Archives of Biochemistry and Biophysics 507 (2011) 271–280

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

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

Virginia L. Gaveglio, Susana J. Pasquaré *, Norma M. Giusto

Instituto de Investigaciones Bioquímicas de Bahía Blanca, Universidad Nacional del Sur and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), C.C. 857, B8000FWB Bahía Blanca, Argentina

ARTICLE INFO

Article history:
Received 29 October 2010
and in revised form 29 December 2010
Available online 7 January 2011

Keywords:
Diacylglycerol
Lysophosphatidic acid
Monoacylglycerol
Nuclei
Phosphatidic acid

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

* Corresponding author. Fax: +54 291 4861200.
E-mail address: pasquare@criba.edu.ar (S.J. Pasquaré).

[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.

0003-9861/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2011.01.001

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