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Phosphatidic acid metabolism in rat liver cell nuclei



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ABSTRACT

The aim of the present research was to analyze the pathways for phosphatidic acid metabolism in purified nuclei from liver. Lipid phosphate phosphatase, diacylglycerol lipase, monoacylglycerol lipase and PA-phospholipase type A activities were detected. The presence of lysophosphatidic acid significantly reduced DAG production while sphingosine 1-phosphate and ceramide 1-phosphate reduced MAG formation from PA. Using different enzymatic modulators (detergents and ions) an increase in the PA metabolism by phospholipase type A was observed. Our findings evidence an active PA metabolism in purified liver nuclei which generates important lipid second messengers, and which could thus be involved in nuclear processes such as gene transcription.

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1. Introduction

Many evidences suggest that phospholipids as well as their polyunsaturated fatty acids are involved in several events inside the nucleus [1]. The nuclear envelope has been identified as the primary place of nuclear lipids although a few early studies have also suggested the presence of phospholipids as a component of chromatin [2]. In eukaryotic cells, PA is a key precursor for the synthesis of major glycerophospholipids and neutral lipids as well as a major signaling lipid [3–5]. PA can be dephosphorylated by the action of phosphatidate phosphatase 1 (PAP1) which is also known as lipin [6,7]. A second type of PA phosphatase that controls the signaling pools of PA and DAG is known as either phosphatidate phosphohydrolase 2 (PAP2) or lipid phosphate phosphatase (LPP) [4].

Abbreviations: 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]; LPA, lysophosphatidic acid; LPP, lipid phosphate phosphatase; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; NEM, N-ethylmaleimide; PA, phosphatidic acid; PAP1, NEM-sensitive Mg²⁺-dependent phosphatidate phosphohydrolase; PAP2, NEM-insensitive Mg²⁺-independent phosphatidate phosphohydrolase; PC, phosphatidylcholine; PI, phosphatidylinositol; PLA, phospholipase A; PLC, phospholipase C; S1P, sphingosine 1-phosphate; TLC, thin layer chromatography; WSP, water soluble products

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Several nuclear enzymes involved in phospholipid metabolism have been described and characterized, and there is evidence that some might have direct regulatory roles in various aspects of nuclear function [8]. Taking into account the multitude of functions of PA and DAG in biosynthetic and signaling pathways and because little is known on PA metabolism in hepatocyte nuclei, our study aims at studying the enzymatic mechanisms through which PA-removal operates in liver nuclei.

2. Materials and methods

2.1. Materials

[2-³H]glycerol (200 mCi/mmol) and Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA, USA). DAPI, sphingosine 1-phosphate, ceramide 1-phosphate from bovine brain, oleoyl-1-α-lysophosphatidic acid, D-sphingosine, and non-hydroxy fatty acid ceramide from bovine brain were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies anti Calnexin (sc-11397) and anti LAP2 (611000) were from Santa Cruz Biotechnology, INC. and BD Transduction Laboratories, respectively. All the other chemicals used were of the highest purity available.

2.2. Purified nuclear fraction preparation

Male Wistar-strain rats were kept under constant environmental conditions and fed on a standard pellet diet. Animal handling was carried out in agreement with the standards stated in the

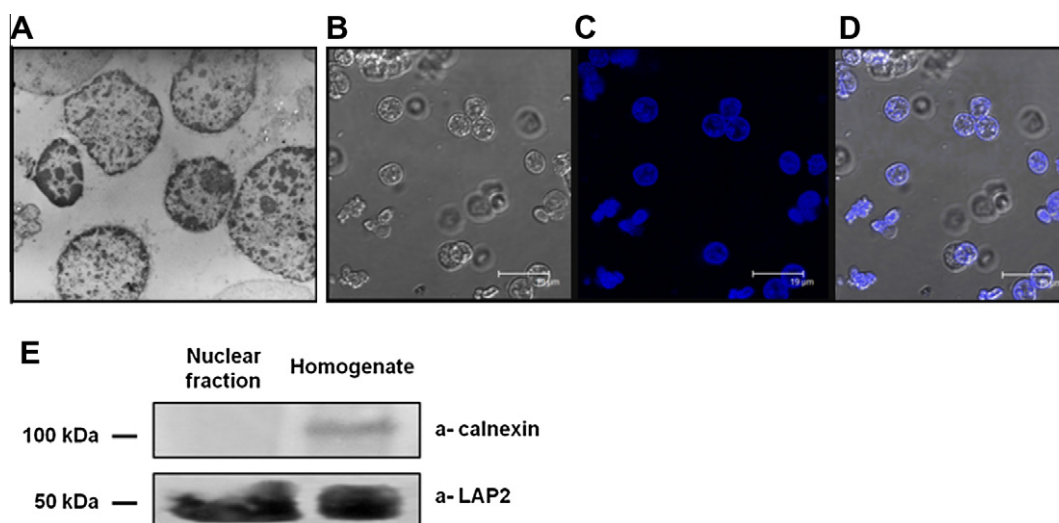


Fig. 1. Purity and integrity of nuclear preparations from rat liver. Electron micrographs of isolated nuclei prepared as described in Section 2, (A) 5000 times (scale bar 3.3 μm); (B) 600 times (scale bar 19 μm). (C) DAPI stained, isolated liver nuclei are in blue, 600 times (scale bar 19 μm), (D) merge, 600 times (scale bar 19 μm), (E) immunoblot analysis of CNX and LAP2 in homogenate and purified liver nuclei. Proteins (50 μg) were boiled in Laemmli buffer, resolved in a 10% SDS-PAGE and transferred to a PDVF membrane for further WB assays. Membranes were blocked and incubated with primary and secondary antibodies as detailed in Section 2. Immunoreactive bands were detected by enhanced chemiluminescence. Numbers on the left indicate molecular weights.

NIH Guide for the Care and Use of Laboratory Animals. Adult rats (4-month-old) were killed by decapitation and liver was immediately dissected (2–4 min after decapitation). The essential procedure for the isolation of nuclei was followed – with minor modifications – as described elsewhere [9,10]. The purified nuclear fraction was suspended in: (i) TKM to quantify proteins and DNA, (ii) 100 mM Tris-HCl, pH 8.5 to determine 5' nucleotidase, (iii) 0.2 M Na-phosphate pH 7 to determine NADH cytochrome c reductase, (iv) the buffer adequate to 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.

2.3. Evaluation of nuclear purity

The purity of nuclear preparations was assessed by transmission electron microscopy using a JEOL 100 CXII microscope operated at 80 kV, determining DNA content by diphenylamine assay [11] and measuring the activities of the marker enzymes 5' nucleotidase [12] and NADH cytochrome c reductase [13]. Calnexin (CNX), an unglycosylated resident ER transmembrane protein, was also determined in purified nuclei [14]. Integrity was assessed by nuclear staining with DAPI.

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

Radioactive PA was obtained from [2-³H]glycerol-PC ([³H]PC) which was synthesized from bovine retinas incubated with [2-³H]glycerol (200 mCi/mmol) as previously described [15]. [³H]PA (0.1 mM, 0.1–0.2 mCi/mmol) was prepared by sonicating in buffer solution containing 5.56 mM EGTA and 5.56 mM EDTA [16].

2.5. Enzymatic assays

2.5.1. LPP assay

LPP activity was determined in an assay medium containing 50 mM Tris-maleate buffer, pH 6.5, 1 mM EDTA plus EGTA, 4.2 mM NEM, 40 μg of nuclear protein and 100 μM [³H]PA, in a volume of 100 μl. When LPP activity was analyzed in the presence of LPA, S1P or C1P, the reaction was started by adding 100 μM [³H]PA (prepared as was specified in Section 2.4.) simultaneously with

different concentrations of unlabelled LPA, S1P or C1P (previously re-suspended in the assay buffer containing 1 mM Triton X-100) [16,17]. Radiolabeled PA was dried and re-suspended in the buffer assay and sonicated until clarity was reached.

2.5.2. PA-PLA assay

PA-PLA activity was determined as described previously [18]. Highly purified nuclei (40 μg of protein) were incubated in 100 mM Tris-HCl, pH 7.5, buffer containing 4 mM CaCl₂, 0.1% cholic acid and 0.2 mM of [2-³H]PA in a final volume of 100 μl, at 37 °C for 1, 3, 5, 10, 15 and 30 min.

LPP and PA-PLA enzymatic assays were slowed by the addition of chloroform:methanol (2:1, v/v). Blanks were prepared in the same way as each enzyme assay except that the nuclear fraction was inactivated by heating at 100 °C or by the addition of C:M (2:1, v/v).

Lipids were extracted with chloroform:methanol (2:1, v/v) and washed with 0.2 volume of CaCl₂ (0.05%) [19]. LPA, PA, MAG and DAG, lipids were chromatographed by TLC on silica gel G or H using different developing solvents as was described previously [10,20]. Chromatograms were visualized by exposure to iodine vapors and scraped off for counting by liquid scintillation. The aqueous phase from Folch extraction containing radiolabel water soluble products (WSP) 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).

2.6. SDS-PAGE and immunoblot

SDS-PAGE was carried out using 10% gels according to Laemmli [21]. Resolved proteins were transferred to immobilon P membranes using a Mini Trans-Blot cell electro blotter (BIO-RAD Life Science Group, CA, USA) for 90 min. Membranes were blocked for 2 h with Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl) pH 7.5, containing 0.1% Tween 20 (TTBS) and 5% milk or crystalline grade bovine serum albumin (BSA). Incubations with primary antibody anti-calnexin (1:2000) were carried out at room temperature for 3 h and with anti-LAP2 (1:2000) at 4 °C overnight. Membranes were washed with TTBS and subsequently exposed to the appropriate HRP-conjugated secondary antibody (anti-rabbit or anti-mouse)

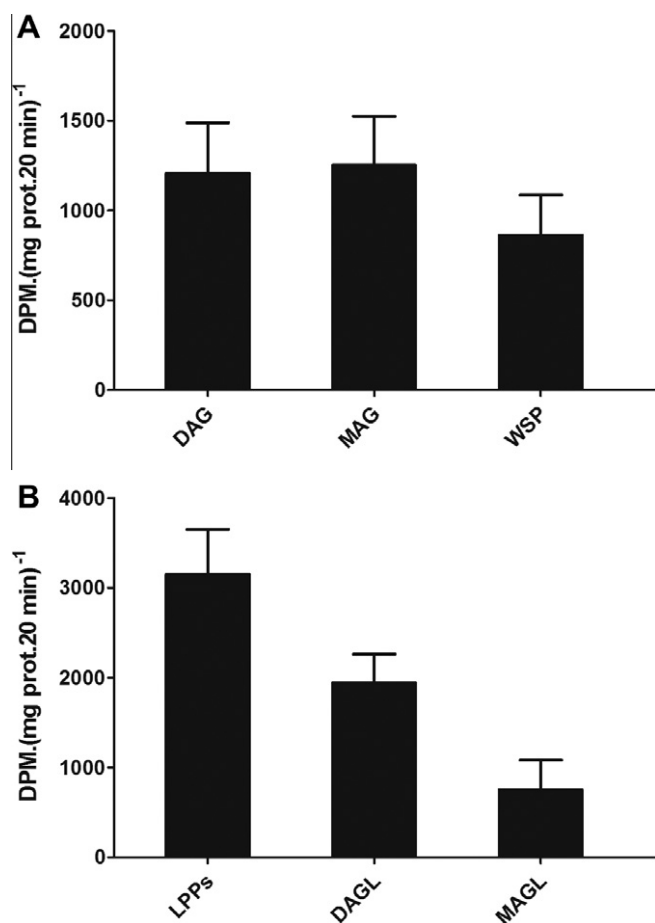


Fig. 2. Metabolism of [³H]-PA in isolated nuclei of rat liver. LPPs, DAGL and MAGL activities were determined using 80 μM [³H]PA and 40 μg of nuclear protein in a final volume of 100 μl, as was described in Section 2.5.1. DAGL activity was determined by monitoring the formation rate of monoacyl[2-³H]glycerol using, as substrate, diacyl[2-³H]glycerol derived from LPP action on [³H]PA. MAGL activity was determined by monitoring the formation rate of [2-³H]glycerol-WSP using monoacyl[2-³H]glycerol derived from diacyl[2-³H]glycerol. The subsequent procedure is specified in Section 2. In (A) and (B) results represent the mean ± S.D. of 11 individual samples.

for 2 h. The membranes rewashed with TTBS and immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences) using standard X-ray film (Kodak X-Omat AR).

2.7. Other methods

Protein and lipid phosphorus were determined according to Bradford [22] and Rouser et al. [23], respectively.

2.8. Statistical analysis

All data are given as means ± S.D. 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, CA, USA, www.graphpad.com). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Nuclear purity

The purity of the nuclear fraction was analyzed by morphological and biochemical studies. The electron microscopy analysis

from the isolated nuclei showed an intact nuclear envelope (Fig. 1A and B) and absence of contamination by non-nuclear membranes (Fig. 1A). Fig. 1C shows DAPI-stained nuclei, thus confirming nuclear integrity. The nuclear fraction could also be identified by its DNA content, having a DNA/protein ratio of 302 μg DNA/mg protein. Because LPP is originally located at the plasma membrane, we analyzed 5' nucleotidase activity as a marker of this fraction. Based on this activity, cross-contamination with plasma membrane was observed to be lower than 1% (Ht = 457 μM Pi/mg prot in homogenate and 4 μM Pi/mg protein in nuclei). The specific activity of LPP in nuclei was found to be 30% with respect to that determined in homogenate (10466 DPM (mg prot 20 min)⁻¹). Furthermore, NADH cytochrome c reductase activity, as a marker of endoplasmic reticulum [24], was assayed in all steps of nuclear purification. The activity of this enzyme in liver purified nuclei represents an 8% with respect to the total liver homogenate (363.4 and 29.3 nmol (min mg prot)⁻¹ in homogenate and nuclei, respectively). WB analysis, revealed the absence of CNX, an ER transmembrane protein, in purified liver nuclei. This fraction was verified by the presence of the nuclear protein marker LAP2 (Fig. 1E). Although the yield of nuclei (24%, based on DNA recovery; 1798 μg DNA/g tissue in homogenate vs 437.9 μg DNA/g tissue in nuclei) was low, the isolation procedure followed in this study was found to be the most adequate of all the methods tested. Taking into account the above-mentioned morphological and biochemical studies, isolated nuclei were considered to be highly pure.

3.2. Metabolism of phosphatidic acid in isolated nuclei

When nuclei were incubated with [³H]PA, DAG, MAG and WSP were detected. With 80 μM of [³H]PA, DAG and MAG formation was similar and WSP represented 68% of DAG and MAG (Fig. 2A). This is indicative of the presence of LPP, DAGL and MAGL in liver nuclei (Fig. 2B).

Previous studies showed that PAP1/lipins have cytosolic localization and that they translocate onto membranes in order to dephosphorylate PA [25,26]. Although has been confirmed the presence of this protein at nuclear level in the hepatic tissue [27], we were unable to detect lipin activity under the two typical assay conditions used (Supplementary data).

3.3. PA metabolism as a function of PA concentration, protein, time and pH

Under our assay conditions, DAG and MAG, and WSP production could be observed at PA concentrations of 50 and 80 μM, respectively (Fig. 3A). The rate of the product formation increased with PA concentrations up to 150 μM (Fig. 3A). The production of DAG, MAG and WSP was linear for 3 min (Fig. 3C) up to a protein concentration of approximately 40 μg (Fig. 3B). Optimum pH was around 6.5 (Fig. 3D). Subsequent experiments were carried out using 80 μM of PA and 40 μg of proteins, in which only DAG, MAG and WSP were formed from PA.

3.4. DAG and MAG production as a function of LPA, S1P and C1P concentrations

The data shown in Fig. 4 indicate the rate of DAG and MAG formation in the presence of LPP alternative substrates. LPA significantly decreased DAG production from [2-³H]PA (Fig. 4A). LPA diminished DAG formation by 39% at concentrations ranging from 25 to 100 μM and MAG formation showed a 41% decrease at 100 μM (Fig. 4A). MAG formation was found to decrease by 33% in the presence of 10 μM of S1P (Fig. 4B). An inhibitory tendency was observed in DAG production in the presence of 50 and 100 μM of C1P (Fig. 4C). MAG formation was diminished by 33%

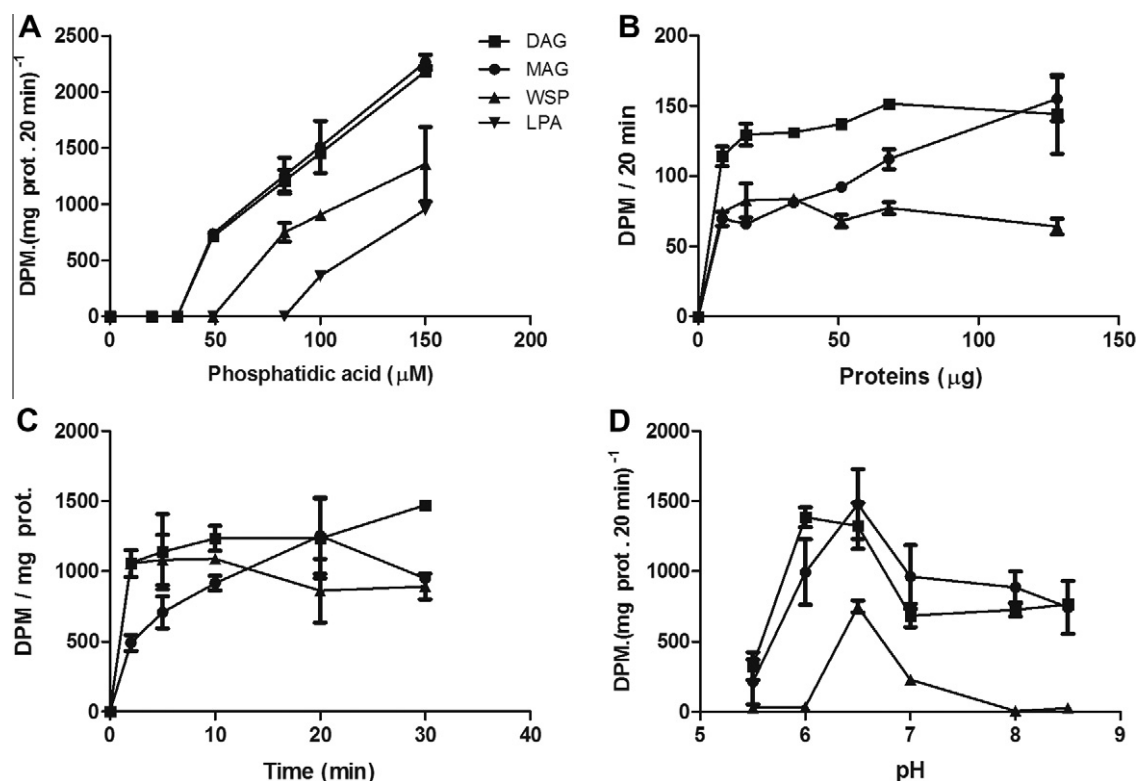


Fig. 3. DAG, MAG and WSP production as a function of PA concentration, protein, time and pH in isolated nuclei of rat liver. DAG, MAG and WSP formation was measured as a function of PA concentration (A), protein concentration (B), time (C) and pH (D). 40 μg of protein for 20 min; 80 μM [³H]PA for 20 min and 80 μM [³H]PA and 40 μg of protein, were the assay conditions used in (A), (B) and (C), and (D), respectively. Results represent the mean ± S.D. of three individual samples.

at 10 μM of C1P, its formation being recovered at 100 μM of C1P. WSP formation underwent no changes with LPP alternative substrates (data not shown).

3.5. Effects of detergents and ions on DAG, MAG and WSP

DAG and MAG production was analyzed by varying the molar ratio of detergent (used in its critical micellar concentration) with respect to PA, which was maintained at 80 μM. When Triton X-100 was used at a molar Triton X-100/PA ratio close to 2.5 (0.2 mM Triton X-100), DAG production remained unmodified whereas MAG and WSP production increased by 67% (Fig. 5A). At a non-ionic detergent/PA ratio close to 12.5 (1 mM Triton X-100) only DAG formation was stimulated (50%) (Fig. 5A). Ca²⁺ (1 mM) and Mg²⁺ (4 mM) ions stimulated MAG formation by 54% and 69%, respectively (Fig. 5B). Ca²⁺ (10 mM) and Mg²⁺ (4 mM) ions stimulated WSP production by 115% and 62%, respectively (Fig. 5B). DAG production was not affected by Ca²⁺ whereas Mg²⁺ (4 mM) ions inhibited DAG production by 31% (Fig. 5B). NaF at 50 mM concentration diminished DAG and MAG generation by 69% (Fig. 5B) whereas a 146% stimulus was observed in WSP (Fig. 5B).

3.6. PA-PLA activity

Under our assay conditions at 1 min incubation time for the determination of PA-PLA activity, LPA formation as well as MAG and WSP formation were observed (Fig. 6). In addition, the formation pattern of products generated by PA-PLA activity revealed an increase in WSP in parallel to a decrease in LPA and MAG as a function of time (Fig. 6).

4. Discussion

Different roles have been attributed to nuclear lipids in the organelle functionality including DNA stabilization, stimulus of RNA and DNA polymerases and initiation of apoptosis [2]. Enzymatic activities in nuclei are either related to lipid metabolism and/or involved in nuclear signaling [2,28,29].

PA and its dephosphorylation product, DAG, are second messengers in agonist-stimulated cell activation. They have been detected in nuclei and have been found to increase during cell proliferation [30]. LPA, the other product generated from PA, behaves as an agonist [31] and its receptors localize at the cell nucleus [32].

LPPs which are integral membrane enzymes mainly located at the plasma membrane, are relatively non-selective for their substrate as they act on other phosphorylated lipids apart from PA [33]. LPPs are involved in lipid signaling in mammalian cells as part of phospholipase D and LPP pathway [4,33].

Our data revealed an active PA metabolism involving the presence of LPP, PLA, LPA phosphohydrolase, LPA phospholipase, DAGL and MAGL activities in liver nuclei. PC has been reported to be the principal source of DAG generated from entirely intact isolated nuclei [34]. The DAG signal turning off mechanism could be fulfilled by DAGL [10] and DAGK [35].

On the other hand, rat liver nuclei have enough enzymes to free fatty acids from phospholipids [36] as well as to incorporate exogenous saturated and unsaturated fatty acids into lipids [37]. In this respect, our results showed that under PLA assay conditions LPA is formed from PA and that it could be rapidly converted into MAG and glycerol-3-phosphate by PLA or LPP and by LPA-phospholipase, respectively. This also agrees with other observations [18] according to which most of the PA generated by treatment with PLD in cells expressing PA-PLA forms is converted into LPA. Some of the

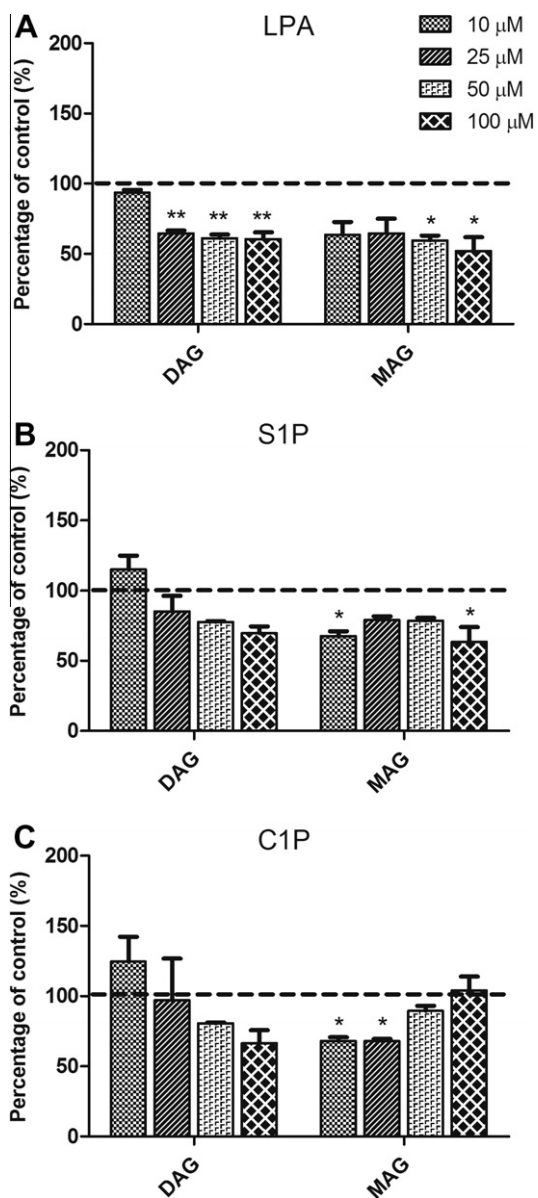


Fig. 4. Metabolism of [³H]PA in the presence of LPA, S1P and C1P in isolated nuclei of rat liver. The effects of LPA (A), S1P (B) and C1P (C) on PA hydrolysis were evaluated using 80 μM [³H]PA in the presence of LPA, S1P or C1P at the indicated concentrations. The subsequent procedure is specified in Section 2. Results represent the mean ± S.D. of three individual samples. **P* < 0.05, ***P* < 0.005 with respect to the absence of LPA, S1P or C1P.

PLA activities which hydrolyze either PC or PE have been identified in nuclei but they do not hydrolyze PA [36,38]. Furthermore, PA-PLA activities in liver nuclei seem to be key as they are likely to generate long chain fatty acids, which are endogenous ligands with high affinity for nuclear receptors [39,40]. This interaction initiates the transcription of multiple genes involved in lipid and glucose metabolism [41,42], activities inherent to the hepatic function.

On the other hand, LPP dephosphorylates not only PA but also LPA, S1P and C1P. Each of these lipid phosphates competitively inhibits the use of PA as substrate by phosphohydrolase. In the present study, a marked competitive effect could be observed between PA and LPA. This seems to be indicative of the presence of LPP1 and LPP2 in purified nuclei in agreement with observations from Roberts et al. [43]. Thus, LPPs could be interpreted to have an important role in attenuating LPA signal through its nuclear

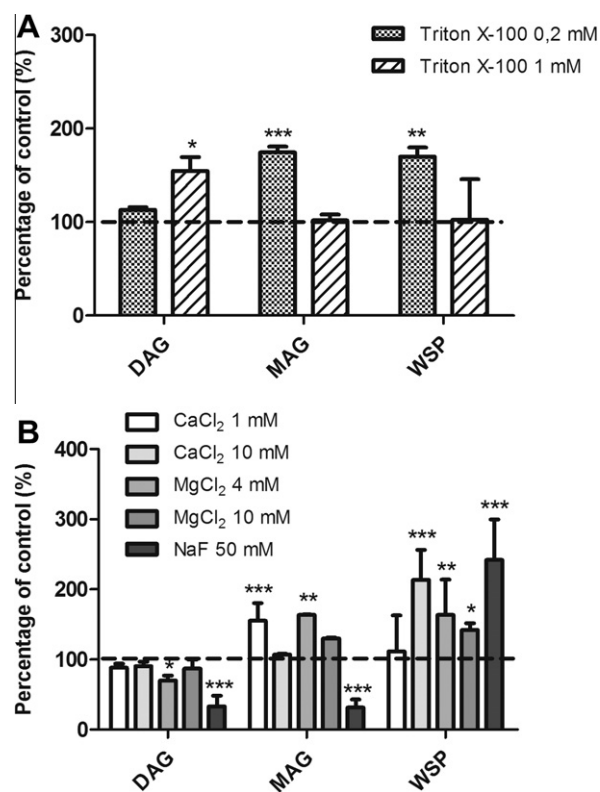


Fig. 5. Effects of detergents and ions on DAG and MAG production in isolated nuclei of rat liver. DAG and MAG production was determined in the presence of the non-ionic detergent Triton X-100 (A) and in the presence of Ca²⁺, Mg²⁺ or NaF (B). All assays were conducted under the same conditions as described in Fig. 2. Results are indicated as a percentage of control values (controls represent 100%) and they represent the mean ± S.D. of three individual samples. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005 with respect to control values.

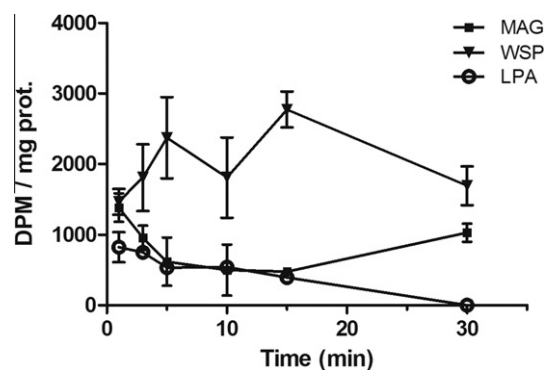


Fig. 6. PA-PLA activity in isolated nuclei of rat liver. MAG, WSP and LPA were determined incubating 40 μg of nuclear protein and 0.2 mM of [³H]PA as a substrate in the corresponding assay buffer, as function of time. Results represent the mean ± S.D. of three individual samples.

receptor type 1 [32]. The C1P effect observed on MAG production could be due to an inhibition on LPA phosphohydrolase in agreement with results from Baker et al. [44].

MAG and WSP can be formed from PA by the action of (i) LPP/DAGL/MAGL, (ii) PLA/LPP/MAGL, and (iii) PLA/LPA phosphohydrolase/MAGL. With the purpose of evaluating this metabolism, we analyzed the production of DAG, MAG and WSP using different modulators of these enzymatic pathways. Ca²⁺ and Mg²⁺ ions significantly stimulate MAG and WSP production without affecting DAG generation. This suggests that the contribution to MAG and WSP formation occurs not only through LPP/DAGL/MAGL pathway

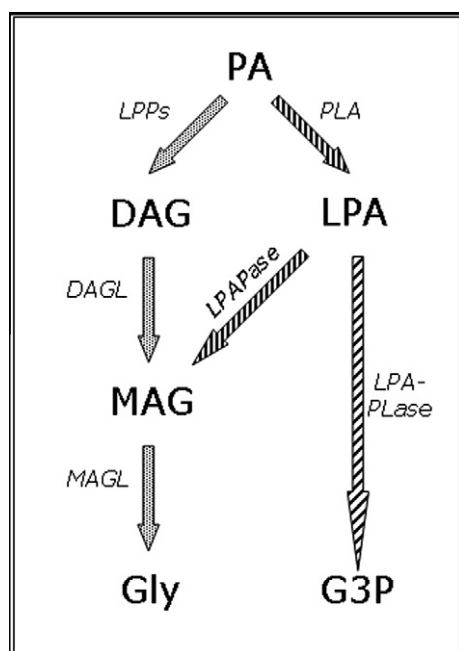


Fig. 7. Phosphatidic acid metabolism in rat liver nuclei. The enzyme name is in italic script. LPP: lipid phosphate phosphatases; DAGL: diacylglycerol lipase; MAGL: monoacylglycerol lipase; PLA: phospholipase A; LPAPase: lysophosphatidic acid phosphohydrolase; LPAase: lysophosphatidic acid phospholipase.

but also through (ii) and (iii) pathways in liver nuclei. Under our assay conditions it was not possible to detect LPA, which indicates that LPA formed by PLA could be rapidly metabolized by LPA lyso-phospholipase [45] and LPA phosphohydrolase [44]. In the presence of NaF, DAG and MAG diminution suggest that LPP/DAGL is the principal enzymatic pathway for MAG formation; while the increase in WSP production could indicate a high PA availability for PLA activity.

Triton X-100 produced a dual effect on PA metabolism. Triton X-100 (0.2 mM), did not affect DAG generation by LPP, thus suggesting that MAG and WSP production could be due to a stimulus exerted on PLA/LPA phosphohydrolase/MAGL pathway. At 1 mM Triton X-100, however, LPP activity was stimulated while MAG and WSP production was not modified, thus indicating an inhibition on DAGL/MAGL and/or PLA/LPA phosphohydrolase/MAGL activities.

The formation of DAG from PA by LPP coupled with a nuclear PLD, a pathway different from PC-PLC, may be highly relevant in nuclei. In this respect, it has been reported that PKC ζ may translocate to the nucleus in response to mitogenic signals by a binding mechanism through which PKC binds to DAG [46].

Summing up, our findings reinforce the hypothesis that liver nuclei have the necessary enzymes to form a PA signaling system. We therefore propose that PA metabolism occurs in liver nuclei as shown in Fig. 7. The interaction among the enzymes controlling the level of PA and the other molecules derived from it, the identification of PA- and DAG-regulated proteins and the presence of receptors that respond to signals derived from PA, all open interesting avenues for further research on PA in nuclei and its pathophysiological role in liver.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2013.01.074>.

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