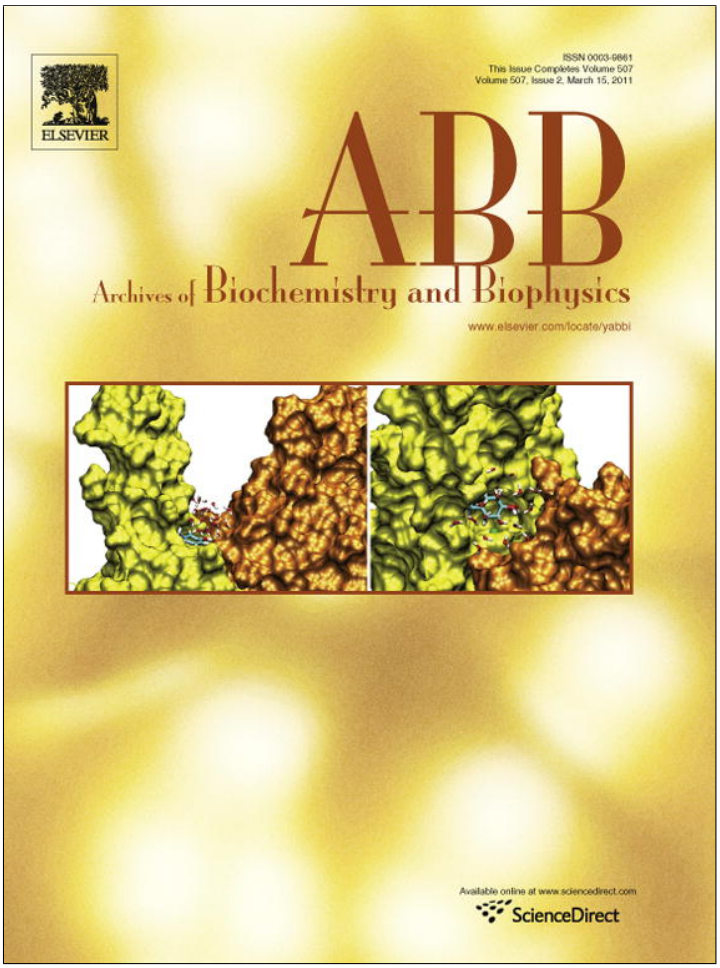

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Metabolic pathways for the degradation of phosphatidic acid in isolated nuclei from cerebellar cells

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ABSTRACT

The aim of the present research was to analyse the pathways for phosphatidic acid metabolism in purified nuclei from cerebellar cells. Lipid phosphate phosphatase and diacylglyceride lipase activities were detected in nuclei from cerebellar cells. It was observed that DAGL activity makes up 50% of LPP activity and that PtdOH can also be metabolised to lysophosphatidic acid. With a nuclear protein content of approximately 40 μg, the production of diacylglycerol and monoacylglycerol was linear for 30 min and 5 min, respectively, whereas it increased with PtdOH concentrations of up to 250 μM. LysoPtdOH, sphingosine 1-phosphate and ceramide 1-phosphate, which are alternative substrates for LPP, significantly reduced DAG production from PA. DAG and MAG production increased in the presence of Triton X-100 (1 mM) whereas no modifications were observed in the presence of ionic detergent sodium deoxycholate. Ca^{2+} and Mg^{2+} stimulated MAG production without affecting DAG formation whereas fluoride and vanadate inhibited the generation of both products. Specific PtdOH-phospholipase A1 and PtdOH-phospholipase A2 were also detected in nuclei. Our findings constitute the first reported evidence of active PtdOH metabolism involving LPP, DAGL and PtdOH-selective PLA activities in purified nuclei prepared from cerebellar cells.

Introduction

Lipids are present not only in the nuclear envelope but also inside the nucleus as a component of chromatin, and there is growing evidence of the importance of internal nuclear lipid metabolism. The inositol lipid cycle and its role in nuclear signal transduction have been extensively studied. The nucleus contains lipids such as phosphatidylcholine, phosphatidylethanolamine, phosphoinositides, phosphatidylserine, sphingomyelin, fatty acids, eicosanoids [1] and enzymes related to lipid metabolism [2]. One peculiarity of nuclear lipids is that their metabolism differs from that of lipids in the plasma membrane or in other cellular organelles [3–5]. Nuclear metabolism gives rise to several lipid second messengers within the nucleus which seem to be involved in the regulation of nuclear structure and gene expression. In this respect, it has been reported that (i) sphingomyelin protects RNA from RNase digestion [6,7]; (ii) phosphatidylserine stimulates both RNA and DNA polymerases [8]; (iii) phosphatidylcholine is involved in cell proliferation by PtdCho-PLC activation and diacylglycerol production [4]; and (iv) phosphoinositides favour PKC translocation inside the nucleus through DAG generated by PtdIns-PLC [4].

DAG pools from two different sources have been described in nuclei, one from PtdCho [9] and the other from inositides [10]. The PtdCho source seems to indicate the presence of phosphatidate phosphohydrolase activity in nuclei. Phosphatidate phosphohydrolase occurs in different isoforms such as NEM-sensitive Mg^{2+}-dependent activity (PAP1) in cytosol and microsomal membranes involved in lipid metabolism, and NEM-insensitive Mg^{2+}-independent activity (PAP2) associated with the plasma membrane and involved in signal transduction [11,12]. PAP2 has been renamed lipid phosphate phosphohydrolase (LPP)[1] because of its ability to dephosphorylate lipid phosphates other than PtdOH, such as LysoPtdOH, C1P and S1P [13]. Based on the involvement of PtdOH, other PtdOH-generated lipids and their related enzymes in cellular

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[1] *Abbreviations used:* AACOCF3, arachidonoyl trifluoromethylketone; BEL, bromoenol lactone; C1P, ceramide 1-phosphate; DAG, diacylglycerol; DAGL, diacylglycerol lipase; DAPI, 4,6-diamidino-2-phenylindole; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid; G-3-P, glycerol-3-phosphate; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; LysoPtdOH, Lysophosphatidic acid; LPP, lipid phosphate phosphatase; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; NEM, *N*-ethylmaleimide; PtdOH, phosphatidic acid; PAP1, NEM-sensitive Mg^{2+}-dependent phosphatidate phosphohydrolase; PAP2, NEM-insensitive Mg^{2+}-independent phosphatidate phosphohydrolase; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PLA, Phospholipase A; PLC, phospholipase C; S1P, sphingosine 1-phosphate; TLC, thin-layer chromatography.

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signalling events, our study focuses on the enzymatic mechanisms of PtdOH-removal in nuclei from cerebellar cells.

Experimental procedure

Materials

[2-^{3}H]Glycerol (200 mCi/mmol) and Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA, USA). AACOCF3, BEL, DAPI. Sphingosine 1-phosphate, ceramide 1-phosphate from bovine brain and oleoyl-L-α-lysophosphatidic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of the highest purity available.

Purified nuclear fraction preparation

Male Wistar-strain rats were kept under constant environmental conditions and fed on a standard pellet diet. All procedures were carried out in accordance with the guidelines issued by the Animal Research Committee of the Universidad Nacional del Sur (Argentina) in accordance with the Guide of the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research (ILAR) of the National Academy of Science (Bethesda, MD). Adult rats (four-month-old) were killed by decapitation and the cerebellum was immediately dissected (2–4 min after decapitation). The basic procedure for the isolation of nuclei was followed – with minor modifications – as described elsewhere [14,15]. Cerebellums from 12 animals were pooled and homogenized in a 1:8 (w/v) ratio in 0.25 M sucrose TKM (50 mM Tris–HCl, pH 7.5, 25 mM KCl, 5 mM Mg Cl_2 and 0.2 mM $CaCl_2$) in the presence of 0.1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 2 μg/ml leupeptin. Homogenization was carried out in a Potter–Elvehjem homogenizer using 10 up-and-down strokes at 600 rpm. The homogenate was filtered through four layers of cheesecloth and two volumes of 2.3 M sucrose in TKM were added to reach a sucrose concentration of 1.6 M. The filtered homogenate was layered over 2.3 M sucrose in TKM and centrifuged at 100,000*g* during 80 min. The purified nuclear fraction was pelleted below the 2.3 M sucrose layer. The nuclei were washed in TKM containing protease inhibitors and centrifuged at 28,000*g* for 30 min. The nuclear pellets were suspended in: (i) TKM to quantify proteins and DNA, (ii) Tris–HCl 100 mM pH 8.5 to determine 5′ nucleotidase, (iii) Na-phosphate 0.2 M pH 7 to determine NADH cytochrome *c* reductase, (iv) the appropriate buffer for the enzymatic assays, (v) cacodylate buffer for electron microscopy, (vi) paraformaldehyde (2%) and Triton-X 100 (0.1%) in phosphate buffer saline (PBS) for DAPI staining, and (vii) chloroform:methanol (2:1, v/v) for phospholipid content determination.

Criteria for nuclear purity

Nuclear preparations were checked for purity by electron microscopy, DAPI stain, DNA content and the marker enzymes 5′-nucleotidase and NADH cytochrome c reductase determinations. For electron microscopy studies, nuclear preparation was suspended in a medium containing 0.12 M cacodylate buffer, pH 7.4, 2.5% glutaraldehyde and 2% (w/v) sucrose, incubated for 1 h at room temperature, and centrifuged at 12,000*g* for 10 min. The pellet was post-fixed in 1% osmium tetraoxide in 0.12 M cacodylate buffer, pH 7.4 for 1 h, dehydrated with serial ethanol concentrations and propylene oxide, embedded in spurr resin and then cut into thin sections. The latter were examined using a JEOL 100 CXII microscope operated at 80 kV [10]. Nuclear integrity was evaluated after staining cell nuclei with DAPI, a fluorescent dye that binds to DNA. Briefly, nuclei were permeated with 0.1% Triton X-100 in PBS, washed with PBS and incubated with DAPI for 20 min. DNA extraction was carried out using saturated phenol in trizma base, pH 8.0. DNA was precipitated with absolute ethanol, dissolved with distilled water and its content assessed by diphenylamine assay [16]. The 5′-nucleotidase and NADH cytochrome c reductase activities were performed according to Widnell [17] and van Gelder [10], respectively.

Preparation of radioactive 1,2-diacyl-sn-glycerol-3-phosphate

Radioactive PtdOH was obtained from [2-^{3}H]glycerol-PtdCho synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described [18]. Lipids were extracted from the tissue as described elsewhere [19]. [^{3}H]PtdCho was isolated by mono-dimensional thin-layer chromatography (TLC) and eluted therefrom [20]. [^{3}H]PtdCho was then hydrolyzed with phospholipase D [21] and the hydrolysis product [^{3}H]PtdOH was purified by one-dimensional TLC on silica gel H developed with chloroform:methanol:acetic acid:acetone:water (9:3:3:12:1.5, v/v). In this solvent system [^{3}H]PtdOH migrates with an Rf of 0.5 and [^{3}H]PtdCho with an Rf of 0.25. The substrate was eluted from silica gel with neutral solvents to avoid the formation of lysophosphatidic acid and was subsequently converted into free acid by washing it twice using an upper phase containing 0.1 M sulfuric acid and then an upper phase containing water. Radioactivity and phosphorus content [22] were measured to determine specific radioactivity. [^{3}H]PtdOH (0.1 mM, 0.1–0.2 mCi/mmol) was prepared by sonication in buffer solution containing 5.56 mM EGTA and 5.56 mM EDTA [23,24].

Preparation of radioactive 1,2-diacyl-sn-glycerol

DAG was obtained from [^{3}H]PtdCho (0.15 μCi/μmol) after hydrolysis by phospholipase C from *Clostridium welchii* (Grade B, calbiochem, Los Angeles, USA). [^{3}H]PtdCho (3 mg) was dried under a stream of nitrogen and redissolved in 2% ethanol in diethyl ether (v/v) [25]. The enzyme was dissolved in 50 mM Tris–HCl buffer, pH 7.3, containing 3 mM $CaCl_2$ and was added to the solution of lipid and the mixture incubated at room temperature for 3 h. DAG was extracted from the hydrolysis mixture with diethyl ether containing 1% water and it was isolated by one-dimensional TLC on silica gel G in the solvent, hexane:diethyl ether:acetic acid (35:65:1, v/v). DAG was extracted from silica gel with *n*-hexane:2-propanol (3:2 v/v) and stored at −20 °C. [^{3}H]DAG specific activity was 0.15 mCi/mmol.

Enzymatic assays

PAP1 and LPP activity assays

PAP1 activity was determined in an assay containing 50 mM Tris-maleate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA and 1 mM EGTA, 0.2 mM Mg^{2+}, and 40 μg of nuclear protein in a volume of 100 μl. The reaction was started by adding 80 μM of [^{3}H]PtdOH plus 55 μM PtdCho. Parallel incubations were carried out after preincubating the enzyme with 4.2 mM NEM for 10 min. The difference between these two activities was labeled as PAP1 activity.

For the determination of LPP activity, each assay contained 50 mM Tris-maleate buffer, pH 6.5, 1 mM EDTA plus EGTA, 4.2 mM NEM, and 40 μg of nuclear protein in a volume of 100 μl. The reaction was started by adding 100 μM [^{3}H]PtdOH. When LPP activity was evaluated in the presence of LysoPtdOH, S1P or C1P, the reaction was started by adding 100 μM [^{3}H]PtdOH/Triton X-100 (1:50 M ratio) mixed micelles in the presence of different concentrations of LysoPtdOH, S1P or C1P (previously re-suspended in the assay buffer containing Triton X-100) [23]. In this case, radiolabel PtdOH was mixed with unlabeled substrates before

drying and re-suspension. This aqueous microdispersion was sonicated in a sonication bath until clarity.

DAGL activity assay

DAGL activity was determined by monitoring the formation rate of monoacyl[2-^{3}H]glycerol ([^{3}H]MAG), using diacyl[2-^{3}H]glycerol ([^{3}H]DAG) generated by LPP action on [^{3}H]PtdOH as substrate. The specific activity of DAG was calculated under all experimental conditions as the ratio between the dpm of [^{3}H]DAG generated and the sum of the nmol of labeled DAG plus the concentration of DAG in nuclei (in nmol). The incubation medium, protein concentration, time and final volume of incubation were the same as those described for LPP assays [26]. PAP and DAGL assays were conducted at 37 °C for 20 min.

DAGL activity was also determined using exogenously added [^{3}H]DAG as substrate. Assays using [^{3}H]DAG were performed at 37 °C in 50 mM MOPS buffer (pH 7.4) containing 0.25% fatty acid-free bovine serum albumin (BSA). DAGL assay (40 μg nuclear protein per assay), having a total volume of 100 μl, was initiated with the addition of suspensions of 300 μM [^{3}H]DAG. Suspensions of [^{3}H]DAG were prepared by separately sonicating with equimolecular concentrations of lysoPtdCho in 0.05 M MOPS buffer (pH 7.4).

PtdOH-selective PLA1 and PLA2 assays

PtdOH-selective PLA1 activity was determined by incubating 40 μg of nuclear protein in 100 mM Tris–HCl (pH 7.5) buffer containing 4 mM $CaCl_2$ and 0.1% cholic acid in a final volume of 100 μl. The PLA2 assay was carried out using the same protein concentration as that for PLA 1 but in 100 mM Tris–HCl buffer pH 6.0 containing 10 mM EDTA. The reactions were started by adding 0.1 mM [^{3}H]PtdOH and continued at 37 °C for 30 min.

LPP, DAGL, PtdOH-PLA1 and PtdOH-PLA2 enzymatic reactions were stopped by adding chloroform:methanol (2:1, v/v). Blanks were prepared identically to each enzymatic assay except that the membrane fraction was either boiled for 5 min or inactivated by the addition of chloroform:methanol (2:1, v/v) before use. Blank values were subtracted from each enzymatic activity.

Lipids were extracted with chloroform:methanol (2:1, v/v) and washed with 0.2 volume of $CaCl_2$ (0.05%) [19]. DAG was separated by gradient-thickness thin-layer chromatography on silica gel G [27] and developed with hexane:diethyl ether:acetic acid (35:65:1, v/v). In this system, solvent DAG migrates to the three fourths of the plate and PtdOH and MAG stay in the origin. To separate MAG from PtdOH, the chromatogram was rechromatographed up to the middle of the plate by using hexane:diethyl ether:acetic acid (20:80:2.3, v/v) as developing solvent. In other experiments aimed at separating LysoPtdOH, PtdOH, MAG and DAG, lipids were chromatographed by TLC on silica gel H and developed with chloroform:acetone:methanol:acetic acid:water (30:40:10:10:4, v/v) up to the middle of the plate and then the chromatogram was rechromatographed using hexane:diethyl ether:acetic acid (45:55:1.5, v/v) as developing solvent. The chromatograms were visualised by exposure to iodine vapors and scraped off for counting by liquid scintillation. To determine cerebellum nuclei lipid composition, lipids were separated according to Rouser [22]. The aqueous phase from Folch extraction containing radiolabel water-soluble products was concentrated to dryness and counted by liquid scintillation. Radiolabel samples were counted after the addition of 0.4 ml water and 10 ml 5% Omnifluor in toluene/Triton X-100 (4/1, v/v).

Other methods

Protein and lipid phosphorus were determined according to Bradford [28] and Rouser [22], respectively.

Statistical analysis

All data are given as means ± SD. Statistical analyses were evaluated by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, and were performed using GraphPad software (San Diego, California, USA, www.graphpad.com). Statistical significance was set at $p < 0.05$.

Results

Integrity and purity of nuclei

The cerebellar cortex includes three layers: molecular, Purkinje cells and granular. There are five cellular elements present in the cerebellum: Purkinje cells, granular cells, Golgi cells, star-shaped cells and basket cells.

In our experimental conditions we started from a cerebellar homogenate including all the above-mentioned cellular types from which nuclei were isolated and purified. Nuclear fraction is produced by sedimentation through very high-density sucrose solutions which effectively remove low-density membranes. The method followed to obtain the nuclear preparation [15] produces two nuclear populations, namely, a minority population N1, obtained at the interface of the sucrose buffer, and a majority population N2 as the pellet. Electron microscopy shows that the N1 nuclear preparation consists of small nuclei (data not shown). Since no LPP or PAP1 activity was detected in this fraction (data not shown), the results of the present paper are based on the N2 nuclear population. Morphological and biochemical criteria were used to assess the purity of nuclei prepared from rat cerebellum. The N2 population of isolated nuclei were examined by electron microscopy and could be clearly seen to have an intact nuclear envelope consisting of the characteristic double membrane (Fig. 1A and B). The examination at a lower-power magnification revealed no evidence of contamination by non-nuclear membranes such as endoplasmic reticulum and mitochondria (Fig. 1A). The different sizes of nuclei could be attributed to the fact that they derive from different cell types (Fig. 1A). Fig. 1C shows DAPI-stained nuclei evidencing nuclear integrity. The nuclear fraction could also be distinguished by its DNA content, having a DNA/protein ratio of 321 μg DNA/mg protein. In our experiments the plasma membrane was likely to be the most troublesome nuclei contaminant on account of the fact that it is the original cellular localization of LPP [29]. We thus evaluated 5′ nucleotidase activity as a marker of this fraction. Based on this activity, there was only 6% cross-contamination with plasma membrane (1820 ± 155 μM Pi/mg protein in homogenate and 107 ± 8.5 μM Pi/mg protein in nuclei). The specific activity of LPP in nuclei is 29% of that determined in homogenate (14,466 ± 820 DPM (mg prot. 20 min)$^{-1}$). Because the outer nuclear membrane is continuous with the endoplasmic reticulum [30], NADH cytochrome c reductase was assayed in all steps of nuclear purification. Based on this activity, the cross-contamination with the endoplasmic reticulum was 10% (190 ± 7.4 nmol (min mg prot.)$^{-1}$ and 19.7 ± 0.75 nmol (min mg prot.)$^{-1}$ in homogenate and nuclei, respectively). It has been demonstrated that PAP1 enzyme has a high activity in the cytosol and in microsomes [29]. However, no PAP1 activity was detected in our purified nuclei, indicating that there was no contamination by cytosol and reticulum. In spite of the low yield of nuclei (37%, based on DNA recovery; 3011 μg DNA/g tissue in homogenate vs. 1104 μg DNA/g in nuclei), the isolation procedure followed in the present study proved to be the most adequate of all methods tested. Based on the above-mentioned morphological and biochemical assays, the isolated nuclei were considered to be highly pure.

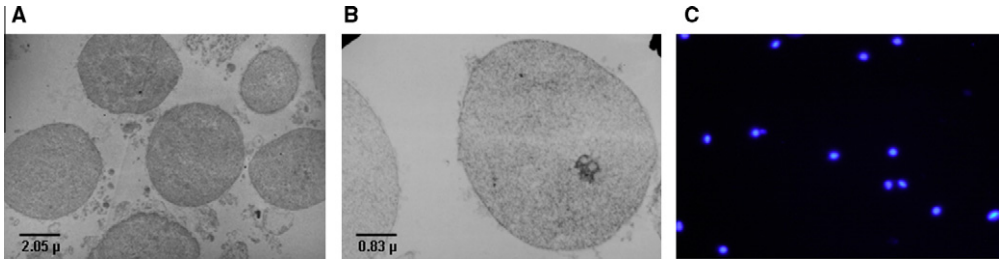

Fig. 1. Purity and integrity of nuclear preparations from rat cerebellum. Electron micrographs of isolated nuclei prepared as described in Section "Materials and methods", (A) 5000× (scale bar 2.05 µm); (B) 10,000× (scale bar 0.83 µm). (C) DAPI stained, isolated cerebellum nuclei are in blue (magnification, 400×). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Phospholipid composition

Table 1 shows the phospholipid composition of intact nuclei. It also indicates that nuclei of rat cerebellum cells are characterised by a high concentration of phosphatidylcholine, followed in decreasing order of concentration by phosphatidylethanolamine, sphingomyelin, phosphatidylserine and phosphatidylinositol.

Metabolism of phosphatidic acid in isolated nuclei

LPP and DAGL activities were detected in cerebellar nuclei by means of [^{3}H]PtdOH sonicated dispersion, as was demonstrated by DAG and MAG formation (Fig. 2). The acyl group composition of PtdOH used as substrate contained 34%, 19%, 18% and 17% of 16:0, 18:0, 18:1 and 22:6, respectively, with negligible amounts of 20:4 [18]. Nuclear fractions were pre-treated with NEM since phosphatidic acid phosphohydrolase activities have been classified as NEM-sensitive (PAP1) and – insensitive (LPP) [12,29]. Nuclei showed no NEM-sensitive activity, the detected rates all being the same with NEM pre-treatment in the presence of 0.2 mM Mg^{2+} and with NEM pre-treatment alone (data not shown). With 80 µM of [^{3}H]PtdOH, DAGL activity made up 50% of LPP activity (Fig. 2A). [^{3}H]PtdOH could also be metabolised to [^{3}H]LysoPtdOH. Under our assay conditions, this product resulted at a concentration of 100 µM [^{3}H]PtdOH, at which LysoPtdOH and MAG levels were similar and made up to 40% of DAG (Fig. 2B).

DAG and MAG production as a function of time, protein and PtdOH concentration, and pH

Results are shown in Figs. 3A–D, 5 and 7 obtained at an 80 µM [^{3}H]PtdOH concentration, at which no LysoPtdOH levels could be detected.

Table 1
Phospholipid composition of isolated rat cerebellum nuclei.

PL (nmol/mg prot)	349.6 ± 6
PL (nmol/mg DNA)	1087 ± 3
Phospholipids (%)	
PtdCho	42.4 ± 2.1
PtdEtn	31.5 ± 1.8
SM	13.3 ± 1.0
PtdSer	7.2 ± 0.0
PtdIns	5.6 ± 0.2
LysoPtdCho	2.5 ± 0.4
PtdOH	1.1 ± 0.1

Phospholipid composition was determined in rat cerebellum nuclei by quantification of phosphorus content as described in Section "Materials and methods". Values are expressed as the mean ± SD of 4 individual samples.

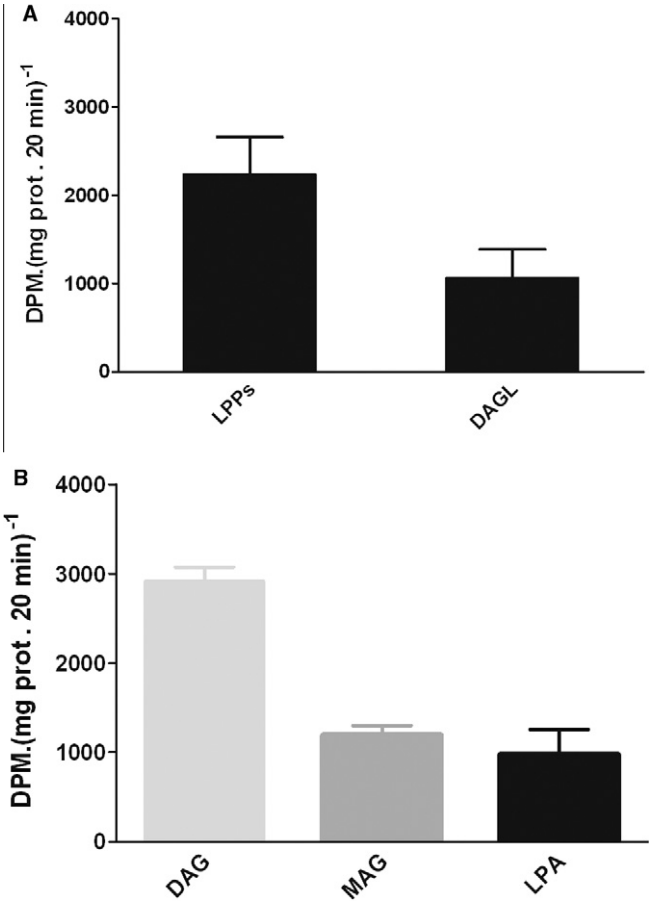

Fig. 2. Metabolism of [^{3}H]-PtdOH in isolated nuclei of rat cerebellum. (A) Lipid phosphate phosphatases and diacylgliceride lipase activities were determined using 80 µM [^{3}H]PtdOH in an assay containing 50 mM Tris-maleate buffer, pH 6.5, 1 mM EDTA plus EGTA, 4.2 mM NEM, and 40 µg of nuclear protein in a volume of 100 µl. DAG lipase activity was determined by monitoring the formation rate of monoacyl[2-^{3}H]glycerol using diacyl[2-^{3}H]glycerol derived from LPP action on [^{3}H]PtdOH as substrate. After 20 min of incubation, at 37 °C the enzymatic reactions were stopped by adding chloroform/methanol (2:1, by vol). The subsequent procedure is specified in the Section "Materials and methods" section. Results represent the mean ± S.D. of 11 individual samples. (B) The assay conditions are the same as those specified in (A) but in this case 100 µM [^{3}H]PtdOH were used. Results represent the mean ± S.D. of 3 individual samples.

The production of DAG and MAG was linear up to about 40 µg nuclear protein in the reaction mixture (Fig. 3B) for 30 min and 5 min, respectively (Fig. 3A). The rate of DAG and MAG production increased with PtdOH concentrations of up to 250 µM. LPPs activity expressed as the sum of [^{3}H]DAG and [^{3}H]MAG. (mg prot. 20 min)$^{-1}$ was presented as a function of PtdOH concentrations (Fig. 3C). From the double reciprocal plot an apparent Michaelis

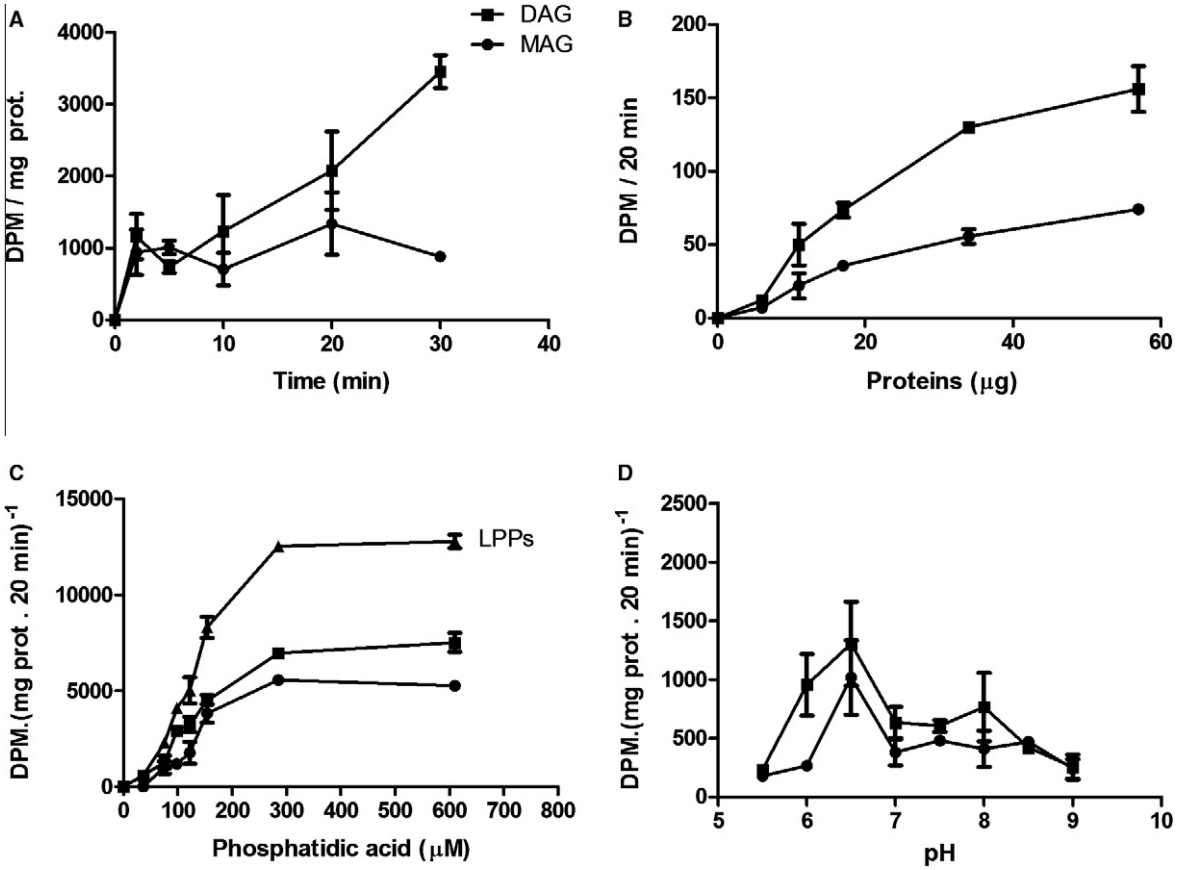

Fig. 3. [^{3}H]DAG and [^{3}H]MAG production as a function of time, protein and PtdOH concentration, and pH. DAG and MAG formation was measured as a function of incubation time (A), protein concentration (B), PtdOH concentration (C) and pH (D). 80 μM [^{3}H]PtdOH and 40 μg of protein; 80 μM [^{3}H]PtdOH for 20 min.; and 40 μg of protein for 20 min., were the assay conditions used in (A–C), respectively. In panel (D), assay conditions were specified in Fig. 2. LPPs activity is expressed as the sum of DPM [^{3}H]DAG and [^{3}H]MAG. (mg prot. 20 min)$^{-1}$. Results represent the mean ± S.D. of 3 individual samples.

constant (km) of 167 μM and V_{max} of 30 nmol (mg prot. 20 min)$^{-1}$ were calculated for LPP. MAG could be detected from PtdOH concentrations of approximately 50 μM PtdOH (Fig. 3C). The optimum pH was around 6.5 (Fig. 3D). Subsequent experiments were carried out using 80 μM of PtdOH and 40 μg of proteins, in which only DAG and MAG were formed from PtdOH.

MAG production as a function of time, protein and DAG concentration

The MAG production using exogenously added DAG as substrate was also evaluated (Fig. 4). MAG formation increased up to two minutes and remained constant for 30 min (Fig. 4A). This product was increased up to about 50 μg of proteins (Fig. 4B). The rate of MAG production increased with DAG concentrations up to 700 μM (Fig. 4C). Interestingly, DAGL activity could be observed even at concentrations as low as 60 μM, when DAG generated from PtdOH was used (Fig. 4D). However, when the enzymatic assay was carried out with DAG exogenously added, MAG formation occurred at a higher concentration of DAG (150 μM) (Fig. 4C). Under these assay conditions, DAGL showed an apparent km of 571 μM with a V_{max} of 12 nmoles (mg prot. 20 min)$^{-1}$. In contrast, using DAG generated from PtdOH as substrate, km and V_{max} were 402 μM and 7 nmoles (mg prot. 20 min)$^{-1}$.

DAG and MAG production as a function of LysoPtdOH, S1P and C1P concentrations

Data shown in Fig. 5 indicate the rate of DAG (square symbols) and MAG (circle symbols) formation in the presence of the alternative substrates of LPP. LysoPtdOH, S1P and C1P significantly decreased DAG production from [^{3}H]PtdOH (Fig. 5A–C). LysoPtdOH diminished DAG formation in percentages varying from 42% to 66% at concentrations ranging between 10 and 100 μM and MAG formation showed a 43% decrease at 100 μM (Fig. 5A). A significant decrease in DAG production was observed at low concentrations of S1P reaching 55% at 10 μM of S1P (Fig. 1B). At 100 μM of S1P, DAG formation was diminished by 34%. MAG formation was not significantly modified by the presence of S1P (Fig. 5B). C1P exerted an inhibitory effect (45%) on DAG formation at concentrations ranging from 10 to 100 μM (Fig. 5C).

Effects of non-ionic and ionic detergents on DAG and MAG production

DAG and MAG production was analysed by varying the molar ratio of detergent with respect to PtdOH, which was maintained at 80 μM. When non-ionic detergent Triton X-100 was used at a molar Triton X-100/PtdOH ratio close to 2.5 (0.2 mM Triton X-100), DAG production remained unmodified whereas MAG production increased by 219% (Fig. 6). In contrast, at a non-ionic detergent/PtdOH ratio close to 12.5 (1 mM Triton X-100), DAG and MAG formation increased by 94% and 113%, respectively (Fig. 6). The ionic detergent sodium deoxycholate did not modify DAG and MAG production at any of the two concentrations used (Fig. 6).

Effects of Ca^{2+}, Mg^{2+}, NaF and vanadate on DAG and MAG production

Ca^{2+} ions stimulated MAG formation at 1 mM and 10 mM concentrations by 206% and 188%, respectively. Mg^{2+} ions at 4 and 10 mM concentrations stimulated MAG production to a similar ex-

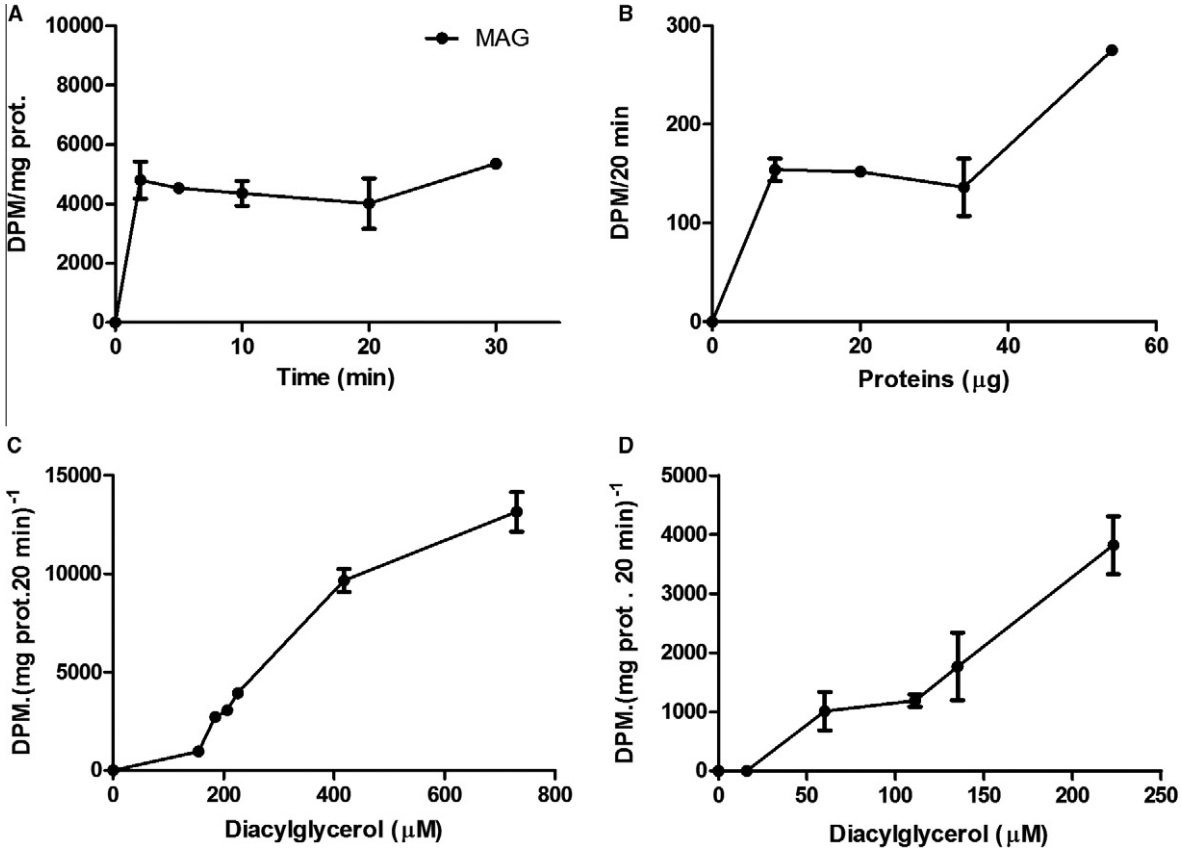

Fig. 4. [^{3}H]MAG production as a function of time, protein and DAG concentration. MAG formation, using DAG exogenously added, was measured as a function of incubation time (A), protein concentration (B), and substrate concentration (C). Panel (D) shows MAG generated from PtdOH. 80 μM [^{3}H]DAG and 40 μg of protein; 80 μM [^{3}H]DAG for 20 min; and 40 μg of protein for 20 min, were the assay conditions used in (A–C), respectively. In panel (D), the assay conditions were specified in the Section "Materials and methods". Results represent the mean ± S.D. of 3 individual samples.

tent: 244% and 231%, respectively (Fig. 7A). DAG production was not affected by Ca^{2+} and Mg^{2+} ions (Fig. 7A). NaF at 50 mM concentration diminished DAG and MAG generation by 92% and 98%, respectively (Fig. 7B), whereas vanadate decreased DAG production by 42% and produced a 30% inhibition in MAG production (Fig. 7B).

The presence of AACOCF3, the inhibitor of cytosolic PLA2, and BEL, the inhibitor of Ca^{2+}-independent PLA2, did not modify MAG production (data not shown).

PtdOH-selective PLA1 and PLA2 activities

When PtdOH-selective PLA1 or PLA2 were assayed, water-soluble products production was identical in both cases. However, DAG and MAG formation was only observed when PtdOH-selective PLA2 was assayed. LysoPtdOH was not detected under these assay conditions (Fig. 8).

Discussion

The role of the cerebellum has traditionally been considered to be restricted to the coordination of voluntary movement, gait, posture, speech, and motor functions. There is also evidence that it plays an important role in cognition, emotion and behavioural control [31], which could be severely affected in neurodegenerative processes such as those of Alzheimer's disease, schizophrenia, dementia, and other psychiatric disorders. The cerebellum also appears to have relevance in ageing mechanisms [32]. Previous reports of our laboratory have demonstrated that lipids derived from PtdOH are important messengers involved in all the above-mentioned processes [33–37].

It has also become clear that lipids and lysophospholipids are key regulators of a vast array of cellular functions and that they have a crucial role in many signal transducing pathways. Apart from the lipids present in the nuclear envelope, there are also lipids located inside the nucleus [38]. As a consequence, inside nuclei there are phospholipid-dependent receptors, transcription factors [39] and a signalling system derived from phospholipids [40]. The roles of nuclear lipids include DNA stabilization, stimulus of RNA and DNA polymerases and initiation of apoptosis [4]. Nuclei contain different enzymatic activities related to lipid metabolism and are involved in nuclear signalling, such as CTP:phosphocholine cytidyltransferase [41], PtdIns-PLC [42], DAGK [43], PtdIns-3Kinase [38], PtdCho-PLD [44], PtdCho-PLC [45], PLA2 [2] and acyltransferase [46].

A growing body of evidence suggests that PtdOH and its dephosphorylation product, DAG, are second messengers in agonist-stimulated cell activation. PtdOH and DAG have been detected in nuclei and have been found to increase during cell proliferation [47]. LysoPtdOH, the other product generated from PtdOH, behaves as an agonist [48] and its receptors localize at the cell nucleus [49]. These findings suggest that it would be of interest to learn more about how PtdOH signals are attenuated and how other signals in nuclei are generated from PtdOH. LPP and PAP1 exhibit nonoverlapping subcellular localization, the original cellular localization of LPP being in plasma membrane [50]; only a trace has been found in the microsomal fraction and none in the cytosol [29]. PAP1 activity

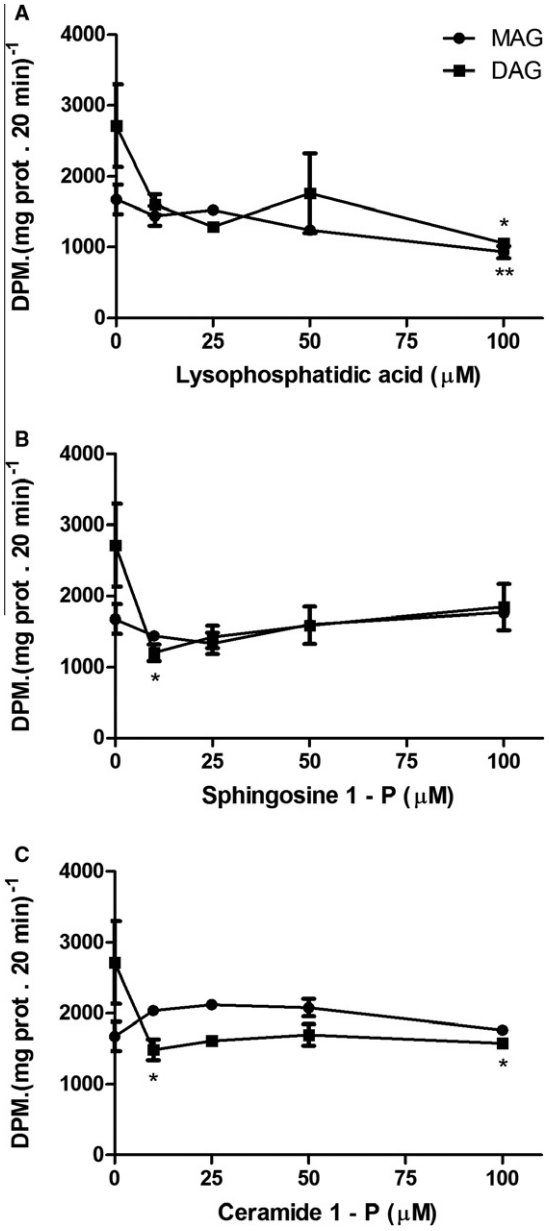

Fig. 5. Metabolism of [^{3}H]PtdOH in the presence of LysoPtdOH, S1P and C1P in neuronal cerebellum nuclei of rats. DAG and MAG were determined using isolated nuclei of rat cerebellum as enzyme source. The effects of LysoPtdOH (A), S1P (B) and C1P (C) on PtdOH hydrolysis were evaluated using 100 μM [^{3}H]PtdOH/Triton X-100 (1:50 M ratio) mixed micelles in the presence of LysoPtdOH, S1P or C1P at the indicated concentrations. All assays were conducted at 37 °C for 20 min. The enzymatic reactions were stopped by adding chloroform/methanol (2:1, by vol). The subsequent procedure is specified in Fig. 2. Results represent the mean ± S.D. of 3 individual samples. $^*p < 0.05$, $^{**}p < 0.005$ with respect to "0".

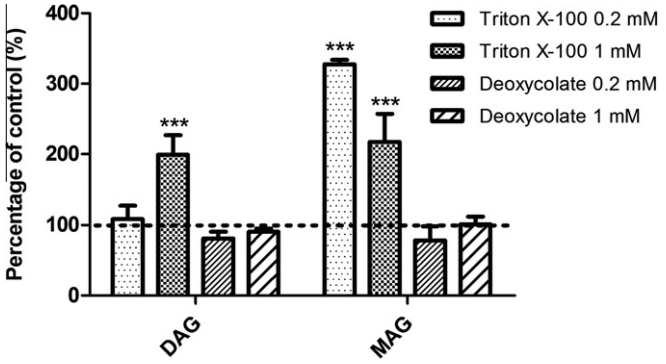

Fig. 6. Effects of non-ionic and ionic detergents on DAG and MAG production. DAG and MAG production was determined in the presence of two different tensioactive agents, Triton X-100 (non-ionic) and sodium deoxycholate (ionic). Two different concentrations of each were used. These assays were conducted in the same manner as described in Fig. 2. Results are indicated as a percentage of control values (controls represent 100%) and represent the mean ± S.D. of 3 individual samples. $^{***}p < 0.0005$ with respect to control values.

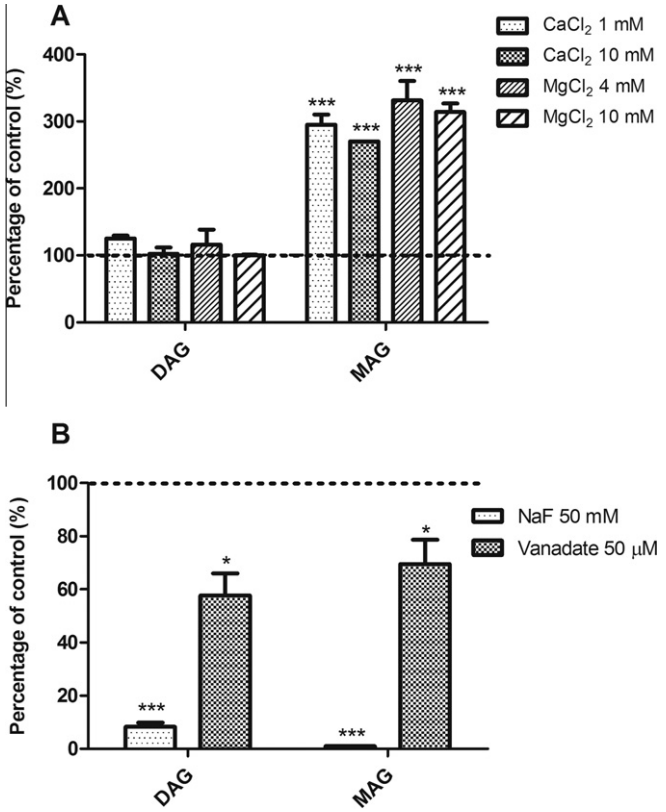

Fig. 7. Effects of Ca^{2+}, Mg^{2+}, NaF and vanadate on DAG and MAG production. DAG and MAG production was determined as specified in Fig. 2, in this case including Ca^{2+} or Mg^{2+} (A) and NaF or sodium vanadate (B) in the buffer assay. All assays were conducted under the same conditions as described in Fig. 2. Results are indicated as a percentage of control values (controls represent 100%) and they represent the mean ± S.D. of 3 individual samples. $^*p < 0.05$, $^{***}p < 0.0005$ with respect to control values.

is localized in the cytosol and transiently interacts with the cytosolic surface of the endoplasmic reticulum [51]. Based on the localization of LPP in the plasma membrane, we evaluated 5′ nucleotidase activity as a marker of this fraction. Minimal contamination (6%) of nuclei by plasma membrane was found. The evaluation of NADH cytochrome c reductase demonstrates a slight (10%) contamination of nuclei by endoplasmic reticulum membranes. PAP1 enzyme has been detected in cytosol and endoplasmic reticulum [29,33]. We previously observed PAP1 activity in crude nuclear fraction obtained from cerebellum homogenate [34]. This, together with the fact that purified nuclei did not contain any detectable PAP1 activity, argues in favour of the absence of reticulum and cytosol contamination. The possibility of mitochondrial contamination in purified nuclei was discarded both on the basis of electronic microscopy observations and the absence of cardiolipin (mitochondrial lipid marker) in purified nuclei when lipid composition was determined. The nuclear content of DNA/mg protein is a further parameter of nuclear purity. The amount of DNA and the protein/DNA ratio in the purified nuclear fraction are comparable

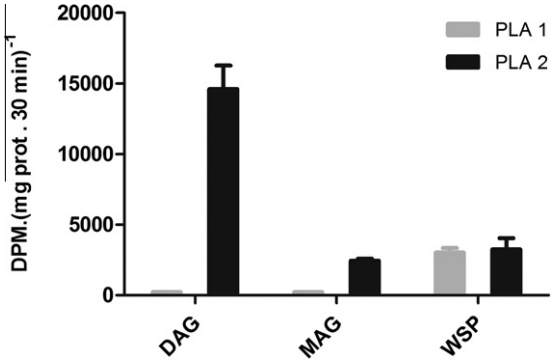

Fig. 8. PtdOH-selective PLA1 and PLA2 activities. DAG, MAG and water-soluble products were determined incubating isolated nuclear fraction and using 0.1 mM of [^{3}H]PtdOH as a substrate in each corresponding assay buffer, at 37 °C for 30 min. Lipid extraction and separation are specified in the Materials and methods section. The aqueous phase from Folch extraction containing radiolabel water-soluble products was concentrated and counted by liquid scintillation. Results represent the mean ± S.D. of 3 individual samples.

with the values for isolated nuclei given in the literature [46,52–54]. Furthermore, in agreement with Kanfer's findings [44], our nuclei preparations showed a 6.5-fold enrichment in DNA/mg prot in isolated nuclei compared with the starting homogenate. Based on the morphological and biochemical criteria used to assess the purity of nuclei prepared from rat cerebellum, the enzymatic activities evaluated in the present paper cannot be attributed to contamination with other subcellular fractions.

Our findings on phospholipid content evidence higher values with respect to those for nuclei from cerebral cortices [55] and for nuclei from rat liver [56]. DAG generation from PtdOH and its immediate partial degradation by DAGL has been thoroughly studied in the nervous system [18,26,57,58]. PAP1 and LPP forms have also been characterised taking into account Mg^{2+}-dependence and NEM-sensitivity [11,12]. Using these criteria we found no PAP1 activity in nuclei from cerebellar cells. Lipins, which belong to a novel family of PAP1 enzymes, localize at the cytoplasm but can also be found in the nucleus in adipocytes and hepatocytes [59]. Our data indicate the presence of LPP and DAGL activities in nuclei of cerebellar cells. The high percentage of MAG derived from DAG (50%) is indicative of a significant level of DAGL activity.

Phosphatidylcholine has been reported to be the principal source of DAG generated from entirely intact isolated nuclei [9,41]. This finding and the fact that PtdCho-PLD activity was found in neuronal nuclei [44] gives support to the presence of LPP in cerebellar nuclei. Divecha [60] suggests that nuclear DAG serves as a chemoattractant for PKC translocation to the nuclear compartment and that a mechanism to turn off the signal therefore follows. This role seems to be fulfilled by DAGL and DAGK, the latter of which has been described in nuclei [43,61].

On the other hand, it has been extensively reported that endogenously produced DAG is further hydrolyzed to MAG and glycerol [26,62]. In addition, it has been demonstrated that exogenously added [^{3}H]-diacylglycerol or [^{3}H]-diacylglycerol co-emulsified with PtdCho are poor substrates for DAGL [63]. The extremely hydrophobic nature of DAG molecules induces their localization inside vesicles from which they are inaccessible to the enzyme [64]. All these findings indicate that the enzymatic generation of DAG by LPP action provides a substrate with a suitable physical state for the expression of DAGL activity. In our work, DAGL activity was assayed taking into account all the above-mentioned premises. A traditional Michaelis–Menten kinetic analysis and time and protein curves were also performed using exogenously added DAG (Fig. 4). For a better interpretation of our results, exogenous DAG containing molecular species similar to DAG generated from PtdOH, was used. Although the kinetic parameters were similar assaying the enzyme in both ways at a low concentration of DAG, MAG production was recorded only from DAG generated by LPP action. These results corroborate the fact that the enzymatic generation of DAG by LPP action provides an appropriate substrate for the expression of DAGL activity.

Interestingly, our results show LysoPtdOH formation from PtdOH in cerebellum nuclei when PtdOH concentration is higher than 100 μM. This finding could therefore constitute the first evidence of PLA activity on PtdOH in nuclei. PLA 2 activity in nuclei hydrolyzes PtdCho and PtdEth at neutral pH [65]. Two phosphatidic-acid selective phospholipases, A1 α and β, have been characterised in plasma membrane [66]. Furthermore, a PtdOH-hydrolyzing PLA2 was purified from homogenate rat brain. This enzyme is PtdOH-selective and has a low measurable catalytic activity when PtdCho or PtdEth is used as substrate [67]. Several PLA activities have been identified in different subcellular fractions from mammalians, including nuclear fraction [68]. None of these enzymes, however, displays a preference for PtdOH as substrate. In order to determine whether or not PLA activity acting on PtdOH in our nuclear preparations is more similar to either PtdOH-specific PLA1 or PLA2 activity, we assayed each activity at its characteristic pH using the appropriate detergents and ions. Our results demonstrate that under PLA1 assay conditions, PtdOH is converted into LysoPtdOH and that it rapidly metabolises to glycerol-3-phosphate by the same PLA or by some other isoform present in nuclei. This also agrees with Hiramatsu's observations [66] according to which most of the PtdOH generated by treatment with PLD in cells expressing PtdOH-PLA1 forms, is converted into LysoPtdOH. However, under PtdOH-PLA2 assay conditions, PtdOH could be metabolised not only by PLA2 but also by LPP and water-soluble products could be formed by (i) LPP/DAGL/MAGL, (ii) PLA2/LPA phosphohydrolase/MAGL activities or (iii) PLA2/LPA phospholipase. As LPP and PtdOH-PLA2 assay conditions were similar, the fact that water-soluble products were only observed when PtdOH-PLA2 activity was evaluated could indicate that water-soluble products formation occurs mainly via pathway (iii). Since PtdOH can metabolise to LysoPtdOH, the lower availability of PtdOH as a result of the action of LPP will lead to the formation of less LysoPtdOH and thus a lower level of activation of the nuclear LysoPtdOH 1 receptor. The presence of LysoPtdOH 1 receptor was described in nuclei [49]. Polyunsaturated LysoPtdOH can also stimulate PPARγ receptors [69].

LPP dephosphorylates not only PtdOH but also other phosphorylated substrates such as LysoPtdOH, S1P and C1P. Each of these lipid phosphates competitively inhibits the use of PtdOH as substrate by the phosphohydrolase. We observed a marked competitive effect between PtdOH and LysoPtdOH, S1P and C1P by LPP at equimolecular concentrations of the alternative substrates for the enzyme. LPP1, LPP2 and LPP3 show the maximal catalytic efficiency for LysoPtdOH, PtdOH and S1P, respectively [70]. The degree of competitiveness observed between PA/LPA and PA/S1P suggests that LPP1 and LPP3 are the most active isoforms in nuclei from cerebellar cells.

There are three possible pathways of MAG formation using PtdOH as substrate, namely (i) by the action of LPP/DAGL, (ii) by the action of phospholipase A/LPP, and (iii) by the action of phospholipase A/LPA phosphohydrolase. In order to evaluate the contribution of each pathway to MAG formation, we analysed DAG and MAG formation in the presence of the different modulators of these pathways. The fact that Ca^{2+} and Mg^{2+} ions significantly stimulate MAG production without affecting DAG generation seems to suggest that MAG is originated not only by the LPP/DAGL pathway but also by phospholipase A/LPA phosphohydrolase or by phospholipase A/LPP pathways in nuclei from cerebellar cells. This is supported by evidence of an NEM-insensitive, Mg^{2+}-dependent LysoPtdOH phosphohydrolase in nuclei of cerebral cortex [46]. As

under our assay conditions LysoPtdOH formed by PLA was rapidly metabolised to MAG, it was not possible to detect LysoPtdOH because of its short half-life [49]. This is at least in part because accumulation of LysoPtdOH, as it occurs with other lysophospholipids [71], induces cell lysis. Nuclear LysoPtdOH removal occurs by LysoPtdOH lysophospholipase [72] and LysoPtdOH phosphohydrolase [46]. The absence of water-soluble products under our assay conditions may indicate that LysoPtdOH lysophospholipase cannot be detected. Similar DAG and MAG diminution by NaF and vanadate suggests that LPP/DAGL is the principal pathway of MAG formation under our assay conditions. Since LPP activity is suggested to be regulated by a phosphorylation/dephosphorylation mechanism and phosphoprotein phosphatase can be inhibited by fluoride, the effect of fluoride through the phosphorylation–dephosphorylation mechanism itself cannot be disregarded [73].

Previous studies have shown that MAG, a phosphohydrolase product, is rapidly degraded by MAGL, an enzyme that is more active than phosphohydrolase and sensitive to inhibition by AACOCF3 [72]. No MAGL activity was observed under our assay conditions, nor was MAG formation modified with respect to the controls in the presence of the MAGL inhibitors AACOCF3 or oleate (data not shown). However, using either MAG or 2-arachidonoylglycerol, the corresponding substrates for MAGL, we observed glycerol formation (13 ± 2 and 6.4 ± 0.6 nmol (15 min $\times$ mg prot.)$^{-1}$). This suggests that the MAG activity generated from PtdOH under our assay conditions is not sufficient to detect MAGL activity.

A dual effect was observed in the presence of Triton X-100. At a molar Triton X-100/PA ratio close to 2.5 (0.2 mM Triton X-100), no effect was observed on DAG generation by LPP, suggesting that the stimulus exerted on MAG production must have been due to the pathway involving phospholipase A/LPA phosphohydrolase and/or phospholipase A/LPP. At a detergent/PA ratio close to 12.5 (1 mM Triton X-100), however, LPP and DAGL activities were stimulated to a similar extent, indicating that the effect on DAGL could be a consequence of the higher availability of DAG, the substrate for DAGL. The different effects observed between Triton X-100 and deoxycholate could be due to the critical micellar concentration (CMC). Based on the detergent concentrations used, Triton X-100 was in its CMC (0.2–0.9 mM) whereas deoxycholate was below CMC (2–6 mM).

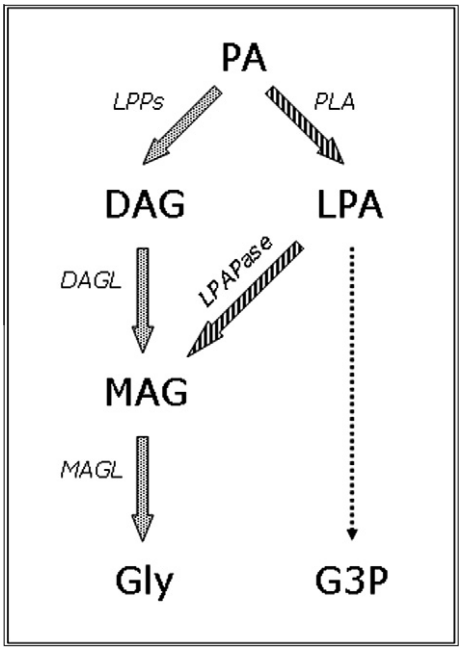

Fig. 9. Phosphatidic acid metabolism in cerebellum nuclei. Dashed lines represent a possible pathway to glycerol-3-phosphate (G3P) formation. The enzyme name is in italic script. LPP: lipid phosphate phosphatases; DAGL: diacylglycerol lipase; MAGL: monoacylglycerol lipase; PLA: Phospholipase A; LysoPtdOH Pase: lysophosphatidic acid phosphohydrolase.

PtdCho is a major source of lipid-derived second messengers molecules that function in both intracellular and extracellular signalling. PtdCho-PLD, LPPs and DAGL could be pivotal enzymes in this signalling system in nuclei and they could act in series to balance the biologically active lipids PA and DAG. DAG-binding proteins such as protein kinase *C* seems to constitute the target for the DAG generated by PLD/LPP pathway in nuclei.

Taken together, our findings reinforce the hypothesis that cerebellar nuclei have enzymes necessary to form a PtdOH signalling system. We therefore propose that PtdOH metabolism occurs in cerebellar nuclei as shown in Fig. 9. The interaction among enzymes controlling the level of PtdOH, the identification of PtdOH and DAG-regulated proteins, and the overlap between PtdOH metabolism and the signalling process, all open interesting avenues for further research on PtdOH in nuclei.

Acknowledgments

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