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ORIGINAL PAPER

Involvement of Lysophosphatidic Acid, Sphingosine 1-Phosphate and Ceramide 1-Phosphate in the Metabolization of Phosphatidic Acid by Lipid Phosphate Phosphatases in Bovine Rod Outer Segments

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Abstract The aim of the present research was to evaluate the generation of [2-^{3}H]diacylglycerol ([2-^{3}H]DAG) from [2-^{3}H]-Phosphatidic acid ([2-^{3}H]PA) by lipid phosphate phosphatases (LPPs) at different concentrations of lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and ceramide 1-phosphate (C1P) in purified ROS obtained from dark-adapted retinas (DROS) or light-adapted retinas (BLROS) as well as in ROS membrane preparations depleted of soluble and peripheral proteins. Western blot analysis revealed the presence of LPP3 exclusively in all membrane preparations. Immunoblots of entire ROS and depleted ROS did not show dark–light differences in LPP3 levels. LPPs activities were diminished by 53% in BLROS with respect to DROS. The major competitive effect on PA hydrolysis was exerted by LPA and S1P in DROS and by C1P in BLROS. LPPs activities in depleted ROS were similar to the activity observed in entire DROS and BLROS, respectively. LPA, S1P and C1P competed at different extent in depleted DROS and BLROS. Sphingosine and ceramide inhibited LPPs activities in entire and depleted DROS. Ceramide also inhibited LPPs activities in entire and in depleted BLROS. Our findings are indicative of a different degree of competition between PA and LPA, S1P and C1P by LPPs depending on the illumination state of the retina.

Keywords Rod outer segments · Lipid phosphate phosphatases · Phosphatidic acid · Diacylglycerol

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Abbreviations

C1P	Ceramide 1-phosphate
DAG	Diacylglycerol
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid
LPA	Lysophosphatidic acid
LPPs	Lipid phosphate phosphatases
NEM	*N*-ethylmaleimide
PA	Phosphatidic acid
PAP2	NEM-insensitive phosphatidate phosphohidrolase
PC	Phosphatidylcholine
PMSF	Phenylmethylsulfonylfluoride
S1P	Sphingosine 1-phosphate
TLC	Thin layer chromatography

Introduction

Lipid phosphate monoesters, including phosphatidic acid (PA), lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P), are intermediaries in phospho- and sphyngolipid biosynthesis and they also play important roles in intra- and extracellular signaling. Dephosphorylation of these lipids terminates their signaling actions and generates products with additional biological activities or metabolic fates [1]. The key enzymes responsible for the dephosphorylation of these lipid phosphate substrates are termed lipid phosphate phosphatases (LPPs). The latter display isoforms and cell specific localization patterns which are distributed between endomembrane compartments and the plasma membrane. The role of LPPs in intracellular lipid metabolism and in the regulation of both intra- and extracellular signaling

pathways that control different cellular functions have been described [2–5].

Rod outer segments (ROS) have the ability to adapt the sensitivity and speed of their responses to ever changing conditions of ambient illumination. Recent evidence has demonstrated that a major contributor to this adaptation is the light-driven translocation of key signaling proteins into and out of ROS, which constitute the cellular place where phototransduction occurs [6]. It has also been reported that transducin, arrestin and recoverin [7–10] are proteins involved in this mechanism. Previous studies revealed the presence of phosphatidate phosphohydrolase 2 (PAP2, renamed as LPPs) and its regulation in isolated ROS from bovine retina [11–14]. In this work, the generation of [2-^{3}H]DAG from [2-^{3}H]PA was analyzed either in the absence or in the presence of LPA, S1P or C1P, all of which are alternative substrates for LPPs. The presence of LPP3 isoform in isolated ROS was also determined by western blot.

It has been extensively reported that the activity of enzymes involved in ROS phospholipid turnover such as phospholipase C [15, 16], phospholipase A2 [17], phosphatidylethanolamine *N*-methyltransferase [18], diacylglycerol kinase [19], PAP2 [14], phosphoinositide-3-kinase [20, 21], and phospholipase D [22] is modulated by light. This fact and the evidence that light induces a redistribution of proteins involved in phototransduction open an attractive field for the study of PA hydrolysis by LPPs under illumination conditions. We have also characterized LPP activities in the presence of [2-^{3}H]PA co-incubated with LPA, or S1P or C1P in ROS prepared from dark-adapted retinas (DROS) as well as in ROS prepared from light-adapted retinas (BLROS). Our results provide evidence that PA is differently hydrolyzed by LPPs in the presence of LPA, S1P, and C1P. Light participation in the competition of LPA, S1P and C1P on PA metabolization is also demonstrated.

Experimental Procedure

Materials

[2-^{3}H] Glycerol (200 mCi/mmol) was obtained from New England Nuclear-Dupont, Boston, MA. Sphingosine 1-phosphate, ceramide 1-phosphate from bovine brain, oleoyl-L-α-lysophosphatidic acid, D-sphingosine, and non-hydroxy fatty acid ceramide from bovine brain were obtained from Sigma-Aldrich, St. Louis, MO, USA. Monoclonal antibody against rhodopsin, Rho4D2, was generously supplied by Robert Molday from the University of British Columbia, Vancouver, Canada. The secondary antibody used by the rhodopsin immunoblots was HRP-conjugated anti-mouse.

Anti-PAP2B (anti-LPP3) was from Upsate biotechnology (catalog number 07-142). The secondary antibody used for anti-PAP2b detection was HRP-conjugated anti-rabbit. All the other chemicals were of the highest purity available.

Dark and Light Adaptation Procedure

Bovine eyes were obtained from a local abattoir, placed on ice within 10 min of the animal's death, and they were kept in darkness for 2 h. Retinas were dissected from the eyes after dark- or light adaptation. Dark-adapted bovine ROS (DROS) and light-adapted bovine ROS (LROS) were prepared under dim red light from DROS. The bleaching process was carried out after removing the cornea and the lens from the eyeballs and by exposing them to light (300 W) at 30 cm for 30 min at room temperature [20]. Controls were performed under the same conditions but under darkness (DROS).

Rod Outer Segment Isolation

The subsequent procedures for ROS preparation were conducted under dim red light for DROS and LROS, and under room light for BLROS and they were carried out at 2–4°C. To isolate ROS, retinas were removed and shaken twice in a 40% sucrose solution containing 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 1 μg/ml aprotinin, and 2 μg/ml leupeptin in 70 mM sodium phosphate buffer (pH 7.2). The remains of retinas were sedimented at 2,200*g* for 4 min and the supernatants containing ROS were diluted 1:2 with sucrose-free buffer and then centrifuged during 30 min at 35,300*g*. ROS were purified by a discontinuous gradient of sucrose [23], yielding: (i) a ROS band (Band I) retained at the 0.84/1.00 M density interface; (ii) a band (Band II) retained at the 1.00/1.14 M density interface of broken ROS contaminated with mitochondria and RIS, and (iii) a pellet composed of non-ROS membranes. The purity of ROS membrane preparations was monitored by electron microscopy. Electron micrographs from purified ROS (Band I) showed intact ROS with their typical structures and no other membrane material was observed (data not shown). The purity of Band I was also controlled by the determination of the absorbance ratio at 278 and 500 nm of solubilized membranes in 70 mM potassium phosphate buffer (pH 7) containing 1% emulphogene. Values of 2.3 ± 0.2 were typically obtained for this ratio. In addition, the purity of membranes was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [24]. Even in overloaded gels (80 μg of ROS protein), rhodopsin comprised 85–90% of photoreceptor integral membrane proteins. Moreover, thin-layer chromatography of

photoreceptor membrane lipids in overloaded plates showed no cardiolipin, thus suggesting non-detectable contamination with mitochondria. Instead, an enrichment of long-chain polyunsaturated fatty acids esterified to di-polyunsaturated molecular species of PC was observed. This enrichment, which is characteristic of bovine ROS [25]. Purity of ROS populations, particularly batches, was determined by assaying marker enzymes activities in all fractions of the gradients. NADPH-cytochrome *c*-reductase (microsomal marker) and cytochrome *c*-oxidase (mitochondrial marker) activities were measured in Band I, Band II and pellet of the gradient. Cytochrome *c*-oxidase was more enriched in Band II and pellet whereas the microsomal marker activity was very low in Band I and evidenced the highest activity in the pellet. These results led us to confirm that Band I (purified ROS) contamination with microsomes or mitochondria was lower than 5% [26, 27].

Soluble and Peripheral Protein Extraction from ROS with Low Ionic Strength Buffer

ROS pellets from DROS or BLROS were resuspended (1 mg protein per ml) in low ionic strength buffer prepared with 5 mM Tris–HCl (pH 7.4), containing 0.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin and 1 μg/ml of pepstatin, followed by 15 passages through a G25 5/8 needle. The membrane suspensions were centrifuged at 35,300*g* for 30 min. The supernatants were removed and centrifuged again in order to ensure that all particulate material was sedimented and membrane pellets were extensively washed. Both membranes and the clear supernatants were analyzed to determine the polypeptide composition, the presence of LPP3 and the activity of LPPs.

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

Radioactive phosphatidic acid was obtained by the action of phospholipase D on [2-^{3}H]-phosphatidylcholine [28]. [2-^{3}H]-phosphatidylcholine was synthesized from bovine retinas incubated with [2-^{3}H] glycerol (200 mCi/mmol) as previously described [11]. Lipids were extracted from the tissue following Folch et al. procedure [29]. [2-^{3}H]-Phosphatidylcholine was isolated by mono-dimensional TLC and eluted there from [30]. [2-^{3}H]PA, the hydrolysis product of [2-^{3}H]-PC, 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). The substrate was eluted from silica gel with neutral solvents to avoid the formation of lysophosphatidic acid. It 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 phosphorous content [31] were measured to determine specific radioactivity. [2-^{3}H]PA with a specific radioactivity of 0.1–0.2 μCi/μmol was obtained.

Determination of LPPs Activities

For the determination of LPPs activities the assay contained 50 mM Tris–maleate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA plus 1 mM ethylene glycol-bis(*β*-aminoethylether *N*,*N*,*N*′,*N*-tetraacetic acid (EGTA), 4.2 mM NEM and 100 μg of ROS membrane proteins in a volume of 200 μl. The reaction was started by the addition of 100 μM of [2-^{3}H]-phosphatidate/Triton X-100 mixed micelles in a constant 1:50 molar ratio of lipid to Triton X-100 [32]. [2-^{3}H]-phosphatidate was dried under a stream of nitrogen and resuspended in 30 μl of buffer assay containing Triton X-100, this aqueous microdispersion was sonicated in a sonication tip until clarity. The effect of the alternative substrates on PA metabolization by LPP activities, was evaluated using 100 μM [2-^{3}H]PA/Triton X-100 mixed micelles in the presence of LPA, S1P or C1P (previously resuspended in the buffer assay containing Triton X-100) at the indicated concentrations [32, 33]. Sphingosine and ceramide were solubilized in 0.1% of dimethyl sulfoxide (DMSO) as vehicle; the results obtained were compared against controls performed with the vehicle. The assays for the determination of LPPs were conducted at 37°C for 30 min under dim red light (DROS), under room light (LROS) or under 300 W light (BLROS). The enzymatic assay was stopped by adding chloroform/methanol (2:1, v/v). Blanks were prepared identically, except that membranes were boiled for 5 min before being used. PA products generated by LPP activities were isolated and measured as described below. The enzymatic activity was expressed as the sum of nmol of [2-^{3}H] diacylglycerol and [2-^{3}H] monoacylglycerol $\times$ (h mg protein)$^{-1}$.

Extraction and Isolation of Lipids

Lipids were extracted with chloroform/methanol (2:1, v/v) and washed with 0.2 volumes of $CaCl_2$ (0.05%) [32]. Neutral lipids were separated by gradient-thickness thin-layer chromatography on silica gel G [34] and developed with hexane/diethyl ether/acetic acid (35:65:1, v/v). To separate monoacylglycerol (MAG) from phospholipids, 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. Once the chromatogram was developed,

[2-^{3}H] PA and phospholipids were retained at the spotting site. Lipids were visualized by exposure of the chromatograms to iodine vapors, scraped off the plate, and quantified by liquid scintillation spectroscopy.

SDS–PAGE and Immunoblot Analysis

SDS–PAGE was performed using 7.5% or 10% gels according to Laemmli [24]. Resolved proteins were transferred to immobilon P membranes using a Mini Trans-Blot cell electro blotter (BIO-RAD Life Science Group, California) for 1 h. Membranes were blocked overnight with Tris-buffered saline (20 mM Tris–HCl, 300 mM NaCl) pH 7.5, containing 0.1% Tween 20 and 5% crystalline grade bovine serum albumin (BSA). Incubations with primary antiserum were performed at room temperature for 2–3 h. Immunoreactions were detected by means of either horseradish peroxidase conjugated to goat antirabbit or goat antimouse IgG followed by enhanced chemiluminescence substrates (ECL; Amersham Biosciences, Inc.). In some experiments, immunoblots were stripped by incubation in 200 mM Tris–HCl buffer, pH 6.7, containing 100 mM β-mercaptoethanol, 2% sodium dodecyl sulfate (SDS) for 1 h at 50°C with gentle agitation. Blots were reblocked with 5% BSA in Tris-buffered saline and probed as described above. Immunoreactive bands were quantified using image analysis software (Image J, a freely available application in the public domain for image analysis and processing, developed and maintained by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA).

Other Methods

Lipid phosphorus and protein were determined as described elsewhere [31, 35].

Statistical Analysis

Statistical analysis was done using Student's *t*-test with the values representing the mean ± SD of the total number of samples indicated in each figure legend.

Results

Hydrolysis of [2-^{3}H]-phosphatidate by LPPs in DROS and BLROS as a Function of LPA, S1P and C1P Concentrations

The DAG formed by LPP activities is partially hydrolyzed by diacylglycerol lipase (DAGL), yielding MAG. DAGL is coupled to LPPs and it seems that these enzyme activities are working in a sort of enzyme complex. Taking this into account the DAG generation and its partial degradation by DAGL immediately occurs. This fact has been extensively described in our laboratory [11, 12]. For this reason the most accurate from to express LPP activity is as the sum of nmol of [2-^{3}H] diacylglycerol and [2-^{3}H] monoacylglycerol × (h mg protein)$^{-1}$.

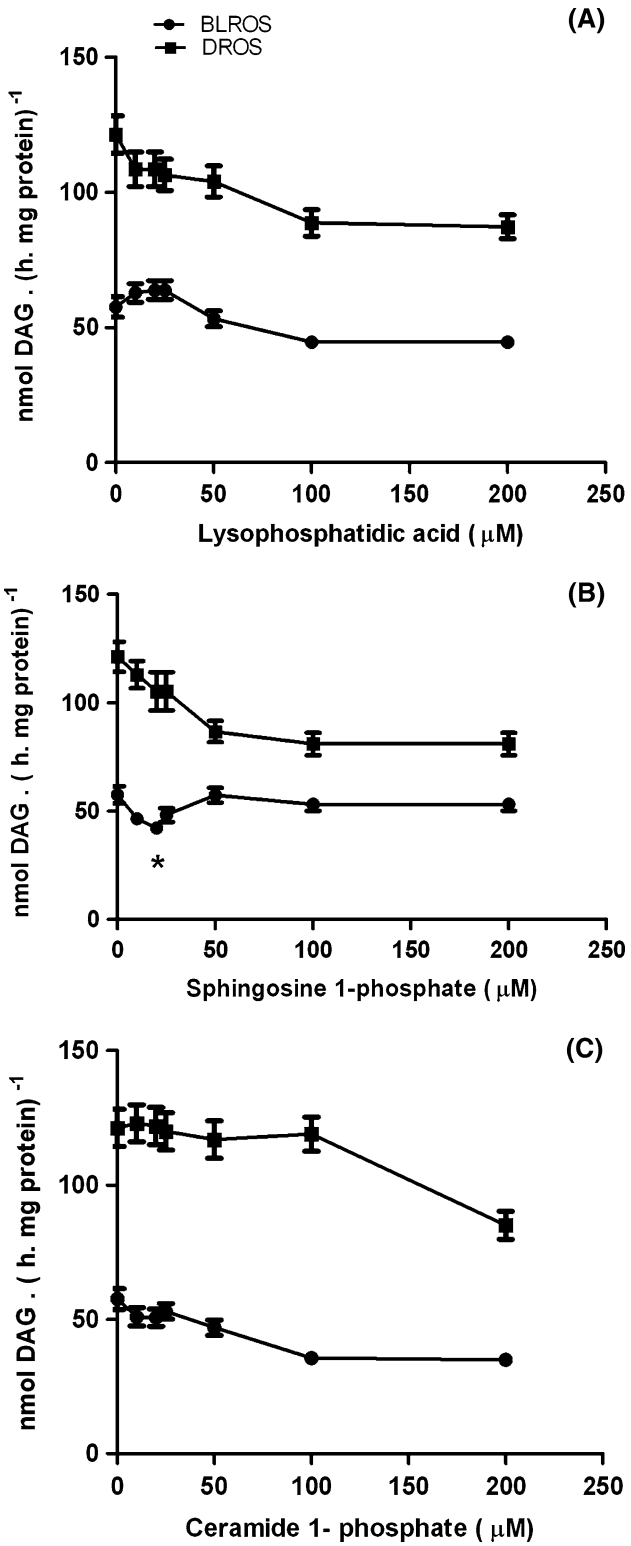
Data presented in Fig. 1 show the rate of DAG formation from [2-^{3}H]-phosphatidate (PA) in the presence of the alternative substrates using two ROS populations: DROS (square symbols) and BLROS (circle symbols). When the light effect was evaluated on LPP activities using only [2-^{3}H]PA as substrate, an inhibition of 53% was observed in BLROS with respect to DROS (Fig. 1). LPA and S1P significantly decreased DAG ($P < 0.025$) production from [2-^{3}H]PA in a concentration-dependent manner in DROS (Fig. 1, square symbols). LPA diminished DAG formation by 15% at concentrations ranging from 10 to 50 μM and by 29% at the highest concentration of LPA assayed (200 μM) (Fig. 1A). MAG generation from LPA could also be catalyzed by LPA phosphohydrolase. The presence of this enzyme activity has not been described in isolated ROS. Moreover, in our experiments with non-labeled LPA it is not possible to evaluate the presence of this enzyme.

An important diminution in DAG production was observed at low concentrations of S1P reaching 27% at 50 μM of S1P (Fig. 1B). C1P exerted an inhibitory effect (30%) on DAG formation at concentrations higher than 100 μM (Fig. 1C).

In order to determine the effect of light (Fig. 1, circle symbols) in the competition of LPA, S1P and C1P in PA hydrolysis, LPP activities were also evaluated in BLROS in the presence of increasing concentrations of the alternative substrates for the enzyme. In BLROS a 22% decrease in DAG formation was observed at 100 μM of LPA (Fig. 1 A). In BLROS PA dephosphorylation was diminished by 19% at 20 μM of S1P whereas at 100 μM of S1P, DAG levels returned to basal levels (Fig. 1B). On the other hand, C1P diminished DAG production at all the concentrations assayed, being highest (41%) at 200 μM (Fig. 1C).

Hydrolysis of [2-^{3}H]-phosphatidate by LPPs in LROS and BLROS in the Presence of LPA, S1P and C1P

For the experiments aimed at the description of LPP activities under different illumination conditions we used three distinct ROS populations: (i) DROS: obtained from dark-adapted retinas and purified under dim red light, (ii) LROS: obtained from DROS and exposed to room light for the enzyme assays, (iii) BLROS: obtained from light-adapted retinas and purified under room light.



◀ **Fig. 1** Hydrolysis of [2-^{3}H]-phosphatidate (PA) by LPPs in DROS and BLROS as a function of lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P) concentrations. LPPs activities were determined using, as an enzyme source, either purified ROS from dark-adapted retinas (DROS) or bleached ROS (BLROS) from DROS whose eye cups were exposed to room light (300 W at 30 cm) for 30 min as specified in Experimental Procedure. The effect of the alternative substrates on PA hydrolysis by LPP activities, was evaluated using 100 μM [2-^{3}H]PA/Triton X-100 mixed micelles in the presence of LPA (**A**), S1P (**B**) or C1P (**C**) at the indicated concentrations. The enzymatic assay was made under dim red light for DROS or under 300 W light for BLROS. Incubation products were subsequently extracted and separated by gradient-thickness thin-layer chromatography and visualized after exposure to iodine vapor. The bands corresponding to PA, DAG and MAG were scraped and quantitated by liquid scintillation spectroscopy. Results represent the mean ± SD of nine individual samples. (*$P < 0.025$)

Figure 2 shows the percentage of LPPs activities in LROS and BLROS with respect to the activities determined in DROS (considered as 100%) in the absence or in the presence of 20 or 100 μM of LPA, S1P or C1P. LROS were obtained, exposing DROS to room light. Under these experimental conditions an inhibition of 24% in LPPs activities with respect to DROS was observed (Fig. 2). LPA and C1P did not modify LPPs activities from LROS (Fig. 2A, C), whereas in the presence of S1P, LPPs activities were stimulated by 13% (Fig. 2B). A differential effect of alternative substrates in PA hydrolysis pattern was observed between LROS and BLROS. In all cases, LPPs activities were higher in LROS than in BLROS. Based on the results shown in Fig. 2 a ratio between the percentages of LPPs activities from LROS and BLROS was determined. This ratio was 1.6 in the presence of PA as unique substrate, 1.3 in the presence of LPA (20, 100 μM), 2.2 and 1.3 in the presence of either 20 or 100 μM of S1P, respectively, and it was 1 in the presence of 100 μM of C1P.

SDS–PAGE and Immunoblot Analysis

We determined the protein composition of entire DROS and BLROS preparation and of DROS and BLROS subject to successive washes with low ionic strength buffers. Low ionic buffer treatment renders a ROS membrane preparation depleted of soluble and peripheral proteins. Both the membrane and soluble fractions obtained there from were used for SDS–PAGE analysis. The levels of arrestin were higher in entire BLROS than in DROS (Fig. 3A). The major polypeptides present in the ROS soluble fraction (supernatant obtained after washing ROS preparations with low ionic buffer) were α and β subunits of transducin (Gα, Gβ), arrestin and phosphodiesterase (PDE) (Fig. 3A). The effect of GTP concentrations, and the rate of GTP hydrolysis participates in Gt translocation [6]. In our experimental setup it seems that the initial concentration of GTP at the moment of ROS isolation is enough for allowing a significant light-mediated translocation of Gt. Immunoblot analysis using RGD domain anti-phosphatidic acid phosphatase 2b (anti-LPP3) revealed that this isoform

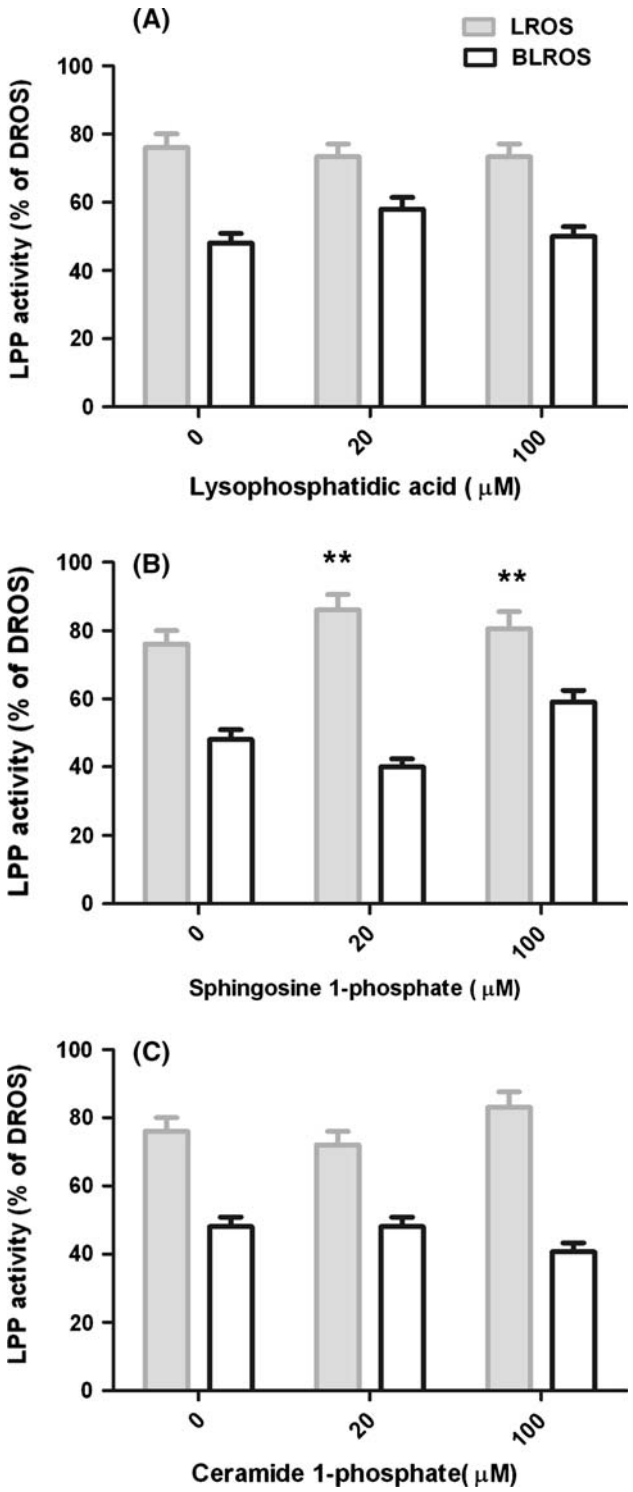


Fig. 2 Hydrolysis of [2-^{3}H]-phosphatidate by LPPs in LROS and BLROS in the presence of LPA, S1P and C1P. BLROS were obtained as was specified in Fig. 1. LROS were prepared following the same procedure as that of DROS but the enzymatic assay was made under room light. Results are expressed as the percentage of LPP activities in LROS or BLROS with respect to DROS and they represent the mean ± SD of nine individual samples. (**$P < 0.01$)

was present in the membrane fractions while it was absent in the soluble fraction (data not shown). No light–dark differences in LPP3 protein levels were observed either in entire ROS or in depleted membranes (Fig. 3B). Opsin immunoblots demonstrated that LPP3 co-localizes with the membrane fraction and that the protein is absent in the soluble fractions. Densitometry ratios (LPP3/opsin) showed no differences in LPP3 levels when entire ROS and depleted ROS were compared (Fig. 3C).

Hydrolysis of [2-^{3}H]-phosphatidate by LPPs in DROS and BLROS Depleted of Peripheral and Soluble Proteins. Effect of LPA, S1P and C1P

In order to evaluate the effect of peripheral and soluble proteins depletion on LPPs activities, enzyme assays were carried out in membranes from DROS and BLROS washed with low ionic strength buffer (Fig. 4). The same LPPs activities and similar dark/light differences were observed both in ROS and in the membranes treated with 5 mM buffer (Fig. 4). No enzyme activity was detected in the soluble fraction obtained from depleted DROS and BLROS (data no shown). DAG generation from [^{3}H]PA in depleted DROS in the presence of LPA, S1P or C1P was 57%, 61% and 86% with respect to the activity determined in the presence of [^{3}H]PA alone. In depleted BLROS membranes, DAG generation in the presence of LPA and S1P was 64% and 50% in the presence of C1P with respect to the activity found in the presence of [^{3}H]PA alone.

Hydrolysis of [2-^{3}H]-phosphatidate by LPPs from DROS and BLROS in the Presence of Sphingosine and Ceramide

In order to determine if the effect of S1P and C1P on DAG production was due either to their competitive characteristics and/or to the sphingosine and ceramide, the respective dephosphorylation products of S1P or C1P by LPPs, sphingosine or ceramide were included in PA hydrolysis assays. Sphingosine and ceramide were added using DMSO as vehicle. ROS were preincubated with sphingosine and ceramide but similar results were observed when sphingosine and ceramide were co-incubated with PA (data not shown). In entire DROS, sphingosine and ceramide inhibited DAG production by a similar percentage (18%) at all concentrations assayed (Fig. 5A, B). In entire BLROS sphingosine (100 μM) significantly inhibited DAG production by 23% and ceramide (50, 100, 300 μM) yielded the same inhibition percentage as that of

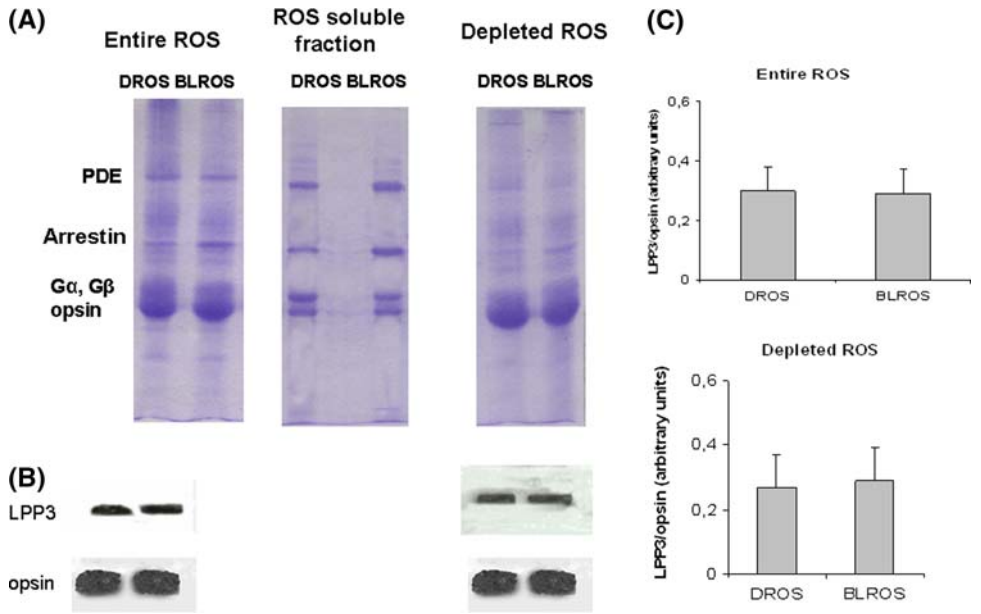


Fig. 3 SDS–PAGE and immunoblot analysis. ROS depleted of peripheral and soluble proteins were obtained from DROS or BLROS. Supernatants were removed (soluble ROS fraction) and membrane pellets (depleted ROS) were analyzed by SDS–PAGE and immunoblot. (**A**) Commassie Blue-stained gel of entire ROS, ROS soluble fraction and depleted ROS either from dark- or light-adapted retinas. (**B**) Immunoblot of entire ROS, ROS soluble fraction and depleted ROS from dark- or light-adapted retinas with RGD domain anti-phosphatidic acid phosphatase 2b (anti-LPP3) (upper panel). Immunoblots obtained with anti LPP3 antibodies were stripped and reprobed with anti-opsin antibody. Each lane contains 30 μg of protein. (**C**) LPP3/opsin ratios (expressed as arbitrary units) were calculated after densitometry analysis of immunoblots

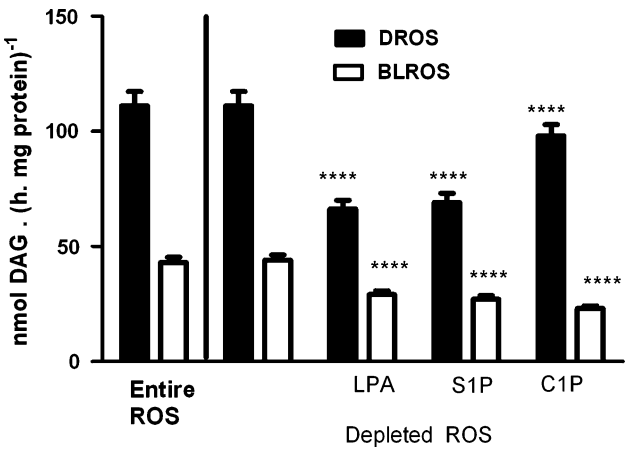


Fig. 4 Hydrolysis of [2-^{3}H]-phosphatidate by LPPs in DROS and BLROS depleted of peripheral and soluble proteins. Effect of LPA, S1P and C1P. LPPs activities were assayed in depleted ROS obtained from either dark- or light-adapted retinas. Membranes were incubated with 100 μM of [2-^{3}H]PA/Triton X-100 mixed micelle or with [2-^{3}H]PA in the presence of 100 μM of LPA, S1P or C1P as was specified in Fig. 1. Results represent the mean ± SD of nine individual samples (****$P < 0.001$)

sphingosine (Fig. 5B). In ROS depleted of soluble and peripheral proteins sphingosine and ceramide inhibited DAG formation by 48% and 40% in DROS and BLROS, respectively (Fig. 5C).

Discussion

The key enzyme LPPs are the main class of enzymes responsible for the dephosphorylation in vitro of PA and of the other phosphorylated substrates, such as LPA, S1P, and C1P. Three mammalian LPP isoforms termed LPP1 (PAP2a), LPP2 (PAP2c), and LPP3 (PAP2b) have been cloned [29]. These substrates are mutually competitive with each other. In general, LPP1, LPP2, and LPP3 show the major catalytic efficiency to LPA, PA, and S1P, respectively [36, 37]. The primary function of LPPs is a direct consequence of the dephosphorylation of their lipid substrates, altering the balance of bioactive lipid mediators [2, 38, 39]. PA and its dephosphorylated product, DAG, have important functions in signaling and PA itself emerges as a regulator of pleiotropic signaling responses [40].

ROS are responsible for the initial events of vision at low light levels. Light modulation of lipid metabolism and of a number of enzymes that generate second lipid messengers in ROS has been thoroughly studied [14, 16–19, 21, 22]. In addition, light-dependent membrane translocation of phototransduction proteins has been documented in detail [7, 41, 42].

Arrestin, transducin and recoverin redistribute in rods in response to bright light. Arrestin predominates at rod inner segments from DROS shifting to ROS during light exposure. Transducin α and β subunits shift in the opposite

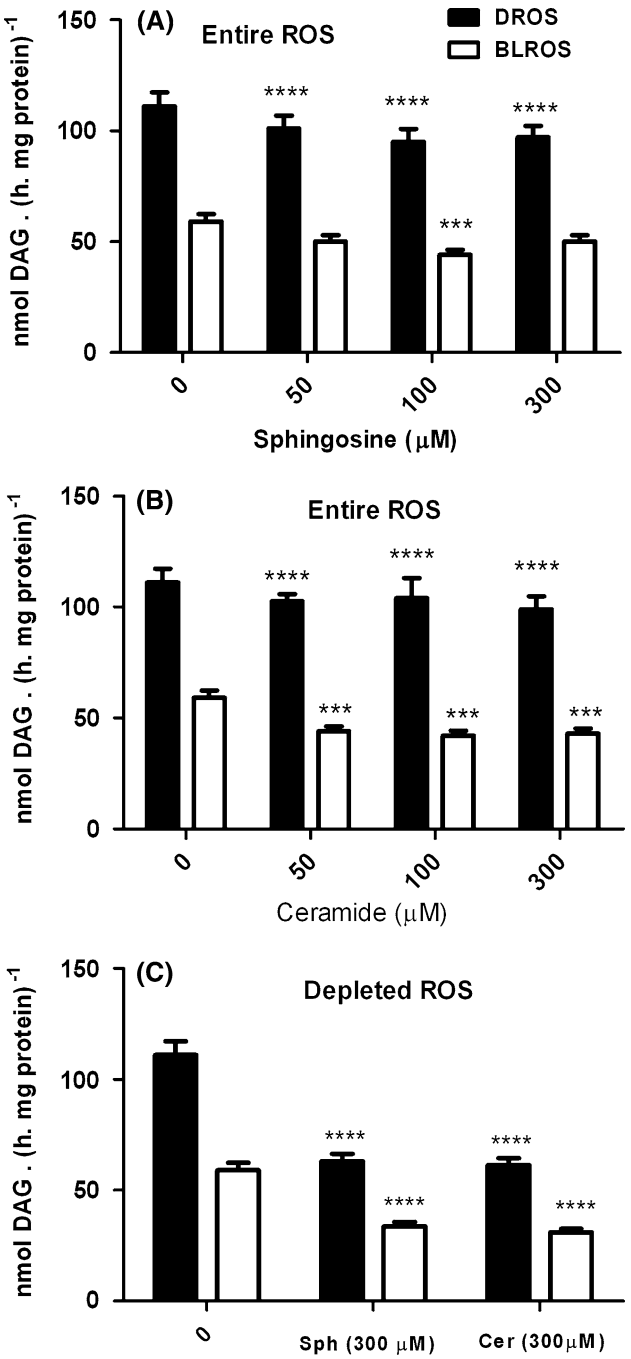


Fig. 5 Hydrolysis of [2-^{3}H]-phosphatidate by LPPs from DROS and BLROS in the presence of sphingosine and ceramide. Entire ROS obtained from dark- or light-adapted retinas were preincubated with sphingosine (50, 100 and 300 μM) or ceramide (50, 100 and 300 μM) for 10 min. Depleted ROS were incubated with sphingosine or ceramide (300 μM). [2-^{3}H]PA (100 μM)/Triton X-100 mixed micelles was subsequently added and the enzymatic activity was determined as specified in Fig. 1. Sphingosine and ceramide were added in DMSO 0.1% and the vehicle always served as control. Results represent the mean ± SD of nine individual samples. (***$P < 0.005$; ****$P < 0.001$ with respect to entire DROS or entire BLROS)

direction in response to light [7, 42] and a significant reduction of recoverin is produced in ROS by light [10]. The differential LPP activity observed in DROS with respect to LROS is only due to the light effect on ROS obtained from DROS. In our study it was observed that LPP activities are strongly inhibited in BLROS (50%). This is indicative of the involvement of bleaching process and light in LPP modulation. The light effect on LPP activities in ROS obtained from DROS has been previously demonstrated in our laboratory [14]. The important inhibition in LPP activities exerted by light and the absence of differences in LPP3 levels between DROS and BLROS could be related either to the absence or to the presence of a specific protein affected by light-driven translocation. These findings agree with our previous observations which demonstrated that light inhibition of LPP activity in ROS is a transducin-mediated mechanism [14]. PLD is inhibited by light as it occurs with LPPs [22]. On the other hand, it has been reported that diacylglyceride kinase (DAGK) [19] is modulated by light in the opposite manner as it occurs with LPPs and PLD. This could be indicating that PA and DAG levels have physiological relevance in ROS under illumination: i.e., under light conditions an increased DAGK activity promotes a major PA availability, under dark conditions an increase in PLD/PAP activities yields a major DAG availability [14, 22].

We have previously described that experimental conditions favoring PKC phosphorylation inhibits LPP in purified ROS [13]. Light stimulates DAG generation by PIP2-PLC activity [43] and thus may activate ROS PKC and this could be at least one of the light-induced mechanisms involved in LPPs modulation. Downstream signals by the action of LPPs may play a key role in light photoreceptor desensitization/adaptation. The functional significance of light-modulated LPP activities in vertebrate photoreceptors has not been fully elucidated to date. However, a role for LPPs in *Drosophila melanogaster* phototransduction has been reported. In *Drosophila* photoreceptors, photoisomerized rhodopsin activates a Gq protein releasing the α subunit, which in turn, activates phospholipase C [44]. PLC activation leads to the opening of two classes of Ca^{2+} permeable channels [45, 46]. In *Drosophila* DAG and PA levels are regulated by the synergistic activity of DA6K and by the recently described LPP gene called Lazaro [47].

In our study, DROS, LROS, and BLROS showed a different PA hydrolysis pattern in the presence of LPA, S1P, and C1P. S1P and LPA in DROS and LPA and C1P in BLROS produced the highest competitive effect on PA hydrolysis. Our study provides the first lines of evidence of the existence of LPP3 (PAP 2b) in ROS. The results

observed in the presence of S1P reinforced the existence of LPP3 in ROS [29]. In view of the results obtained with LPA we cannot discard the presence of LPP1 and LPP2 besides LPP3 in purified ROS. LPP3 has been localized with PLD in caveolin-enriched detergent-resistant microdomains (DRMs) where LPP3 metabolizes phospholipase D2-derived PA [48, 49]. The presence of PLD activity [48] and the existence of DRMs [50] in purified ROS, reinforce a physiological role for LPP3 in this system.

Previous observations suggest that LPP3 protein regulates cell–cell interactions and act as an ecto-enzyme [3, 51]. It was previously demonstrated that LPP from ROS is mainly located in the disk membrane [11]. For this reason ecto- LPP activity could provide lipid messengers in the extracellular medium inside the ROS disks. The pattern observed in the presence of S1P, mainly in DROS, could be either a consequence of a competition with PA by LPPs or it could be due to the formation of sphingosine from S1P, which may inhibit PA hydrolysis. This possibility was corroborated by our observations of the sphingosine effect on DAG generation (Fig. 4). In this respect, it has been reported that sphingosine not only inhibits DAG formation but also stimulates PA formation, inhibiting LPPs and stimulating PLD and DAGK [52, 53].

C1P is a potent inhibitor of protein phosphatases (PP) and PPs have been implicated in the inhibition of LPPs in isolated ROS [13, 54, 55]. Another possibility is that C1P exerts a direct action on LPPs. It has been reported that C1P is required for the activation and translocation of other enzymes involved in lipid metabolism such as cytosolic phospholipase A2 [56]. An inhibition in DAG production from PA by ceramide was also observed. Ceramide itself is an important second messenger in various stress responses and there are several candidates for ceramide-regulated enzymes [57]. Increased intracellular ceramide levels have been involved in the activation of photoreceptor apoptosis [58]. Furthermore, it has been suggested that LPP2 and LPP3 play an important role in apoptotic processes. This is supported by the fact that DAG and sphingosine, the products of LPPs, are involved in the apoptosis induction [39] while S1P and LPA have anti-apoptotic roles [59].

Our results regarding basal LPP activities under dark and light conditions showed no differences between entire ROS and ROS depleted of soluble and peripheral proteins, thus corroborating the fact that LPPs are membrane-associated proteins. On the contrary, it has been reported that DAGK exhibits a different activity in ROS depleted of soluble and peripheral proteins with respect to intact ROS [60]. The effects of LPP alternative substrates obtained in depleted ROS with respect to that observed in entire ROS could be indicating the involvement of soluble and/or peripheral proteins in the regulation of LPPs (Fig. 4). The presence of sphingosine and ceramide strongly inhibited LPPs activities in depleted DROS, being the activity levels similar to those found in depleted BLROS (in the absence of sphingosine or ceramide) (Fig. 5C).

Our findings indicate that the competition between PA and LPA, or S1P or C1P by the active site of LPPs is modulated by ROS illumination state and by ROS protein association/dissociation. The role of LPPs in signaling events and the fact that the products generated by these enzymes control the cell-death decisions in photoreceptor cells, open an interesting field to further studies about the regulation of LPPs by light in these cells.

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