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Diacylglyceride lipase activity in rod outer segments depends on the illumination state of the retina

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ABSTRACT

We have demonstrated that the competition between phosphatidic acid (PA) and lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P) for lipid phosphate phosphatases (LPP) generates different levels of diacylglycerol (DAG) depending on the illumination state of the retina. The aim of the present research was to determine the diacylglyceride lipase (DAGL) activity in purified rod outer segments (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. [2-^{3}H]monoacylglycerol (MAG), the product of DAGL, was evaluated from [2-^{3}H]DAG generated by LPP action on [2-^{3}H]PA in the presence of either LPA, S1P or C1P. MAG production was inhibited by 55% in BLROS and by 25% when the enzymatic assay was carried out in ROS obtained from dark-adapted retinas and incubated under room light (LROS). The most important events occurred in DROS where co-incubation of [2-^{3}H]PA with LPA, S1P or C1P diminished MAG production. A higher level of DAGL activity was observed in LROS than in BLROS, though this difference was not apparent in the presence of LPA, S1P or C1P. DAGL activity in depleted DROS was diminished with respect to that in entire DROS. LPA, S1P and C1P produced a similar decrease in MAG production in depleted DROS whereas only C1P significantly diminished MAG generation in depleted BLROS. Sphingosine and ceramide inhibited MAG production in entire DROS and stimulated its generation in BLROS. Sphingosine and ceramide stimulated MAG generation in both depleted DROS and BLROS. Under our experimental conditions the degree of MAG production depended on the illumination state of the retina. We therefore suggest that proteins related to phototransduction phenomena are involved in the effects observed in the presence of S1P/sphingosine or C1P/ceramide.

1. Introduction

Rod outer segments (ROS) are responsible for the initial events of vision at low light levels. Vertebrate photoreceptors are highly specialized neurons responsible for the reception and primary processing of visual information. The outer segment compartment of the photoreceptor contains large amounts of proteins that are involved in light detection mechanisms as well as in the generation of visual signaling (Arshavsky et al., 2002; Burns and Baylor, 2001; Fain et al., 2001). In dark-adapted rods most of transducin is located in the outer segment. Prolonged exposure to bright light induces massive transducin translocation from ROS to other compartments of the rod cells (Brann and Cohen, 1987; Philp et al., 1987; Sokolov et al., 2002). It has been reported that proteins such as arrestin and recoverin are also involved in this process. This mechanism, which desensitizes rods under conditions of intensive illumination, contributes to rod light adaptation (Broekhuyse et al., 1987; Kerov et al., 2005; Philp et al., 1987; Strissel et al., 2005).

We have previously described diacylglyceride lipase (DAGL) activity in ROS on the premise that the diacylglycerol produced from phosphatidic acid (PA) for lipid phosphate phosphatases (LPP) activity is metabolized to monoacylglycerol (Pasquare de Garcia and Giusto, 1986; Pasquare and Giusto, 1993; Pasquare et al., 2000; Perez Roque et al., 1998). Modulation by light in ROS

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Abbreviations: BLROS, ROS prepared from light-adapted retinas; C1P, ceramide 1-phosphate; DAG, diacylglycerol; DAG lipase, diacylglycerol lipase; DTT, dithiotreitol; DROS, ROS prepared from dark-adapted retinas; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid; LPA, lysophosphatidic acid; LROS, light-adapted bovine ROS; MAG, monoacylglycerol; NEM, N-ethylmaleimide; PA, phosphatidic acid; LPP, lipid phosphate phosphatase; PC, phosphatidylcholine; PMSF, phenylmethylsulfonylfluoride; ROS, rod outer segments; S1P, sphingosine 1-phosphate; TLC, thin layer chromatography.

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lipid metabolism enzymes such as phospholipase C (Ghalayini and Anderson, 1992), phospholipase A2 (Castagnet and Giusto, 1993), phosphatidylethanolamine N-methyltransferase (Roque et al., 1999), diacylglycerol kinase (Huang et al., 2000), PAP2 (Pasquare et al., 2008, 2000), phosphoinositide-3-kinase (Guo et al., 1997; Rajala et al., 2002), and phospholipase D (Salvador and Giusto, 2006) has also been reported. Lipid phosphates initiate key signaling cascades in cell activation. Extracellular lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) stimulate families of G-protein couple receptors that elicit different responses. Inside the cell, PA, LPA, S1P and ceramide 1-phosphate (C1P) behave as signaling molecules with diverse functions including the stimulation of cell division, cytoskeletal rearrangement and membrane movement. The degradation of lipid phosphates by LPP either on the cell surface or inside the cell regulates cell signaling under physiological or pathological conditions. This cell signaling occurs through the attenuation of lipid phosphate signaling and the production of bioactive diacylglycerol, sphingosine and ceramide (Brindley, 2004). Diacylglycerol (DAG) has different roles as a basic component of membranes, as an intermediary in lipid metabolism and as a key molecule in lipid-mediated signaling. In eukaryote cells, DAG generation and/or removal has severe effects on organ development and has been associated with diseases such as cancer, diabetes, immune system disorders and Alzheimeŕs disease (Carrasco and Merida, 2007). The precise control of DAG production and clearance is necessary for the correct functioning of this molecule in the signaling mechanism. DAGL are also strongly linked to the following signaling functions: (a) in platelets, in response to thrombin their combined action with PLC facilitates the release of arachidonic acid (Smith et al., 1991); and (b) in neurons, this activity is necessary for the generation of endocannabinoid 2-arachidonoyl-glycerol during retrograde synaptic transmission (Yoshida et al., 2006). The present study evaluates the metabolization of DAG generated from PA by LPP activity in ROS prepared from dark-adapted retinas (DROS) and in ROS prepared from light-adapted retinas (BLROS). The enzymatic activity was also determined in DROS and BLROS depleted of soluble and peripheral proteins. In all instances, the metabolization of [2-^{3}H]DAG was analyzed in the presence of either LPA, S1P or C1P, all of which are alternative substrates for LPP. Our results demonstrate that light modulates the metabolism of DAG by DAGL activity and show how DAG is used differently by DAGL in the presence of LPA, S1P and C1P.

2. Experimental procedure

2.1. Materials

[2-^{3}H]Glycerol (200 mCi/mmol) was obtained from New England Nuclear-Dupont, Boston, MA, USA. 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. All other chemicals were of the highest purity available.

2.2. Rod outer segment isolation

Bovine eyes were obtained from a local abattoir. They were placed on ice within 10 min of the animal's death and then 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. Bleached ROS (BLROS) were prepared from retinas whose optic cup was exposed under 300 W light at 30 cm for 30 min (Guo et al., 1997), after removing the cornea and the lens from the eyeballs. The subsequent procedures for ROS preparation were conducted under dim red light for DROS and LROS and under room light for BLROS, all 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). Retina remnants were sedimented at 2200 × *g* for 4 min and the supernatants containing ROS were diluted 1:2 with sucrose-free buffer and then centrifuged at 35,300 × *g* during 30 min. ROS were purified by a discontinuous gradient of sucrose (Kuhn, 1982) yielding: (i) a ROS band (Band I) retained at the 0.84/1.00 M density interface, (ii) broken ROS contaminated with mitochondria and RIS retained at the 1.00/1.14 M density interface (Band II), and (iii) a pellet composed of non-ROS membranes. The purity of ROS membrane preparations was monitored by electron microscopy. Electron micrographs of purified ROS (Band I) showed intact ROS with their typical structures, no other membrane material being 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, membrane purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970). Even in overloaded gels (80 μg of ROS protein), rhodopsin reached 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 sterified to dipolyunsaturated molecular species of PC was observed (Aveldano, 1987). The purity of ROS populations was determined by assaying marker enzyme activities in all fractions of the gradients. NADPH-cytochrome *c*-reductase (microsomal marker) and cytochrome *c*-oxidase 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 showed the highest activity in the pellet. These results lead us to conclude that Band I (purified ROS) contamination with either microsomes or mitochondria is lower than 5% (Hodges and Leonard, 1974; Roque and Giusto, 1995).

2.3. Soluble and peripheral protein extraction from ROS with low ionic strength buffer

ROS pellets from DROS or BLROS were re-suspended (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 suspension membranes were centrifuged at 35,300 × *g* during 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 DAGL activity.

2.4. Reconstitution assay

After treatment of DROS, LROS or BLROS with low ionic strength buffer, the membrane pellets were recombined with the soluble fraction obtained therefrom. The "reconstituted" suspension was employed to determine DAGL activity.

2.5. Determination of DAGL activity

DAG lipase activity was determined by monitoring the formation rate of monoacyl[2-^{3}H]glycerol, using diacyl[2-^{3}H]glycerol generated by LPP action on [2-^{3}H]glycerol-PA as substrate (Pasquare and Giusto, 1993). [2-^{3}H]glycerol-PA was prepared as specified elsewhere (11). Briefly, PA was obtained from [2-^{3}H]phosphatidylcholine which had been synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol). Lipids were extracted from the tissue and [2-^{3}H]phosphatidylcholine was isolated by mono-dimensional TLC and eluted therefrom. Next, [2-^{3}H]phosphatidylcholine was hydrolyzed with PLD and the hydrolysis product was purified by one-dimensional TLC. The substrate was eluted from silica gel with neutral solvents and subsequently converted into free acid by washing it twice using an upper phase containing 0.1 sulfuric acid and then an upper phase containing water.

The specific activity of DAG was calculated under all experimental conditions. The specific activity was calculated as the ratio between the dpm of [2-^{3}H]diacylglycerol generated (in each one of the LPA, S1P and C1P concentrations employed) and the sum of the nmol of labeled DAG (in each one of the LPA, S1P and C1P concentrations employed) plus the concentration of DAG in the respective membranes (in nmol). The standard assays contained 50 mM Tris–maleate buffer, pH 6.5, 10 nM of [2-^{3}H]DAG, 1 mM DTT, 1 mM EDTA plus 1 mM EGTA, 4.2 mM NEM and 100 μg of membrane protein in a volume of 100 μl. DAG metabolization by DAGL was evaluated using 100 μM [2-^{3}H]PA/Triton X-100 (1:50 molar ratio) mixed micelles in the presence of LPA, S1P or C1P (previously re-suspended in the assay buffer containing Triton X-100) (Hooks et al., 1998; Roberts et al., 1998). In this case, radiolabel PA was mixed with unlabeled substrates before drying and re-suspension. This aqueous microdispersion was sonicated in a sonication tip until clarity. Sphingosine and ceramide were solubilized in 0.1% of dimethyl sulfoxide (DMSO) as vehicle; the respective controls were carried out with 0.1% of DMSO alone.

The assays for the determination of DAGL activity 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. Enzymatic hydrolysis products were isolated and measured as described below. DAGL activity was expressed as nmol of monoacyl [2-^{3}H]glycerol (h mg protein)$^{-1}$.

2.6. 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%) (Folch et al., 1957). Neutral lipids were separated by gradient-thickness thin-layer chromatography on silica gel G (Giusto and Bazan, 1979) and developed with hexane/diethyl ether/acetic acid (35:65:1, v/v). To separate monoacylglycerol (MAG) from phospholipids, the chromatogram was re-chromatographed up to the middle of the plate by using hexane/diethyl ether/acetic acid (20:80:2.3, v/v) as developing solvent (Pasquare de Garcia and Giusto, 1986). Lipids were visualized by exposure of the chromatograms to iodine vapors. They were then scraped off the plate and quantified by liquid scintillation spectroscopy.

2.7. Other methods

Lipid phosphorus and protein were determined as described elsewhere (Bradford, 1976; Rouser et al., 1970).

2.8. Statistical analysis

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

3. Results

3.1. Hydrolysis of [2-^{3}H]-DAG, generated from [2-^{3}H]-phosphatidate, by DAGL in DROS and BLROS as a function of lysophosphatidic acid, sphingosine 1-phosphate and ceramide 1-phosphate concentrations

DAG resulting from LPP activity is partially hydrolyzed by DAGL, yielding MAG. DAGL is coupled to LPP and these enzymes appear to work like an enzymatic complex. DAG generation and its partial degradation by DAGL immediately occur, a phenomenon that has been extensively described in our laboratory (Pasquare de Garcia and Giusto, 1986; Pasquare and Giusto, 1993). DAGL activity was therefore characterized by using [2-^{3}H]-DAG formed by the action of LPP on [2-^{3}H]-PA. The specific activity of the DAGL substrate (DAG) was calculated in each one of the LPA, S1P and C1P concentrations employed. Three distinct ROS populations were used: (i) DROS obtained from dark-adapted retinas and purified under dim red light, (ii) LROS obtained from DROS and exposed to room light, and (iii) BLROS obtained from light-adapted retinas and purified under room light, as mentioned in Section 2.

Fig. 1 shows the rate of MAG formation in the presence of LPA, S1P and C1P in DROS (square symbols) and BLROS (circular symbols). MAG production was inhibited by 55% in BLROS with respect to DROS (Fig. 1). LPA, S1P and C1P inhibited MAG production in a dose-dependent manner in DROS (Fig. 1A–C). Maximum inhibition at 50 μM of LPA (Fig. 1A), S1P (Fig. 1B) and C1P (Fig. 1C) corresponded to 39%, 51% and 33%, respectively. In BLROS, MAG generation was increased by 22% at 10 μM of LPA, diminishing until reaching the control value (Fig. 1A). A decrease of 48% in MAG formation was observed at 100 μM of C1P (Fig. 1C).

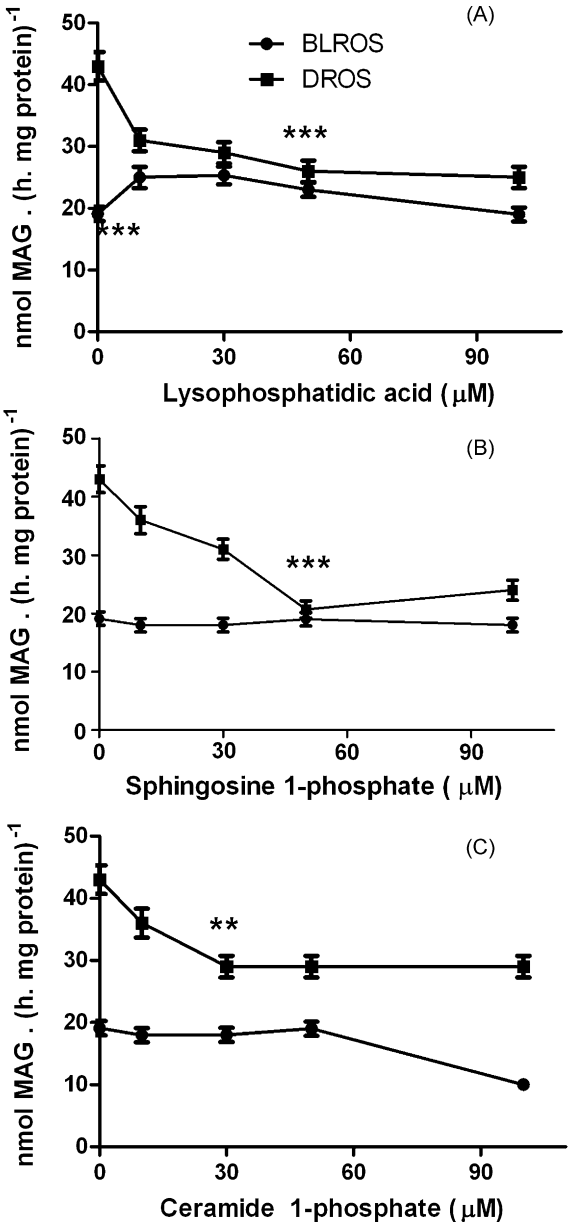

Fig. 1. Hydrolysis of [2-^{3}H]-DAG, generated from [2-^{3}H]-phosphatidate (PA), by DAGL in DROS and BLROS as a function of lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P) concentrations. DAGL activity was determined using, as an enzyme source, either purified ROS from dark-adapted retinas (DROS) or bleached ROS (BLROS) from dark-adapted retinas whose optic cups were exposed to room light (300 W at 30 cm) for 30 min as specified in Section 2. The enzymatic activity was evaluated using [2-^{3}H]DAG generated from [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 carried out 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 enzymatic products were scraped and quantitated by liquid scintillation spectroscopy. The results represent the mean ± S.D. of nine individual samples (***$p < 0.0001$, **$p < 0.005$ with respect to DROS "0").

3.2. Hydrolysis of [2-^{3}H]-DAG by DAGL in LROS in the presence of LPA, S1P and C1P

Fig. 2 shows DAGL activity in LROS in the absence of and in the presence of 20 or 100 μM of LPA, S1P or C1P. An inhibition of 28% was observed in LROS compared with DROS (30 ± 1 nmol MAG (h mg protein)$^{-1}$ vs. 42 ± 2 MAG (h mg protein)$^{-1}$). The presence of LPA (100 μM) increased MAG production by 37% in LROS (Fig. 2A) whereas its production was inhibited by 29% and 38% in the presence of C1P at concentrations of 20 and 100 μM, respectively (Fig. 2C). The presence of sphingosine 1-phosphate did not modify MAG production in LROS (Fig. 2B). A ratio between DAGL activities from LROS and BLROS was determined on the basis of the results shown in Figs. 1 and 2, giving 1.6 in the presence of PA and 1 in the presence of 20 μM of LPA and C1P and in the presence of 100 μM of S1P.

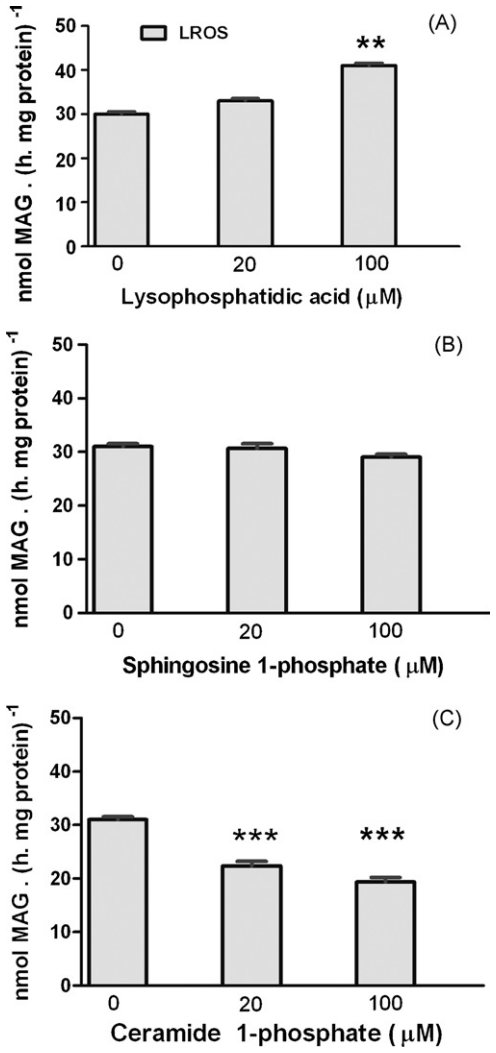

Fig. 2. Hydrolysis of [2-^{3}H]-DAG by DAGL in LROS in the presence of LPA, S1P and C1P. LROS were prepared following the same procedure as for DROS except that the enzymatic assay was performed under room light. The results represent the mean ± S.D. of nine individual samples (***$p < 0.0001$, **$p < 0.005$ with respect to LROS "0).

3.3. Hydrolysis of [2-^{3}H]-DAG by DAGL from DROS and BLROS in the presence of sphingosine and ceramide

In order to assess whether the effect of S1P and C1P on MAG production is due to sphingosine and ceramide generated by LPP on S1P or C1P, we evaluated the effect of sphingosine and ceramide on MAG formation in DROS and BLROS. To this end, sphingosine and ceramide were added using dimethylsulfoxide as vehicle in a concentration that did not modify enzymatic activity. In DROS, sphingosine inhibited MAG production by 15% at 300 μM (Fig. 3A). Ceramide inhibited DAGL activity in a dose-dependent manner: inhibition was 28% and 42% at 50 and 100 μM of ceramide, respectively, this latter level of inhibition being maintained at 300 μM (Fig. 3B). Sphingosine and ceramide stimulated DAGL activity in BLROS by 21% and 27%, respectively, at all the concentrations assayed (Fig. 3A and B).

3.4. Hydrolysis of [2-^{3}H]-DAG by DAGL in entire and depleted DROS, LROS and BLROS

To investigate whether the extraction of peripheral and soluble proteins modifies DAGL activity, we determined the enzymatic

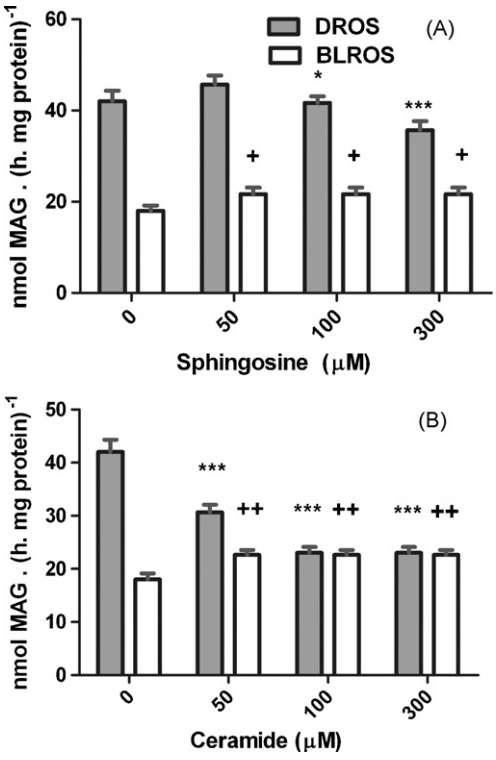

Fig. 3. Hydrolysis of [2-^{3}H]-DAG by DAGL from DROS and BLROS in the presence of sphingosine and ceramide. Entire ROS obtained from dark- or light-adapted retinas were preincubated with sphingosine or ceramide (50, 100 and 300 μM) for 10 min. Sphingosine and ceramide were added in DMSO 0.1%. The enzymatic activity was determined as specified in Fig. 1. The results represent the mean ± S.D. of nine individual samples (***$p < 0.0001$, *$p < 0.05$ with respect to DROS "0"; +$p < 0.05$, ++$p < 0.005$ with respect to BLROS"0").

activity in membranes from DROS, LROS and BLROS treated with 5 mM buffer (Fig. 4).

The successive washes of DROS, LROS and BLROS with low ionic strength buffers rendered an ROS membrane preparation depleted of soluble and peripheral proteins as determined by SDS/PAGE

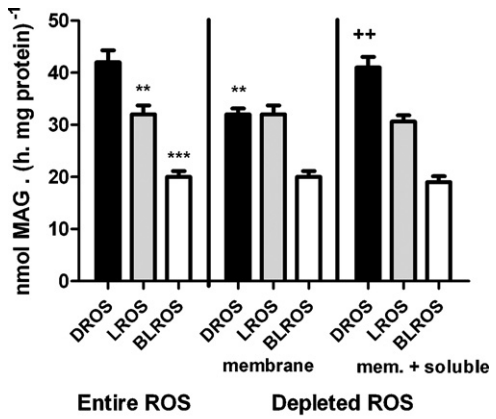

Fig. 4. Hydrolysis of [2-^{3}H]-DAG by DAGL in entire, depleted and reconstituted DROS, LROS and BLROS. DAGL activity was assayed in entire, depleted or reconstituted ROS obtained from either dark- or light-adapted retinas. DROS, LROS and BLROS were obtained as specified in Figs. 1 and 2. After treatment of DROS, LROS or BLROS with low ionic strength buffer, the membrane pellets were recombined with the soluble fraction obtained therefrom. The enzymatic assay was specified in Fig. 1. The results represent the mean ± S.D. of nine individual samples (***$p < 0.0001$,**$p < 0.005$ with respect to entire DROS; ++$p < 0.005$ with respect to membrane DROS).

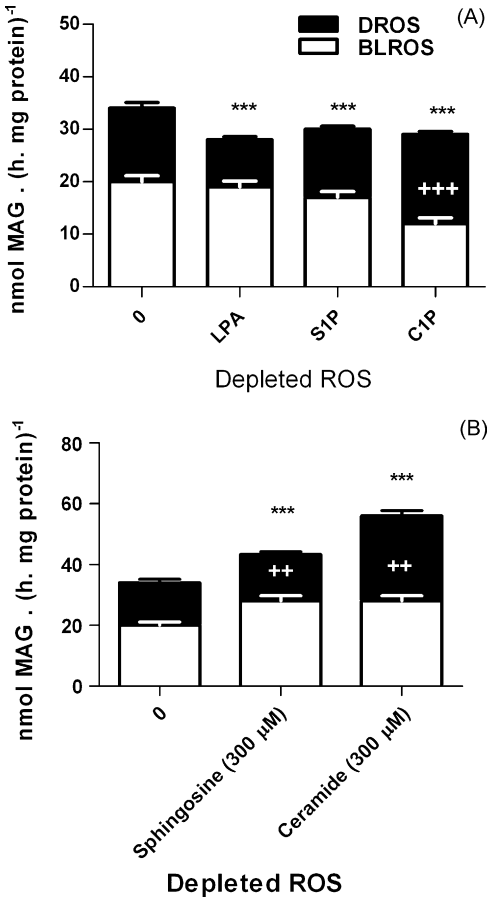

Fig. 5. Hydrolysis of [2-^{3}H]-DAG by DAGL in DROS and BLROS depleted of peripheral and soluble proteins in the presence of LPA, S1P and C1P (A), or sphingosine and ceramide (B). DAGL activity was assayed in depleted ROS obtained from either dark- or light-adapted retinas as specified in Fig. 1. 100 μM LPA, S1P and C1P, was used in (A). The results represent the mean ± S.D. of nine individual samples (***$p < 0.0001$, with respect to DROS "0"; +++$p < 0.0001$, ++$p < 0.05$ with respect to BLROS "0").

analysis (Pasquare et al., 2008). In depleted DROS, DAGL activity was inhibited by 25% with respect to entire DROS, reaching similar values to those in LROS (Fig. 4). Reconstitution experiments where depleted membranes were combined with their respective soluble fractions demonstrated a recovery of the enzymatic activity in depleted DROS (Fig. 4). The combination of depleted LROS and BLROS with their respective soluble fractions did not modify DAGL activity (Fig. 4). No enzymatic activity was detected in the soluble fractions obtained from depleted DROS and BLROS (data not shown).

3.5. Hydrolysis of [2-^{3}H]-DAG by DAGL in DROS and BLROS depleted of peripheral and soluble proteins in the presence of LPA, S1P and C1P or sphingosine and ceramide

We used 100 μM of LPA, S1P and C1P to evaluate their action in depleted membranes since this concentration is equimolecular with [2-^{3}H]-PA and is the concentration at which the maximal effect can be observed in entire ROS (Fig. 5A). In depleted DROS, MAG generation was almost 82% in the presence of LPA, S1P and C1P as compared with their absence. In depleted BLROS, on the other hand, MAG production was 58% in the presence of C1P with no significant differences being observed in the presence of LPA or S1P.

The role of sphingosine and ceramide in depleted ROS was also evaluated. In depleted DROS, sphingosine and ceramide stimulated MAG formation by 27% and 63%, respectively; in depleted BLROS (Fig. 5B) they each stimulated MAG generation to the same extent (38%).

4. Discussion

Diacylglycerol has unique functions as a basic component of membranes, as an intermediary in lipid metabolism and as a key element in lipid-mediated signaling. In addition to the PKC family, an increasing number of proteins are known to be modulated by DAG (Brose et al., 2004; Yang and Kazanietz, 2003). It was observed that in excised patches from frog rod outer segments, DiC8 modulates the gating of the cGMP-gated channel in the absence of a phosphorylation reaction (Gordon et al., 1995). Diacylglycerol lipase is known to be present in neural tissue (Rosenberger et al., 2007) and the cloning and enzymatic characterization of two DAGL isoforms, α and β, have been analyzed (Bisogno et al., 2003). Previous studies from our laboratory demonstrate that in ROS and other subcellular fractions from bovine retina, diacylglycerol produced from phosphatidic acid is additionally metabolized to monoacylglycerol, thus indicating the presence of DAGL activity (Pasquare de Garcia and Giusto, 1986; Pasquare and Giusto, 1993). In the present research, MAG generation was determined using, as substrate, diacyl[2-^{3}H]glycerol generated from [2-^{3}H]glycerol-PA by LPP activity. In other tissues it has been reported that endogenously produced DAG is further hydrolyzed to MAG and glycerol (Ide et al., 1990; Pasquare and Giusto, 1993). In addition, it has been demonstrated that exogenously added [^{3}H]-diacylglycerol or [^{3}H]-diacylglycerol co-emulsified with PC are poor substrates for DAGL (Bisogno et al., 2003). On the other hand, the extremely hydrophobic nature of DAG molecules induces their localization inside vesicles from which they are inaccessible to the enzyme (Pagano and Longmuir, 1985). Studies using detergents have revealed that the binding of the enzyme to the lipid bilayer is a crucial issue in DAGL activity. 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. Under our assay conditions, the hydrolysis of diacylglycerol was observed at a very low substrate concentration (10 nM). This is indicative not only of the physiological importance of the enzyme in ROS but also of the fact that LPP and DAGL work in a coupled form. The acyl chain length and degree of saturation of the fatty acids in the substrate may alter substrate presentation and catalysis (Rosenberger et al., 2007). The composition of the substrate [^{3}H]-DAG derived from [^{3}H]-PA and used in our assays contained 34%, 19%,18% and 17% of 16:0, 18:0, 18:1 and 22:6, respectively, with negligible amounts of 20:4 (Pasquare de Garcia and Giusto, 1986). It has been reported that bovine brain DAGL hydrolyzes DAG with a composition similar to our substrate (Rosenberger et al., 2007).

There are three possible pathways of MAG formation using PA as substrate: (i) LPP/DAGL, (ii) by the action of phospholipase A/LPP, and (iii) by the action of phospholipase A/LPA lysophosphatase. The role of (ii) and (iii) in MAG generation can be discarded because phospholipase A in ROS shows its maximal activity at pH 9.0 with negligible activity at pH 6.5 (Castagnet and Giusto, 1993), the pH at which DAGL activity was assayed in the present study; and LPA lysophosphatase activity has not been described in ROS to date. It can therefore be concluded that MAG generation in ROS occurs exclusively via the LPP/DAGL pathway.

Proteins involved in signal transduction, such as arrestin, transducin and recoverin, redistribute in rods in response to bright light. In addition, arrestin shifts to ROS and transducin α and β shift in the opposite direction during light exposure (Philp et al., 1987; Strissel et al., 2005; Whelan and McGinnis, 1988). In our study, it

was observed that DAGL activity was inhibited under light conditions and in depleted DROS. The activity in depleted DROS reached similar values as those in LROS. Reconstitution experiments where depleted DROS were combined with their respective soluble fractions demonstrated a recuperation of enzymatic activity, suggesting that any soluble or peripheral protein detached from ROS or protein redistribution produced by bleaching could stimulate DAGL activity, or that the bleaching process could induce the detachment of the DAGL enzyme without the substrate concentration being able to detect this in the soluble fraction. Both possibilities are feasible since light-adapted ROS are insensitive to protein depletion (Fig. 4). The functional significance of light modulation in DAGL activity in vertebrate photoreceptors has not been fully elucidated to date. However, evidence of the role of DAGL in *Drosophila* phototransduction has been reported (Huang et al., 2004; Minke and Parnas, 2006). Furthermore, several studies have demonstrated that the enzymes involved in ROS phospholipid turnover are modulated by light (Castagnet and Giusto, 1993; Ghalayini and Anderson, 1992; Giusto et al., 2000; Guo et al., 1997; Huang et al., 2000; Pasquare et al., 2008; Rajala et al., 2002; Roque et al., 1999; Salvador and Giusto, 2006).

In entire DROS, the DAGL substrate (DAG) was diminished in the presence of either LPA or S1P (Pasquare et al., 2008) and MAG production was inhibited by one percentage point higher. In depleted DROS, BLROS and depleted BLROS, DAG was diminished in the presence of LPA or S1P (Pasquare et al., 2008), whereas MAG diminished slightly, was stimulated, or underwent no changes in each of the membrane preparations. A stimulatory effect on DAGL activity therefore occurred in the presence of LPA and S1P. Summing up, DAGL activity was inhibited in the presence of LPA and S1P in entire DROS and stimulated in depleted DROS, entire and depleted BLROS. The fact that S1P and LPA diminish DAGL activity in DROS and that they produce a stimulatory effect on DAGL in ROS membranes where protein redistribution occurs (BLROS) or where soluble or peripheral proteins are detached (depleted DROS) seems to indicate that S1P and LPA produce their effects either by modulating or interacting with a protein involved in the phototransduction cascade that modulates DAGL activity. Interestingly, LROS/BLROS differences in DAGL activity were not observed at low concentrations of LPA or C1P or at high concentrations of S1P. These findings were not observed in LPP (Pasquare et al., 2008), thus suggesting that they are related to DAGL itself and that the high inhibition observed in DAGL activity caused by bleaching is partially compensated by LPA, S1P or C1P. It has been reported that C1P is required for the activation and translocation of other enzymes involved in lipid metabolism (Chalfant and Spiegel, 2005). The effects observed in the presence of LPA could be a consequence of either its detergent-like properties or its role as a lipid mediator with growth factor-like activity (Keller et al., 1997). The latter can only be corroborated with the presence of LPA-receptor in photoreceptor cells, which has not been reported to date. Sphingosine or ceramide generated from S1P and C1P by LPP may modify DAGL activity, as corroborated by our observations of the effect of sphingosine and ceramide on MAG generation in entire DROS. The fact that S1P and C1P in depleted DROS and in entire and depleted BLROS have the opposite effect to sphingosine and ceramide suggests that these lipids act independently on enzymatic activity.

DAGL from DROS diminished in the presence of ceramide, reaching similar values to those of BLROS. Ceramide seemed to induce either protein migration or detachment of DAGL enzyme from ROS membrane to soluble. The cellular ratio between S1P/sphingosine and C1P/ceramide is a critical factor in cell survival/cell death decisions. Ceramide is an important second messenger in stress and there are several ceramide-regulated enzymes (Hannun and Obeid, 2002). It has been reported that DAG, ceramide (German et al., 2006) and sphingosine are involved in apoptosis induction (Pyne et al., 2005) whereas S1P has an anti-apoptotic role (Grey et al., 2002). The concentration of DAG in small membrane areas, its characteristic negative curvature and its lack of charge induce unstable, asymmetric regions in membrane bilayers. Intermediaries with increased curvature minimize this tension and are essential for membrane fusion and fission processes (Lentz et al., 2000). Consequently, DAG may affect physiological processes by altering the membrane structures and fluidity and may favor the shedding of membranous disks. DAG is a precursor for the cannabinoid receptor CB-1 (Sugiura et al., 2002) and MAG by-product of the termination of protein-kinase C/DAG-mediated intracellular signaling (Gammon et al., 1989). In the light of these findings it can be concluded that DAGL activity has an important role in controlling DAG/MAG levels. Taken together, our results also suggest that the metabolism of DAG following light-mediated ROS stimulation plays an active role in organizing signaling responses following the initial light stimulus.

The interaction among enzymes controlling the DAG level; the identification of DAG-regulated proteins; the overlap between DAG metabolism and the signaling process; and the function of MAG as an endocannabinoid, all open interesting avenues for further research into DAG and MAG in photoreceptor cells.

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