston, MA, U.S.A.). Pre-coated TLC plates (Silica gel 60 F254, 20 × 10 cm) were from Merck GmbH (Darmstadt, Germany). PBut, PA and phophatidylserine were from Lipid Products (Surrey, U.K.). Percoll, sn-1-stearoyl-2-arachidonoylglycerol and PDBu were from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). DAG kinase (EC 2.7.1.107) was from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.). ADA (EC 3.5.4.4.) was from Boehringer Mannheim GmbH (Mannheim, Germany). Soluene was from Packard (Meriden, CT, U.S.A.). 1S,3R-ACPD, DHPG, and L-AP3 were from Tocris Cookson (Bristol, U.K.). GF109203X was from LC Laboratories (Nottingham, U.K.). Bio-Rad protein assay kit was from Bio-Rad (Hercules, CA, U.S.A.). All of the other reagents were of the highest quality grade and were purchased from Sigma or BDH (Poole, Dorset, U.K.).

Synaptosome preparation and fractionation

Synaptosomes from the cerebrocortices of 6–8-week-old Wistar rats were prepared on discontinuous Percoll gradients as described (Dunkley *et al*., 1986). In brief, the P2 pellet was gently resuspended in 320 mM sucrose, 0.5 mM EDTA, 5 mM TES (pH 7.4), and layered onto a three step gradient composed of 3, 10 and 23% Percoll, and centrifuged at 25,000 × *g* for 10 min. The interface between the 10 and 23% layers containing the synaptosomes was removed and added to 10 volumes of chilled HEPES-buffered medium, HBM (mM): NaCl 120, KCl 5, HEPES 20, $NaHCO_3$ 5, $MgCl_2$ 1, Na_2HPO_4 1.2 and glucose 10 (pH 7.4), then centrifuged at 25,000 × *g* for 10 min. The final synaptosomal pellet was resuspended in 1 ml HBM per rat and the protein content was determined using Bio-Rad protein assay kit.

Synaptosomes are regarded as intact isolated nerve terminals which retains the machinery necessary for neurotransmitter exocytosis and remain functionally competent for up to 6 h.

Activation of PLD in synaptosomes

PLD activity in synaptosomes was measured following the addition of exogenous butanol, in the presence of which PLD catalyses the transphosphatidylation reaction to give the stable end product, PBut (Morris *et al*., 1997). The PLD assay was based on the method previously described for lymphocytes, except that [^{3}H]-arachidonic acid or orthophosphate were used instead of oleic acid (Gargett *et al*., 1996).

Synaptosomes (1 mg ml^{-1}) were incubated at 37°C for 10 min in HBM with 0.1 % BSA (fatty acid free), spun down and washed twice with HBM. [^{3}H]-arachidonic acid (5 μCi ml^{-1}) or [^{32}P]-orthophosphate (1 mCi ml^{-1}) were used to label synaptosomes at 37°C for 60 min. Radiolabelled synaptosomes were then washed twice with HBM containing 0.1% BSA. Under these conditions, about 20% of the [^{3}H]-labelled arachidonic acid was incorporated into lipid extracts.

Aliquots of synaptosomes (200 μg for [^{3}H]-arachidonic acid labelling and 40 μg for [^{32}P]-orthophosphate) were incubated in the presence of 0.3% butanol with 1.3 mM $CaCl_2$ and 1 mU ml^{-1} ADA in a shaking water bath. The reaction was started by the addition of the stimulants and was terminated after the indicated time by the addition of 1 ml of ice-cold chloroform:methanol:conc. HCl (1 : 1 : 0.006, v v^{-1}). Lipids were extracted by the method of Bligh & Dyer (1959) and were dried under a stream of nitrogen. The lipid film was reconstituted in 20 μl of chloroform : methanol (19 : 1, v v^{-1}) and applied to heat activated (110°C, 30 min) TLC plates. Phospholipids were separated using the organic phase of ethyl acetate:iso-octane:acetic acid:water (26 : 4 : 6 : 20, v v^{-1}). PBut and PA spots were identified by including cold standards which were located by iodine vapour and autofluorography of EN^{3}-HANCE sprayed TLC plates. The [^{3}H]-PBut, [^{3}H]-PA and [^{3}H]-arachidonic acid spots were scraped into vials containing Soluene, and quantified by liquid scintillation counting. The basal activity was calculated as the difference between the presence and the absence of 0.3% butanol. The approximate Rf values obtained for sample spots of PA, PBut and arachidonic acid were 0.4, 0.6 and 0.9 respectively.

Diacylglycerol mass assay

Synaptosomes (1 mg protein ml^{-1}) were resuspended in HBM with 1.3 mM $CaCl_2$ and 1 mU ml^{-1} ADA and stimulations were started by adding the stimulant or vehicle. At the specified time points, samples of 0.08 mg synaptosomes were removed and the reaction was terminated by adding the samples to ice-cold chloroform in methanol solution. After lipid extraction the DAG mass assay was conducted as described (Paterson *et al*., 1991) with some slight modifications. Lipid samples (extracted from 0.02 mg protein per tube), or sn-1-stearoyl-2-arachidonoylglycerol standards (0–500 pmol), were incubated with DAG kinase (5 mU per tube) and 1 mM [γ-^{32}P]-ATP (specific activity = 10 Ci mol^{-1}) in a mixed-micelle preparation [6 mol% phophatidylserine in 0.3% Triton

X-100] in (mM): imidazole 50 (pH 6.6), NaCl 50, $MgCl_2$ 12.5 and EGTA 1.25 at 37°C for 60 min. At the end of the reaction, the resulting lipids were extracted by a modification of the method of Bligh & Dyer (1959). The lower chloroform phases were dried under nitrogen, dissolved in 20 μl of chloroform:-methanol (19 : 1, v v^{-1}) and resolved by TLC. The plates were developed with chloroform:methanol:acetic acid (38 : 9 : 4.5, v v^{-1}), air-dried, and lipid spots located by autoradiography. The radioactive spots corresponding to PA and ceramide phosphate were scraped and quantified by liquid scintillation counting. Other synaptosomal lipids extracted from 0.02 mg protein did not interfere with DAG kinase activity in the DAG range of 50–2000 pmol per assay (data not shown).

Competitive binding assay of IP_3

Synaptosomes remain functionally intact only for periods of up to 6 h (Nicholls, 1993). This life-span may not be sufficient to achieve equilibrium of labelling with [^{3}H]-inositol. In such conditions, the accumulation of inositol phosphates in the presence of lithium may not be an accurate measure of PI-PLC activation due to the uneven incorporation of radiolabel into different inositol lipid pools (Sillence & Downes, 1992) or agonist mediated changes in the rate of radiolabel incorporation (Challiss *et al.*, 1988). Therefore, we employed the mass IP_3 assay as a more reliable measurement of PLC activity in this preparation. Our procedure is based on that described by Palmer *et al.* (1989). The stimulation of synaptosomes was carried out as described for the DAG mass assay. The reaction was stopped by adding ice-cold trichloroacetic acid to give a final concentration of 5% and the samples were centrifuged to separate trichloroacetic acid-soluble and insoluble fractions. Trichloroacetic acid was removed from the water-soluble cellular fraction with H_2O-saturated diethyl-ether. A 20 μl aliquot of the extract, at the appropriate dilution, was incubated for 15 min at 4°C with 40 μl binding buffer consisting of 0.05 M Tris-HCl pH 9.0, 2 mg ml^{-1} BSA, 2 mM EDTA, 0.05 μCi [^{3}H]-IP_3 and 20 μl of 2 mg ml^{-1} IP_3-binding protein. After this period, isotopic binding equilibrium was attained, and unbound radio-label was removed by centrifugation and aspiration of supernatant. The resulting pellet was then resuspended in H_2O and [^{3}H]-IP_3 content determined by liquid scintillation counting.

PA phosphohydrolase assay

PA phosphohydrolase activity in lysed synaptosomes was assayed according to the method described with a slight modification (Balsinde & Dennis, 1996). The substrate [^{14}C]-phosphatidic acid was presented as mixed micells with Triton X-100 at a detergent:phospholipid mole ratio of 10 : 1. The incubation mixture contained in a final volume of 0.1 ml; 100 μM [^{14}C]-PA substrate (0.025 μCi per assay) (mM), Triton X-100 1, Tris-HCl 150, (pH 7.2), beta-mercaptoethanol 10, $MgCl_2$ 2, EDTA 1, EGTA 1 and 20 μg of lysed synaptosomes. After the indicated times the reaction was stopped, and [^{14}C]-PA and [^{14}C]-DAG were separated by TLC using the system chloroform:methanol:acetic acid (90 : 10 : 10 v v^{-1}). Radioactivity on TLC was measured by BAS 2000 system (Fujifilm, Japan).

Data presentation and analysis

The quantities of [^{3}H]-, [^{14}C]- and [^{32}P]-PBut were expressed as a percentage of total [^{3}H]-, [^{14}C]- and [^{32}P]-labelled phospholipids on each lane of the TLC plates. All the assays were performed in triplicate and data are expressed as mean $\pm$ s.e.mean. Statistical analysis of data was by Student's *t*-test or ANOVA with Tukey-Kramer tests, $P<0.05$ being taken as statistically significant.

Results

PLD activators in rat cerebrocortical synaptosomes

The time course profile of PLD stimulation by 1S,3R-ACPD, DHPG and PDBu was conducted using [^{3}H]-arachidonic labelled synaptosomes (Figure 1). The mGluR agonist, 1S,3R-ACPD is reported to activate mGluR1, mGluR2 and mGluR5, while DHPG, activates mGluR1 and mGluR5 only (Conn & Pin, 1997). By expressing the data as per cent of total lipid, a basal unstimulated PtdBut formation is evident which increased over the time course analysed. When the reaction was conducted for 15 min in the absence of butanol, contaminating radioactivity which co-migrated with the PtdBut standard was detected but was significantly lower than that obtained in the presence of butanol. The magnitude of stimulation by the mGluR agonists 1S,3R-ACPD, DHPG and PDBu relative to the basal control value was maximum at 15 min (1.26 ± 0.04, 1.20 ± 0.04 and 2.04 ± 0.07 respectively where $n=3-6$. The 15 min time point was therefore selected for optimal stimulation.

Since mGluR activation of PLD may occur *via* both PKC dependent and independent pathways (Pellegrini-Giampietro *et al.*, 1996), we investigated a potential additivity between the PKC and mGluR activation of PLD in the synaptosomal preparation. In [^{3}H]-arachidonic acid-labelled synaptosomes, the PDBu stimulation of PLD was increased from 1.74 ± 0.04 to 1.90 ± 0.06 fold when PDBu and 1S,3R-ACPD were combined (Figure 2A). The difference between the combination of stimuli versus the individual stimuli achieved statistical significance ($P<0.05$) with Student-Newman-Keuls tests, but did not ($P>0.05$) with the more stringent Tukey-Kramer multiple comparison tests. However, we confirmed this additivity in [^{32}P]-labelled synaptosomes in which the magnitude of stimulation achieved with PDBu and 1S,3R-ACPD (2.58 ± 0.21) was significantly higher than with PDBu on its own (1.9 ± 0.06) (Figure 2B) ($P<0.001$ Tukey-Kramer multiple comparison tests).

In order to investigate further the role of PKC in PLD activation, we examined the effect of 2.5 μM GF109203X, a PKC inhibitor, which is more specific than Ro-31-8220 or staurosporine (Beltman *et al.*, 1996). GF109203X at 2.5 μM induced a partial, but statistically significant inhibition of PDBu-activated PLD ($n=6$). In contrast, it did not have a significant effect on 1S,3R-ACPD-stimulated PLD activity (Figure 3). We have previously demonstrated that GF109203X at 2.5 μM blocked PDBu induced phosphorylation of MARCKS which is a well characterized PKC substrate (McLaughlin & Breen, 1999).

mGluR coupling to PI-PLC in synaptosomes

The additivity of the effect of PDBu and 1S,3R-ACPD, together with the insensitivity of 1S,3R-ACPD-stimulated PLD to inhibition by GF109203X suggests that mGluR activation of PLD is PKC independent. This would suggest that the PLD enzyme(s) in synaptosomes is insensitive to the PKC isoforms stimulated by the physiological activator, DAG, generated by PI-PLC, or that mGluR activation fails to stimulate PI-PLC in synaptosomes. We therefore examined

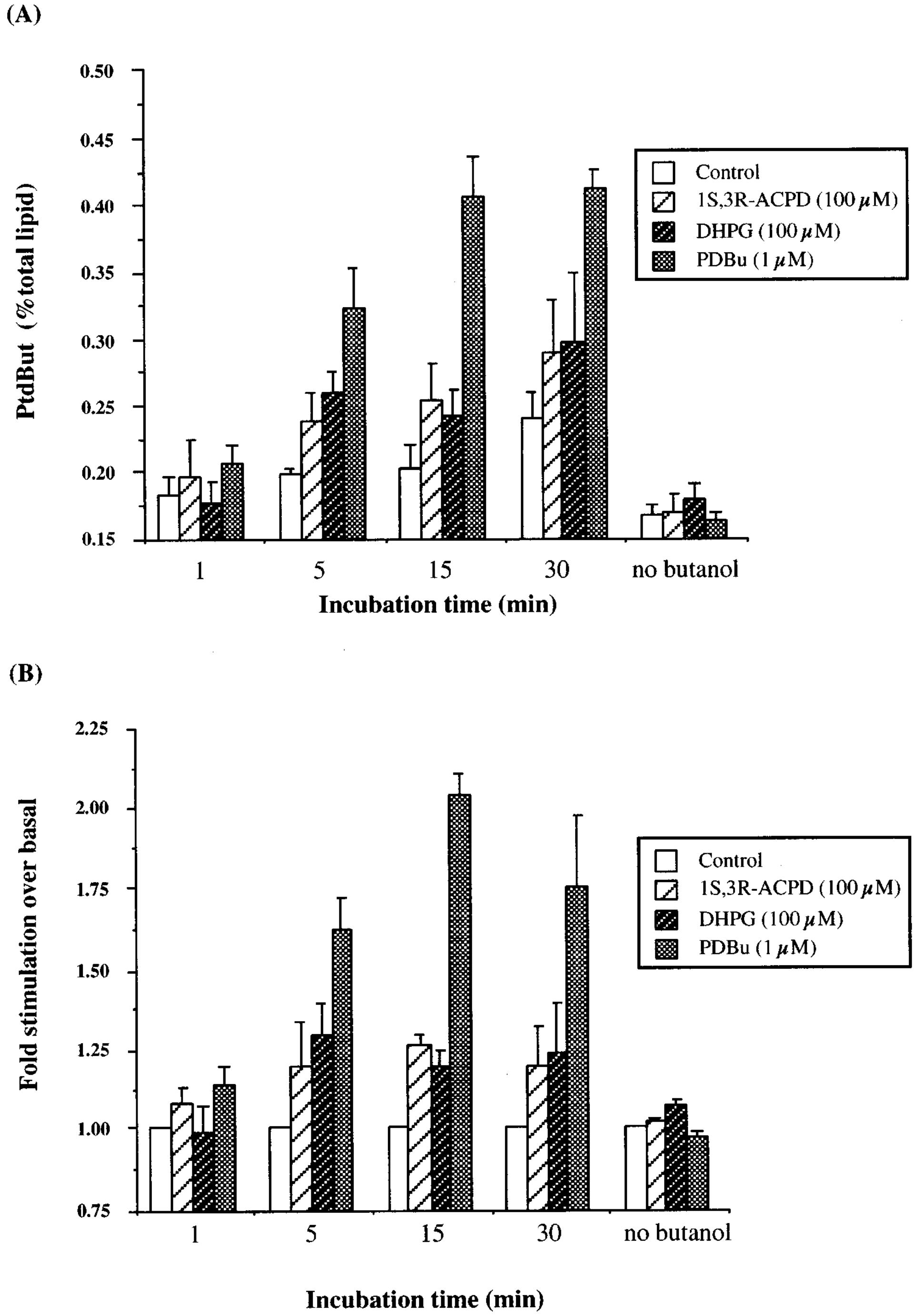

Figure 1 The time course profile of PDBu, 1S,3R-ACPD, and DHPG stimulated PLD-catalysed formation of [^{3}H]-PBut in synaptosomes. Synaptosomes were incubated in HBM with 1.3 mM $CaCl_2$ and 0.3% butanol in the presence of 1 μM PDBu, 100 μM 1S,3R-ACPD or 100 μM DHPG for the indicated times or in the absence of butanol for 15 min. The results are illustrated as the per cent of radioactivity present in the PtdBut spot relative to the total activity in the lipid extract (A) and the per cent stimulation over basal untreated synaptosomes (B). The basal activity was calculated as the difference between the presence and the absence of 0.3% butanol. Data are expressed as mean $\pm$ s.e.mean from 3–6 independent experiments.

mGluR-activated PI-PLC activity. PI-PLC activation is conventionally measured as an increase in IP_3 or DAG concentration, the two products of PIP_2 hydrolysis by PLC. mGluRs activate PI turnover with fast kinetics (Aramori & Nakanishi, 1992; Carruthers *et al*., 1997), therefore, we measured IP_3 after 15 s. However, we could not detect any change in IP_3 levels by receptor stimulation with either 1 mM quisqualate (a non-selective mGluR agonist) or 100 μM 1S,3R-ACPD (Figure 4). This method however has been successfully employed to demonstrate an elevation of IP_3 in carbachol stimulated cerebellar granule cells (del Rio *et al*., 1998).

Effects of mGluR agonists on DAG mass formation

To confirm that PI-PLC activation does not occur in response to mGluR activation we analysed DAG mass at an early time point (15 s) when DAG would most likely be directly generated by PI-PLC activation and at a later time point (15 min) when DAG formation can occur also as a consequence of the hydrolysis of PA generated by PLD activity. There was no significant elevation of DAG in response to 15 s exposure to either 1S,3R-ACPD or DHPG at which time point DAG from PI-PLC activation would be

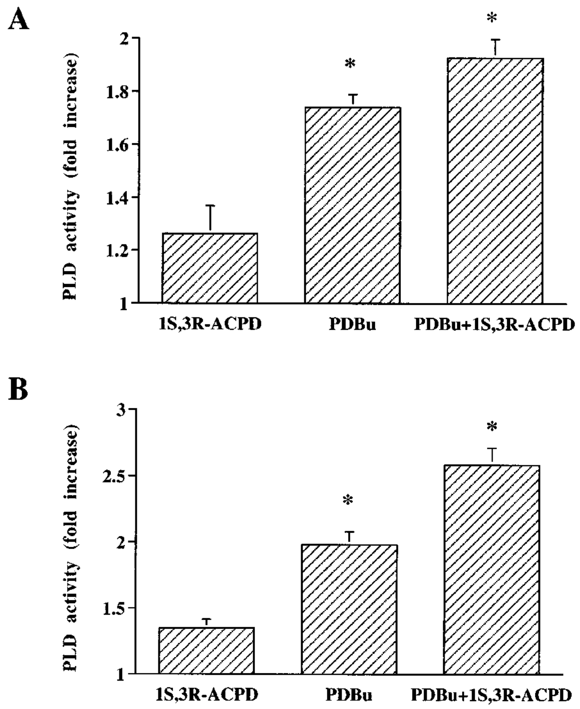

Figure 2 The additive effects of 1S,3R-ACPD and PDBu on PLD-catalysed [^{3}H]- or [^{32}P]-PBut formation in synaptosomes. (A) [^{3}H]-PBut formation after 15 min. Data are expressed as mean ± s.e.mean of three independent experiments each performed in triplicate. Asterisk indicates statistically significant difference between the groups and from control by ANOVA. (B) [^{32}P]-PBut formation after 15 min. [^{32}P]-labelling experiments also showed stimulation of PDBu-induced PLD activation by 1S,3R-ACPD. The control values are 0.28 ± 0.01 (n = 6) per cent of total radioactivity in each lane. Data are expressed as percentage of [^{32}P]-PBut out of total radioactivity in each lane, which are mean ± s.e.mean of two independent experiments each performed in triplicate. The basal activity was calculated as the difference between the presence and the absence of 0.3% butanol. Asterisk denotes statistically significant difference between the groups and from control by ANOVA.

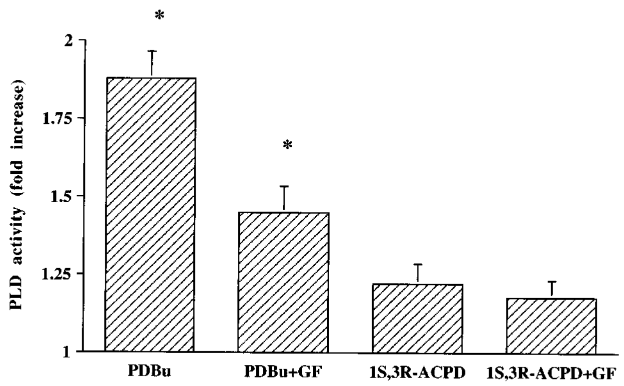

Figure 3 The effects of a PKC-inhibitor, GF109203X on PDBu-induced and 1S,3R-ACPD-induced PLD activity. GF109203X (2.5 μM) was added 5 min before adding 1 μM PDBu or 100 μM 1S,3R-ACPD. The final concentration of DMSO as vehicle was 0.01%. The basal activity was calculated as the difference between the presence and the absence of 0.3% butanol. Asterisk denotes statistically significant difference between the groups and from control by ANOVA.

expected to be produced. The significant peak in DAG was not detected within the first 1 min after the stimulation. On the contrary, both agonists produced gradual increases in DAG, which achieved levels of statistical significance at 15 min (Figure 5). DAG goes down gradually after 15 min (data not

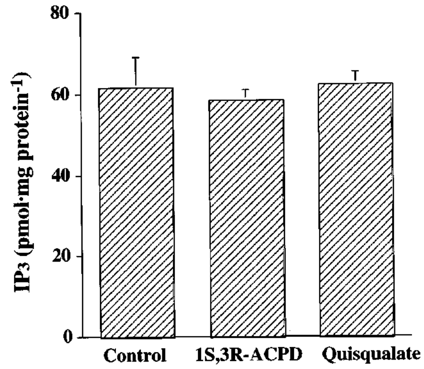

Figure 4 The effects of 100 μM 1S,3R-ACPD and 1 mM quisqualate on IP_3 formation in synaptosomes. The reactions were terminated at 15 s. Data are expressed as mean ± s.e.mean of at least three independent experiments each performed in triplicate. there was no significant difference between the IP_3 quantified in the absence or presence of agonist.

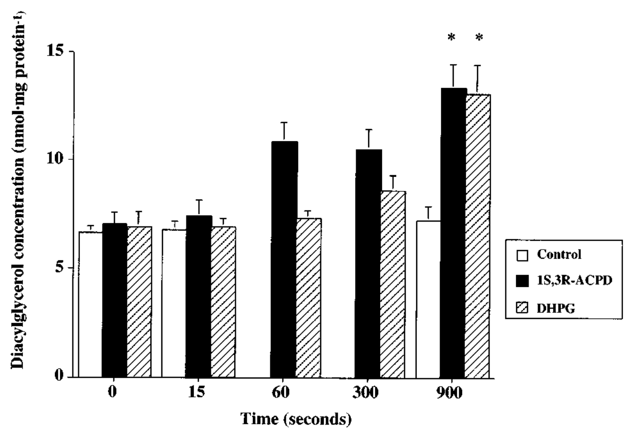

Figure 5 The effects of 100 μM 1S,3R-ACPD, DHPG on DAG mass production. The reactions were terminated at the time points indicated. Lipids extracted from 0.02 mg of synaptosomal protein were assayed respectively. Data are expressed as mean ± s.e.mean of at least three independent experiments assayed in triplicate. Asterisks indicate the statistical significant difference from control value by t-test.

shown). The formation of DAG as a consequence of PLD activity would require the presence of PAP activity. We have shown that PAP activity is detectable in synaptosomal membranes (Figure 6). [^{14}C]-DAG, which was the product of PAP activity, increased up to 130% in 30 min when compared with micelles which lacked synaptosomal membrane.

Discussion

In this investigation we have employed the rat cerebrocortical synaptosome preparation to investigate the signalling pathways coupled to mGluR activation of PLD. While similar experiments have been conducted in tissue slices from various neuroanatomically defined rat brain regions (Boss & Conn, 1992; Holler et al., 1993; Klein et al., 1998; Pellegrini-Giampietro et al., 1996), the analysis of brain slices prevents an accurate assessment of the contribution of the presynaptic versus postsynaptic components to the PLD activities. Thus the synaptosome system allows a focused investigation of presynaptic signalling events which are of particular relevance given that glutamate has been proposed to have an

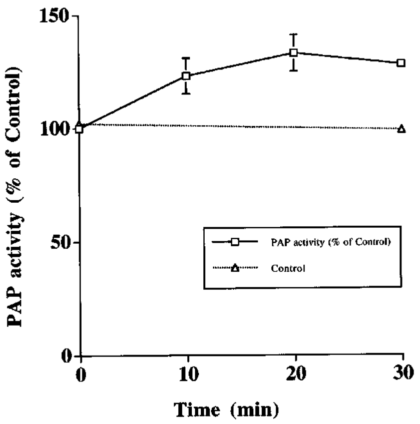

Figure 6 The activity of phosphatidic acid phosphohydrolase (PAP) in synaptosomes. The results are illustrated as fold increases in the [^{14}C]-DAG (per cent of radioactivity in total lane) relative to the time point 0. The reactions were carried out in the presence (□) or in the absence (△) of lysed synaptosomes. The reactions were terminated at the time points indicated. The [^{14}C]-DAG was significantly increased at 10, 20 and 30 min. Data are expressed as mean ± s.e.mean of three independent experiments assayed in triplicate or duplicate.

autoregulatory action on its exocytosis from presynaptic terminals. We have established that the synaptosome preparation employed in the present study is enriched in the small G-protein, Rab3A, and has minimal GFAP immunoreactivity consistent with an enrichment in presynaptic terminals with minimal gliasome contamination (McLaughlin & Breen, 1999). The main findings of the present study are that mGluR agonists can activate synaptosomal PLD in a manner which may be PLC and PKC-independent.

While our observation that mGluRs activate PLD is consistent with previous reports we do observe some differences. While both 1S,3R-ACPD and DHPG stimulate synaptosomal PLD activity, the magnitude of stimulation was lower than that described in other reports (Pellegrini-Giampietro *et al*., 1996). Also, DHPG antagonizes 1S,3R-ACPD stimulated PLD (Pellegrini-Giampietro *et al*., 1996), an effect not observed in this study. The discrepancies between the agonist effects may be accounted for by the different systems employed. Indeed it has been reported that the mGluR subtypes have a distinctive distribution pattern between pre- and postsynaptic elements (Shigemoto *et al*., 1997). Additionally, the relatively small magnitude of PLD activation achieved with mGluR agonists when compared with PDBu in our report may reflect the recent observation that PLD activation by 1S,3R-ACPD is ontogenetically regulated in hippocampal slices, being highest in young animals (Klein *et al*., 1998). It is, however, of interest that the synuclein proteins, which can inhibit PLD (Jenco *et al*., 1998), are abundant in presynaptic terminals. Their role in presynaptic PLD regulation requires further investigation. While it is clear that mGluRs are capable of stimulating PLD in the brain, and that the pharmacology of this response does not conform to the classical subdivisions of these receptors (Conn & Pin, 1997), this may be consistent with a new category of mGluR, which awaits distinctive proof by molecular and pharmacological identification.

We have demonstrated that PLD activation in cerebrocortical synaptosomes may occur independently of PKC, by showing the additivity of PDBu and 1S,3R-ACPD and the insensitivity of 1S,3R-ACPD-induced PLD activation to GF109203X. The linkage of PKC to the PLD signal transduction pathway may depend on the tissue and the stage of development studied (Briscoe *et al*., 1995; Exton, 1997; Klein *et al*., 1997; 1998; Schmidt *et al*., 1996). Additionally, it has been reported that PKC activation of PLD may occur by ATP independent mechanism (Conricode *et al*., 1992; Singer *et al*., 1996), and this mechanism would not be inhibited by the bisindolemalemide family of PKC inhibitors since their action is due to a competitive inhibition of the ATP binding site (Gordge & Ryves, 1994). Our results in cerebrocortical synaptosomes are in agreement with the report by Pellegrini-Giampietro (1996) on PLD activation by 1S,3R-ACPD-sensitive mGluR *via* a PKC-independent pathway in hippocampal slices. Recently Klein *et al*. (1998) have suggested that there are two modes of mGluR-mediated PLD activation, a PKC-dependent one (which predominates in young animals) and a PKC-independent mechanism which predominates in older animals. The mechanism of PLD activation observed in our experiments is compatible with the type of activity present in older animals.

In most systems studied, there are two phases of DAG formation in response to agonists linked to the hydrolysis of PC or PIP_2 (Exton, 1994; Wakelam, 1998). The initial rapid increase usually results from PIP_2 hydrolysis by PLC and is associated with an increase in inositol phosphates and cytosolic Ca^{2+} detectable with 15 s of stimulation. The second increase is slower and reaches a maximum after several minutes, with DAG produced by PLD contributing to the second peak. In this study we have demonstrated that there is no initial peak in DAG, which strongly suggests that mGluRs do not activate PI-PLC in synaptosomes, while the DAG generated at the longer time point may be derived from PAP activity which we have shown to be present in synaptosomes.

The coupling of group I mGluR to PI-PLC, as demonstrated by inositol phosphate measurements, has been shown in many systems (Abe *et al*., 1992; Aramori & Nakanishi, 1992; Carruthers *et al*., 1997; Wakelam, 1998). Here we show that there was no detectable increase in IP_3 in synaptosomes within 15 s of receptor stimulation, a time point at which we can measure an elevated IP_3 response to carbachol in cerebellar granule cells (del Rio *et al*., 1998). To our knowledge at present, this is the first report on the effect of mGluR agonists on IP_3 levels in synaptosomes although laterotoxin has been reported to activate synaptosomal PLC but produce only a small nonsignificant 5% increase in IP_3. (Davletov *et al*., 1998). However, the lack of detectable IP_3 elevation together with the monophasic slow DAG increase suggests that mGluR1 and mGluR5 agonists fail to stimulate PI turnover in rat cerebrocortical synaptosomes. On the other hand, one group has reported mGluR-linked PLC activity in P2 synaptosomes which are less purified than Percoll gradient separated synaptosomes. They reported that DAG was drastically increased by 10–20 fold within 5–15 s and then returned to the basal level in 1 min (Coffey *et al*., 1994; Herrero *et al*., 1994; Sanchez Prieto *et al*., 1996; Vazquez *et al*., 1994). Our observations are inconsistent with these findings. Moreover, there are some unusual features of the previously published work cited above which differ markedly from the experiments we now report. Firstly, the earlier work utilized methods which failed to separate the two products of DAG kinase, ceramide phosphate and PA, making it difficult to compare with our observations. Secondly, it is striking that the report by Coffey *et al*. (1994) (see also its erratum), gave DAG values of 2–3 pmol mg^{-1} protein, more than 1000 fold below the values we now report. Our own basal values, given in Figure 5 (7–8 nmol mg^{-1} protein), are compatible with those reported for other tissues (e.g. bovine parathyroid cells) (McKay & Miller, 1996). We have tried to replicate the synaptosome preparation and DAG measurement procedure used in the studies mentioned above. Regardless of the preparation and the methodology of DAG detection, we have consistently found no significant increase in early time points (within the first minute) in DAG levels, but rather a monophasic rise that plateaus between 1 – 5 min, with a maximum increase of 2 fold over basal.

Taken together, the lack of stimulation of IP_3 or DAG production at early time points, strongly indicates that there is no significant PI-PLC activation by mGluRs in cerebro-cortical synaptosomes. This conclusion is further supported by a report which reveals that mGluR1 and mGluR5 are localized to postsynaptic elements, while other mGluRs are distributed pre- and post-synaptically in hippocampal tissues, as measured using specific anti-mGluR antibodies (Shigemoto *et al*., 1997).

The role of PLD in neuronal model systems requires further clarification. The regulation of PLD can have important consequences because it generates two different second messengers (DAG and PA), each with its own signalling potential (English, 1996; Exton, 1997; Hodgkin *et al*., 1998). In particular, DAGs derived from PI or PC appear to differ in their ability to induce the translocation of selective PKC isoforms and the time scale of the PKC activation (Ha & Exton, 1993). The long-term formation of DAG produced by PLD can lead to the prolonged activation of PKC (Nishizuka, 1995; Wakelam, 1998).

In conclusion, we have presented evidence that PLD activation by 1S,3R-ACPD sensitive mGluRs in rat cerebro-cortical synaptosomes occurs through a PI-PLC- and PKC-independent pathway. Therefore PLD may have significant role in the autoreceptor function of glutamate modulation of exocytosis at the presynaptic terminal.

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