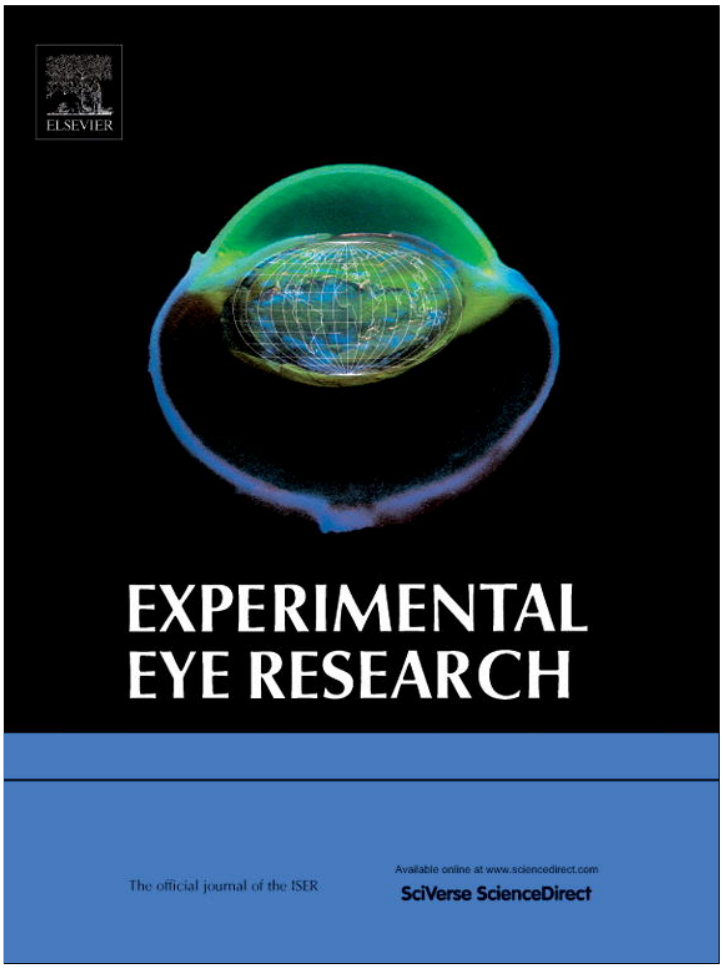

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Diacylglycerol kinase epsilon in bovine and rat photoreceptor cells. Light-dependent distribution in photoreceptor cells

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ABSTRACT

The present study shows the selective light-dependent distribution of 1,2-diacylglycerol kinase epsilon (DAGKε) in photoreceptor cells from bovine and albino rat retina. Immunofluorescence microscopy in isolated rod outer segments from bleached bovine retinas (BBROS) revealed a higher DAGKε signal than that found in rod outer segments from dark-adapted bovine retinas (BDROS). The light-dependent outer segment localization of DAGKε was also observed by immunohistochemistry in retinas from albino rats. DAGK activity, measured in terms of phosphatidic acid formation from a) [^{3}H]DAG and ATP in the presence of EGTA and R59022, a type I DAGK inhibitor, or b) [γ-^{32}P]ATP and 1-stearoyl, 2-arachidonoylglycerol (SAG), was found to be significantly higher in BBROS than in BDROS. Higher light-dependent DAGK activity (condition b) was also found when ROS were isolated from dark-adapted rat retinas exposed to light. Western blot analysis of isolated ROS proteins from bovine and rat retinas confirmed that illumination increases DAGKε content in the outer segments of these two species. Light-dependent DAGKε localization in the outer segment was not observed when U73122, a phospholipase C inhibitor, was present prior to the exposure of rat eyecups (*in situ* model) to light. Furthermore, no increased PA synthesis from [^{3}H]DAG and ATP was observed in the presence of neomycin prior to the exposure of bovine eyecups to light. Interestingly, when BBROS were pre-phosphorylated with ATP in the presence of 1,2-dioctanoyl sn-glycerol (di-C8) or phorbol dibutyrate (PDBu) as PKC activation conditions, higher DAGK activity was observed than in dephosphorylated controls. Taken together, our findings suggest that the selective distribution of DAGKε in photoreceptor cells is a light-dependent mechanism that promotes increased SAG removal and synthesis of 1-stearoyl, 2-arachidonoyl phosphatidic acid in the sensorial portion of this cell, thus demonstrating a novel mechanism of light-regulated DAGK activity in the photoreceptors of two vertebrate species.

1. Introduction

Vertebrate rods are able to adapt their physiological responses to different illumination conditions via multiple molecular and cellular mechanisms that optimize the response speed and sensitivity of photoreceptor cells operating at different light intensities (Arshavsky, 2002). One such adaptation mechanism is the light-dependent translocation of signaling proteins into and out of rod outer segments (ROS) where phototransduction takes place (Arshavsky, 2003; Slepak and Hurley, 2008). Three light-dependent translocating proteins involved in phototransduction have been identified to date: arrestin (Arr), which quenches photoactivated rhodopsin (Rho*); recoverin (Rec), a Ca^{++} binding protein; and α and βγ subunits of transducin, the G-protein mediator in phototransduction. Under light conditions, Arr moves into the outer segments whereas transducin and Rec move in the

Abbreviations: ROS, rod outer segment; BBROS, ROS from bleached bovine retinas; BDROS, ROS from dark-adapted bovine retinas; RBROS, ROS from retinas of light-exposed rats; RDROS, ROS from retinas of dark-adapted rats; LROS, ROS obtained from bovine retinas under room light condition; DAGK, diacylglycerol kinase; DOG, 1,2-dioleoyl-sn-glycerol; SAG, 1-stearoyl-2-arachidonoyl-sn-glycerol; di-C8, 1,2-sn-dioctanoilglycerol; PA, phosphatidic acid; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

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opposite direction. The translocation of these proteins appears to be involved in reduced photoreceptor sensitivity and in the shortening of the response duration, both of which are light adaptation mechanisms. Other proteins not involved in phototransduction are differentially localized under dark or light conditions. RhoA levels were found to be higher in BDROS than in BBROS and there is evidence of the involvement of the small G-protein, RhoA, in the regulation of ROS phospholipase D (PLD), strongly suggesting that this G-protein regulates ROS PLD activity in a light-dependent manner (Salvador and Giusto, 2006). Translocation of type II phosphatidylinositol-5-phosphate 4-kinase (PIPKIIα) in photoreceptor cells from rodent and transgenic frogs has also been recently reported (Huang et al., 2011). Interestingly, light causes a major redistribution of Grb14 which is located mainly in the inner segment, nuclear layer and synapse in dark-adapted rods. In contrast, in light-adapted rods Grb14 is redistributed throughout the entire cell, including the outer segment (Rajala et al., 2009).

Retinal photoreceptor cells from vertebrates have an active phosphoinositide metabolism (Ghalayini and Anderson, 1984; Ghalayini et al., 1998; Ghalayini and Anderson, 1992, 1995; Giusto and Ilincheta de Boschero, 1986; Ilincheta de Boschero and Giusto, 1992). Such studies include phospholipase C (PLC) (Ghalayini and Anderson, 1984; Ghalayini et al., 1998), PI synthetase (Ghalayini and Anderson, 1995), PI 3-kinase (Guo et al., 1997) and diacylglycerol kinase (DAGK) (Giusto and Ilincheta de Boschero, 1986; Ilincheta de Boschero and Giusto, 1992) (Huang et al., 2000). Light activation of phospholipase leads to the hydrolysis of phosphatidylinositol-(4,5)-bisphosphate (PIP2) and the stimulation of protein kinase C (PKC) activity (Kelleher and Johnson, 1985; Newton and Williams, 1991, 1993). PKC activity in ROS is regulated by Ca^{2+} and lipids so this isozyme(s) belongs to the subfamily of conventional PKCs (Williams et al., 1997). Previous studies demonstrated that PIP2-PLCβ and DAGKγ are soluble proteins that are light-activated and photoassociated with disc membranes (Ghalayini et al., 1998; Huang et al., 2000).

Ten mammalian DAGK isozymes have been identified to date. A salient feature of the DAGK family is that all except class II isozymes are highly expressed in the brain, thus indicating their physiological importance in the central nervous system. DAGKε is unique among DAG kinases in that it preferentially phosphorylates DAG with an arachidonate in sn2 position. This selectivity suggests that DAGKε has a more prominent role than other DAGK isoforms in enriching a precursor pool for inositol phospholipid synthesis with unsaturated fatty acids (Cai et al., 2009).

Taking into account that several steps in the PI cycle are stimulated by light and that DAGKε was found in human and rat retina (Kohyama-Koganeya et al., 1997; Tang et al., 1999), the aim of this work was to analyze the presence and light effect of DAGKε in bovine and rat ROS. A differential content of this isoform in ROS from bleached or dark-adapted bovine retinas was observed in IF studies. Light-dependent localization in retina outer segments from rats which had been dark-adapted overnight and subsequently exposed to light for 30 min, was also revealed by IH studies. As previously reported (Slepak and Hurley, 2008), Arr showed a similar light-dependent distribution. Western blot (WB) studies confirmed the enrichment of DAGKε in BROS with respect to DROS from two light/dark models (bovine and rat). Furthermore, light-dependent phospholipase C activation seemed to be involved in DAGKε enrichment in rat ROS. The involvement of phospholipase C activity and PKC regulation of DAGK activity in ROS of bovine retinas exposed to light were also observed. Here, we provide evidence that light induces a redistribution of DAGKε in the photoreceptor cells of two vertebrate species.

2. Materials and methods

2.1. Materials

Bovine eyes were obtained from a local abattoir, placed on ice within 10 min of the animal's death and subsequently kept in darkness for 2 h.

Rat eyes were obtained from albino Wistar rats bred in our own colony. Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG was purchased from Jackson Immuno Research Laboratories, Inc. USA. Biotinylated Goat Anti-Rabbit IgG was generously supplied by Dr. María Marta Facchinetti (INIBIBB). Monoclonal antibody against Rho, Rho-4D2 and anti-visual Arr were generously supplied by Dr. Luis Politi (INIBIBB). Polyclonal antibodies against DAGKε were a generous gift from Dr. Matthew K. Topham (Huntsman Cancer Institute, University of Utah, USA) and Dr. Nicholas Bazan, Neuroscience Center of Excellence at Louisiana State University Health Sciences Center, New Orleans, USA. Antibody for GAPDH and supplies such as U73122, DOG and SAG were also generously provided by Dr. Bazan. The others chemicals were from Sigma (St. Louis, MO, U.S.A.).

2.2. Rod outer segment preparation

ROS from dark-adapted bovine retinas (BDROS) were prepared under dim red light from dark-adapted retinas and ROS from bleached bovine retinas (BBROS) were prepared from retinas whose eyecups had been exposed to 300 W light at 30 cm for 30 min (Guo et al., 1997). The subsequent procedures for ROS preparation were performed at 2–4 °C under either dim red light for BDROS or room light for BBROS. To isolate bovine 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) and, in this manner, bovine ROS were detached. The remainder of the retina was 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* for 30 min. ROS were purified by a discontinuous gradient of sucrose (Kuhn, 1982) yielding a ROS band retained at the 0.84/1.00 M density interface.

ROS from retinas of rats adapted to the dark for 12 h (RDROS) or ROS from retinas of rats exposed to 300 W light at 30 cm (equivalent to 1600 luxes) for 30 min after 12 h of dark-adaptation (RBROS) were obtained as follows: four freshly dissected rat retinas were transferred to 500 μl of ice-cold Hepes–Ringer's buffer (Chen et al., 2008). ROS were detached in 3-s bursts of vortexing (×15) and allowed to settle for 3 min. Crude ROS supernatant was collected (repeated twice), layered on top of a 30 and 40% (wt and vol) discontinuous sucrose gradient in Hepes–Ringer's buffer, and centrifuged for 45 min at 150,000 × g. The interface was collected, diluted 2-fold in buffer, and pelleted at 3000 × g for 20 min. The purity of ROS membrane preparations from bovine and rat retinas was monitored by electron microscopy.

2.3. Immunofluorescence in isolated ROS from bovine retinas

An equal suspension of either BDROS or BBROS was fixed with paraformaldehyde (2%) at room temperature for 30 min and permeabilized with 0.1% of Triton X-100 at room temperature for 15 min. ROS were subsequently washed three times at room temperature for 2 min with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2PO_4, 1.4 mM KH_2PO_4, pH 7.4) and blocked with 5% bovine seroalbumin (BSA). Rabbit polyclonal anti-DAGKε antibody (anti-DAGKε) diluted in 5% BSA (1:100) was added and the mixture was incubated overnight at

4 °C. After four washes with PBS, ROS were incubated at room temperature with goat anti-rabbit secondary antibody coupled to FITC (1:200) for 2 h and after three further washes with PBS, suspended in 50 μl of 30% sucrose. An aliquot of the suspension was analyzed by fluorescence microscopy at 488 nm wavelength. Primary antibody was omitted for the negative control and no specific labeling was detected in this suspension. The positive control was carried out in the presence of anti-Rho-4D2 as primary antibody. Quantification analyses of fluorescent intensities were performed using ImageJ 1.38 software (Abramoff et al., 2004; Rasband, 1997) and multiple selections of the slides were analyzed using the Region of Interest (ROI) tool.

2.4. Dark–light in vivo model

To analyze DAGKε distribution in photoreceptors cells, an *in vivo* model with six-month-old Wistar rats was used to perform immunohistochemistry. After 12 h, dark-adapted rats were killed either under dim red light or after a 30 min exposure to light of 1600 lux intensity. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

For immunohistochemistry, rat eyes were enucleated and fixed by immersion in 10% neutral-buffered formalin in PBS for 24 h. After removing the cornea and lens from the fixed eyeballs, the eyecups were dehydrated via a graded series of ethanol and embedded in paraffin at 56 °C. Paraffin-embedded retinal tissues were cut into 5-μm sections on a rotary microtome (Leica, Nussloch, Germany). The sections were then deparaffinized in xylene at 60 °C for 30 min and rehydrated in a series of decreasingly diluted solutions of ethanol for 5 min. After hydration, the sections were treated with 3% hydrogen peroxide in ethanol 96° for 15 min to block endogenous peroxidase activity, washed in PBS for 15 min, exposed to 2% BSA for 30 min and subsequently incubated overnight at 4 °C with rabbit anti-DAGKε or anti-visual Arr as primary antibodies diluted 1:100 in 2% BSA or 1:750, respectively. After three washes with PBS, the sections were incubated with the secondary antibody biotinylated anti-rabbit IgG (Vector) diluted 1:400 in 2% BSA for 30 min. After three further washes with PBS, avidin-biotin peroxidase complexes were formed using an Elite kit (Vector). The peroxidase reaction was developed with a diaminobenzidine substrate kit (Vector). Sections were mounted before they were counterstained with hematoxylin. In the negative control, primary antibodies were omitted and no specific labeling was detected in these sections.

2.5. Dark–light in situ model

An *in situ* model using immunohistochemistry performed on six-month-old Wistar rats was applied for the analysis of light-dependent phospholipase C activation of DAGKε distribution in photoreceptor cells. After 12 h dark adaptation, eyes were enucleated and the cornea, lens and aqueous humor subsequently removed from the eyeballs under dim red light. The eyecups were placed on ice in appropriate containers and immersed in oxygenated (95% O_2, 5% CO_2) Ames medium with 0.5% glucose and 0.1% DMSO (DMSO controls) or 10 μM U73122 in 0.1% DMSO (PLC inhibition condition), in dim red light. After 15 min incubation, half the material was maintained under dim red light while the other half was exposed to 300 W light at 30 cm for 30 min (Guo et al., 1997). All the material was subsequently fixed by immersion in 10% neutral-buffered formalin in PBS for 24 h and the eyecups were processed as described in 2.4.

2.6. Determination of DAGK activity in ROS

DAGK activity was determined in isolated ROS measuring PA synthesis using [γ^{32}P]ATP or [^{3}H]DAG as radioactive substrates.

2.6.1. DAGK assay using [γ-^{32}P]ATP

When DAGK activity was determined by measuring radioactive phosphate incorporation into PA using 3 μCi [γ-^{32}P]ATP, either endogenous DAG, exogenous DOG or SAG were used as substrates. The standard assay contained either 50 mM OG or 1 mM Na-deoxycholate in 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT and 250 μM ATP in a volume of 200 μl. When DAGK substrate selectivity was measured, exogenous DAG was added to the assay and an appropriate volume of DAG stock solution was evaporated under a stream of nitrogen in a glass test tube. It was then re-suspended in Na-deoxycholate or in OG and the detergent-lipid mixture was sonicated in a test tube placed in a Cup Horn system (Branson Digital Sonifier, model 450). Prior to incubation, ROS membranes (300 μg protein) were sonicated for 15 s with radioactive ATP and detergent-lipid suspension in a water bath (Branson Sonifier). Reactions were performed at 37 °C for 5 min. Blanks were prepared identically, except that membrane fractions were boiled for 5 min before use. Reactions were stopped by adding chloroform/methanol/1 N HCl (2:1:0.2, by volume) and lipids were extracted following Jolles et al. (1981) (Jolles et al., 1981). Five additional washes of the lipid extract for [γ-^{32}P]ATP elimination were carried out using theoretical upper phase. PA, PI(4)P and PI(4,5)P2 were separated by TLC on 1% potassium oxalate in silica gel H developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7.5, by vol) (Ilincheta de Boschero and Giusto, 1992).

Lipids were visualized by exposure of the chromatograms to iodine vapors, identified with appropriate lipid standards and scraped off for counting by liquid scintillation spectroscopy.

2.6.1.1. DAGK assay in presence of PKC activators. ROS obtained from bovine retinas under room light condition (LROS) were subsequently assayed after activation of PKC-dependent protein phosphorylation conditions. The incubation mixtures for ROS phosphorylation by PKC containing 3 mg LROS protein, 60 mM Tris–HCl buffer at pH 7.4, 10 mM KF, 0.5 mM DTT, 7.5 mM magnesium acetate, 2.5 mM ATP, 20 μg/ml PS, 0.8 μg/ml DOG and 0.5 mM $CaCl_2$ in a total volume of 1.72 ml were shaken at 37 °C for 30 min. To study the effect of PKC activators on DAGK, either 1 μM phorbol dibutyrate (PDBu) or 50 μM dioctanoylglycerol (di-C8) was added to the assay medium instead of DOG. PKC activity was inhibited with 100 μM of H7, which was already present 10 min before PDBu addition. Control membranes for these experiments were prepared as described above except that EGTA (0.5 mM final concentration) was included in the assay medium to rule out PKC activation by endogenous calcium (C2). Extreme dephosphorylation conditions (C1 control) were prepared in CAPS buffer pH 8.5 without ATP and KF, and with 3.5 units/ml of alkaline phosphatase.

The reaction was performed and the enzyme reaction products were isolated and quantified as described in 2.6.1.

2.6.2. Preparation of radioactive 1,2-diacyl-sn-glycerol and DAGK assay using [^{3}H]DAG

Radioactive DAG was obtained from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described (Pasquare de Garcia and Giusto, 1986). Lipids were extracted from the tissue as described in Folch et al. (1957). [^{3}H]Diacylglycerol plus Cholesterol was isolated by mono-dimensional TLC in silica gel G plates and with a solvent system of hexane:diethyl ether:acetic acid (60:40:2,3 by volume). Lipids were eluted

(Arvidson, 1968) and diacylglycerol was purified by one-dimensional TLC on silica gel G developed with chloroform:methanol:acetic acid (98:2:1, by volume). The substrate was eluted as above from the silica gel and stored in chloroform solution to avoid the production of 1,3-diacyl-sn-glycerol. An appropriate volume of radioactive diacylglycerol was evaporated under a stream of nitrogen in a glass test tube and then re-suspended in octyl-β-glucopyranoside (OG) at a final assay concentration of 50 mM. Buffer conditions were identical to those previously described (2.6.1). Prior to incubation, ROS membranes (300 μg protein) were sonicated for 15 s with ATP and detergent-lipid suspension in a water bath (Branson Sonifier). Reactions were performed at 37 °C for 10 min. Blanks were prepared identically, except that membrane fractions were boiled for 5 min before use. Reactions were stopped by adding chloroform/methanol/1 N HCl (2:1:0.2, by volume) and lipids were extracted as above. Only one additional wash of the lipid extract for aqueous substance elimination was carried out using theoretical upper phase. Lipid products synthesized from [^{3}H]DAG as precursor were separated by TLC on silica gel G (Giusto and Bazan, 1979) and developed with hexane/diethyl ether/acetic acid (30:70:1, by volume). For [^{3}H]PA and [^{3}H]MAG isolation, the plate was re-chromatographed up to the middle using hexane/diethyl ether/acetic acid (20:80:2.3, by volume) as developing solvent. In this experimental approach [^{3}H] Glycerol was also measured as a product of monoacylglycerol lipase activity in Folch's methanol:water layer in order to ensure that PA synthesis from [^{3}H]DAG was not modified by other enzymatic transformations of substrate. Lipids were visualized by exposure of the chromatograms to iodine vapors, identified with appropriate lipid standards and scraped off for counting by liquid scintillation spectroscopy.

2.6.2.1. DAGK assay in the presence of R59022 and neomycin. A DAGK assay with [^{3}H]DAG as radioactive substrate and 2 mM ATP was performed. BDROS and BBROS (300 μg protein) were incubated in the presence of 10 μM R59022 or 0.2 mM neomycin for 10 min before [^{3}H]DAG addition. As the R59022 vehicle, DMSO was present in the control and under all experimental conditions at a final concentration of 0.1%. Buffer conditions were as previously described (2.6.1) but with the addition of 1 mM EGTA. All subsequent procedures were performed as previously described (2.6.2.).

2.7. Western blotting of proteins from entire ROS

Bovine ROS proteins from dark and bleached conditions (BDROS and BBROS, respectively) or ROS proteins from dark-adapted or light-exposed rat retinas (RDROS and RBROS, respectively) as described in 2.4. were heat-denatured at 95 °C in 4× loading buffer (250 mM Tris–HCl, pH 6·8, 4% (w/v) SDS, 30% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 2% (v/v) bromophenol blue) in a volume ratio 3:1 (sample: buffer). An aliquot of 20 μl (50 μg protein) of each sample was resolved in a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was blocked for 2 h at room temperature with 5% non-fat milk in TBST (5%, w/v non-fat dried milk in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1%, v/v Tween 20) and then incubated overnight at 4 °C in blocking buffer containing rabbit polyclonal anti-DAGKε antibody (1:750) (bovine) and goat polyclonal anti-DAGKε antibody (1:1250) (rat). After four washes in TBST, the blot was incubated for 2 h at room temperature with polyclonal horseradish peroxidase conjugated secondary antibody (anti-rabbit or anti-goat) diluted 1:2000 in blocking buffer and washed as indicated above. Bands were visualized by the ECL method. Quantification analyses of protein bands detected by WB were performed using ImageJ 1.38 software working in a linear range (Abramoff et al., 2004; Rasband, 1997). The amount of DAGKε in each sample of bovine ROS was normalized to the amount of α-actin or GADPH and in each sample of rat ROS to the amount of tubulin. Molecular weight identification was performed with a Sigma high molecular weight range marker (M3788).

2.8. Statistical analysis

Data were analyzed using GraphPad Prism, version 5.0 (San Diego, CA). An unpaired *t*-test was performed to determine the difference between Light/Dark means ± SD (two tail *p* value election) (Figs. 2, 3 and 5) and a one-way ANOVA with Dunnett's post-hoc test to determine the difference between control and PKC activators and inhibitor (H7) treatments (Fig. 6). Two-way ANOVA with the Bonferroni post-test was used to determine the difference between substrates (DOG and SAG) and R59022 treatment and the possible interactions of each (Fig. 1), and between light and dark conditions and inhibitors treatment (Fig. 8). Differences were considered significant at $p < 0.05$.

3. Results

3.1. DAGK in isolated ROS from bovine retinas. Substrate and detergent preferences are features of DAGKε activity

Bovine ROS were prepared under room light from retinas obtained from dark-adapted bovine eyes. Experiments were subsequently carried out to study DAGK activity using [γ-^{32}P]ATP and 1, 2-dioleoylglycerol (DOG) or 1-stearoyl-2-arachidonoylglycerol (SAG) as substrates. A detergent-lipid micellar assay was carried out using OG or Na-deoxycholate.

Although DAGKε has the ability to phosphorylate 1,2-dipalmitoylglycerol (DPG) and other saturated DAGs, it is also highly efficient at 2-arachidonoyl DAGs phosphorylation. This DAGK isoform was also found to show OG preference in a

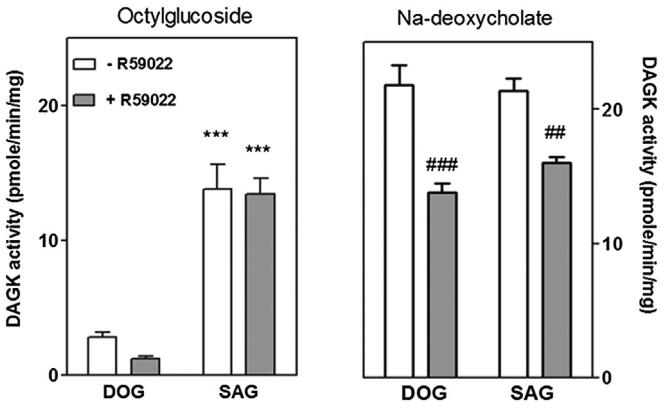

Fig. 1. Substrate and detergent preferences are features of DAGKε activity in isolated ROS from bovine retinas exposed to room light. DAGK activity was measured in the presence of the inhibitor of type I isoforms, R59022. ROS from bovine retinas obtained under room light conditions (LROS) were pre-incubated in the presence of vehicle (DMSO) or R59022 (10 μM) at 37 °C for 10 min followed by incubation with 250 μM of [γ-^{32}P]ATP (3 μCi) and a lipid-detergent suspension with 50 mM OG (left panel) or 1 mM Na-deoxycholate (right panel) as detergents, and 0.5 mM DOG or 0.5 mM SAG, as exogenous substrates. After 5 min incubation at 37 °C the enzyme reaction was stopped, the lipids were extracted and, isolated and PA was separated and quantified as described in Materials and Methods. Results are expressed as pmol PA × (mg protein × min)$^{-1}$ and are means ± SD. Two-way ANOVA with the Bonferroni post-test was used to determine the difference between substrates (DOG and SAG) and R59022 treatment and the possible interactions between each. Asterisks indicate significant differences between substrates (***$p < 0.001$) and numerals indicate significant differences with respect to the vehicle condition (##$p < 0.01$; ###$p < 0.001$).

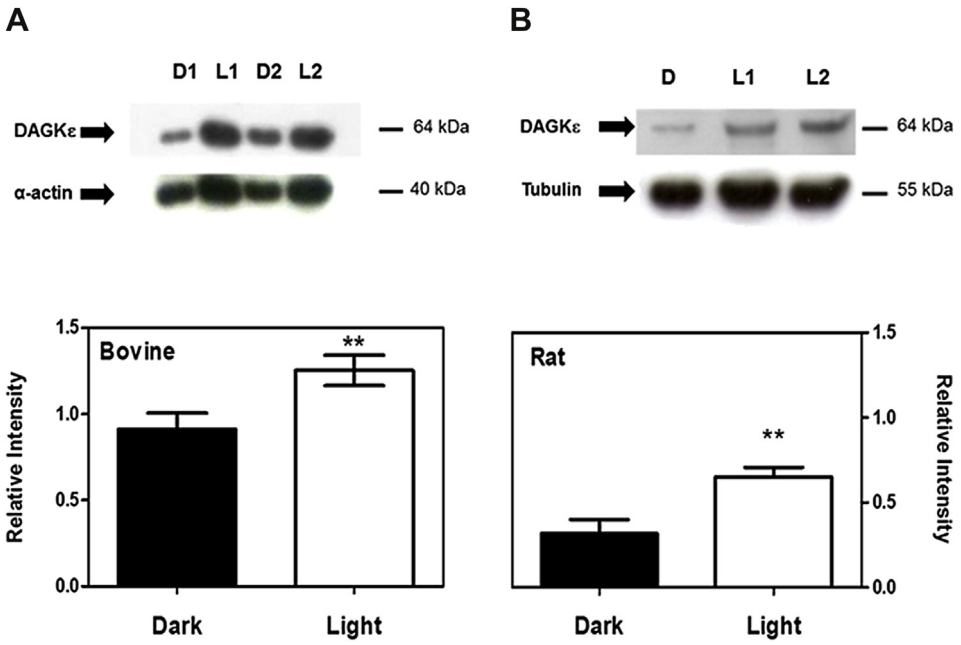

Fig. 2. DAGKε expression in bovine and rat ROS from dark or light adapted retinas. For the WB assay, ROS proteins from bovine (A) and rat (B) retinas (100 μg) were resolved in a 10% SDS–PAGE and transferred to a PDVF membrane. Membranes were blocked and incubated with primary and secondary antibodies as detailed in Materials and Methods. Immunoreactive bands were detected by enhanced chemiluminescene. Numbers to the right indicate molecular weights and the data shown are a representative result of three independent experiments. Bands corresponding to DAGKε were analyzed using ImageJ software (NIH Image, Bethesda, MD). The amount of DAGKε in each sample of bovine ROS was normalized to the amount of α-actin. The amount of DAGKε in each sample of rat ROS was normalized to the amount of tubulin. An unpaired *t*-test was performed to determine the difference between light/dark means (two tail *p* value election). Asterisks indicate significant differences with respect to the dark condition. $^{**}p < 0.01$.

detergent-lipid micellar assay (Milne et al., 2008; Thirugnanam et al., 2001).

It is known that type I DAGK activities (α, β and γ isoforms) are usually measured in the presence of Na-deoxycholate and that they show no preference with regard to DAG acyl composition. Previous studies demonstrate that DAGKγ is a soluble protein that is light-activated and photoassociated with disc membranes from bovine and rat retinas (Huang et al., 2000). It is also known that R59022 and R59949 are potent inhibitors of calcium-dependent isoforms of DAGK such as type I isoforms. Selective inhibition of type I isozymes by R59022, a poor DAGKε inhibitor, has been reported (Jiang et al., 2000; Tang et al., 1996). This experimental tool was also used to indirectly analyze the level of type I isozyme activity such as that of DAGKγ in BBROS and DBROS.

As shown in Fig. 1, in the presence of OG, the ability of DAGK to convert SAG into PA was observed to be 6-fold higher than its ability to convert DOG, an effect that was not inhibited by R59022. However, R59022 was observed to inhibit DOG conversion. Whereas differences between treatments (+R59022/−R59022) were not significant, differences between substrates (DOG/SAG) were extremely significant ($p < 0.0001$).

In contrast, substrate preference was not observed in the presence of Na-deoxycholate and similar levels of PA were synthesized from DOG and SAG. Under this new condition, PA synthesis from DOG and SAG was 100% and 20% higher, respectively, than that observed in the OG assay. Similarly, R59022 produced an approximately 40% inhibition of DOG and SAG conversion by DAGK activity. Differences between treatments (+R59022/−R59022) were extremely significant ($p < 0.001$ with DOG as substrate and $p < 0.01$ with SAG as substrate).

These findings strongly suggest that in bovine ROS obtained from retinas under room light conditions, DAGKε and type I DAGK activity appears to be present. DAGKε presence and light influence in ROS were subsequently tested by WB.

3.2. DAGKε in isolated ROS from bovine and rat retinas. Enrichment in ROS from bleached retinas

Western blot analysis confirmed the higher content of DAGKε in BBROS with respect to BDROS. Bovine ROS were prepared under dim red light from retinas obtained from dark-adapted bovine eyes or those from eyecups exposed to 300 W light at 30 cm (1600 lux or 140 cd) for 30 min in ice-cold water after dark adaptation of excised bovine eyes. Immunoblots of four pools of bovine ROS (3 retinas each from D1, D2 and L1, L2) probed with anti-DAGKε showed a single band corresponding to the reported molecular mass of DAGKε (64 KDa). Fig. 2A shows the WB from this experiment. The entire transferred membrane obtained with these pools is shown in the supplemental material section (Fig. S1). Housekeeping proteins such as α actin (Fig. 2A) or GAPDH are useful as loading controls for WB and protein normalization. The densitometric analysis of the WB from four independent experiments showed that the relative intensity of DAGKε in BBROS was about 40% higher than that in BDROS.

Since the bovine eyes obtained from a local abattoir were dark-adapted for only 2 h, this may have been insufficient time to fully deactivate light-dependent mechanisms. To confirm DAGKε enrichment in BBROS with respect to BDROS, an *in vivo* experimental model with Wistar rats was used.

The right panel of Fig. 2B shows a WB from a representative experiment with rat ROS obtained from four retinas under each condition. The content of tubulin was used as a control of protein loading. The analysis of blots (relative intensity) showed that the amount of DAGKε in RBROS was two-fold higher than that of RDROS. Significant differences between bovine and rat bleached ROS with respect to respective dark controls were obtained (*p* value 0.0018 and 0.0037 respectively).

The presence of DAGKε in purified bovine ROS was also observed by fluorescence microscopy using rabbit anti-DAGKε primary

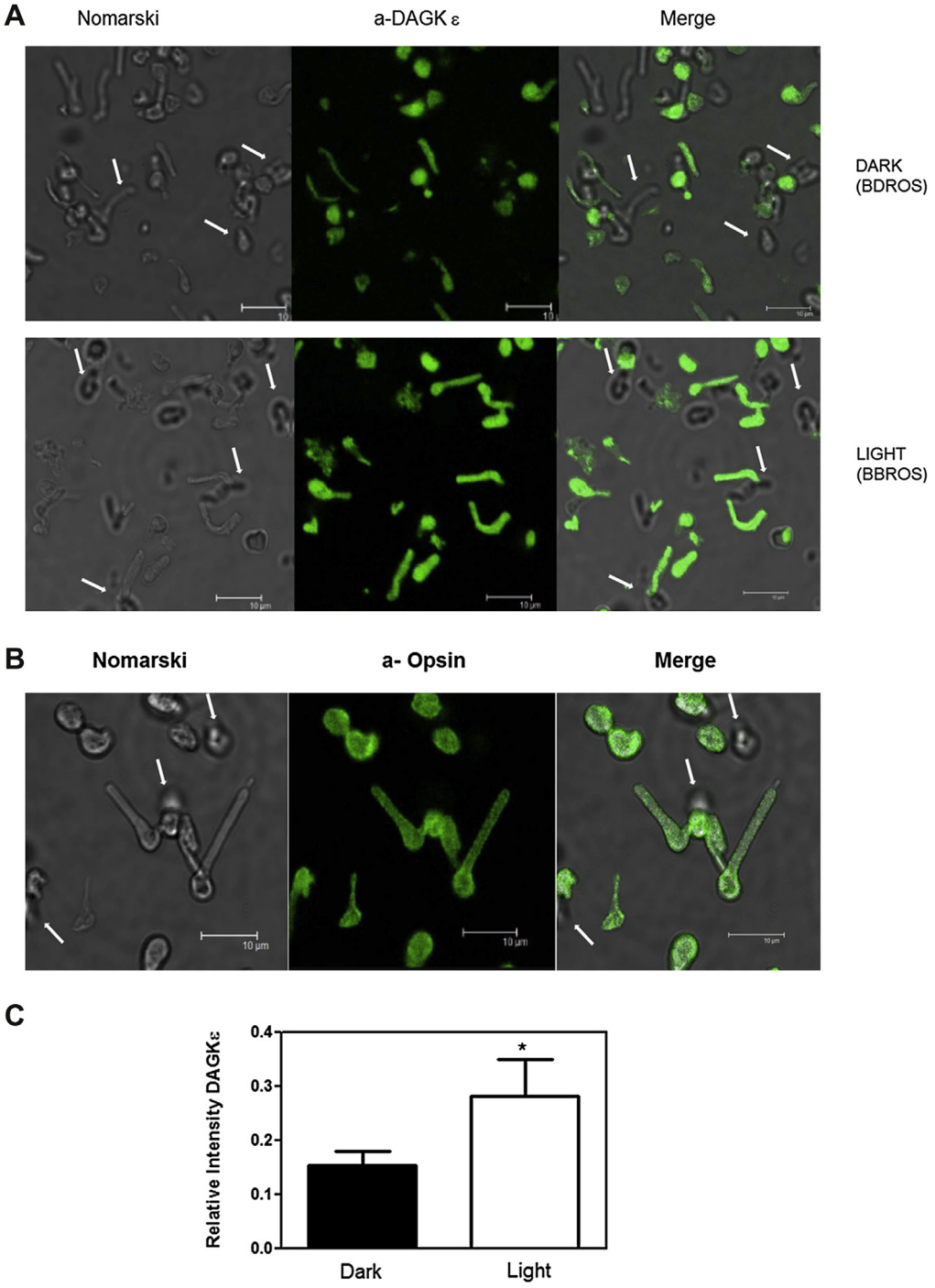

Fig. 3. Presence of DAGKε in Bovine Rod Outer Segment (ROS) from dark- or light-adapted retinas. Bovine dark-adapted ROS (BDROS) or bleached ROS (BBROS) were prepared from bovine retinas as described in Materials and Methods. ROS were treated and incubated with primary and secondary antibodies as detailed in Materials and Methods. An equal suspension of BDROS or BBROS was analyzed to determine DAGKε presence by immunofluorescence at 488 nm wavelength (A). A negative control was carried out by omitting the primary antibody and anti-Rho-4D2 was used as a positive control to identify ROS (B). White arrows were included to indicate unfocussed ROS structures appearing with no IF signal. Images were analyzed (C) using ImageJ software (NIH Image, Bethesda, MD) to compare relative immunolabeling intensity. An unpaired *t*-test was performed to determine the difference between light/dark means (two tail *p* value election). Asterisks indicate significant differences with respect to the dark condition. $^{*}p < 0.05$.

antibody and FITC-secondary antibody (Fig. 3A). Fig. 3 shows representative results from 6 different fields of three different experiments. Lineal and circularized ROS structures could be visualized in bovine ROS preparations. The presence of opsin was detected using rabbit anti-Rho-4D2 and revealed that all the focalized structures visualized by Nomarski (left panel) have positive FITC signals (Fig. 3B). Under control conditions without exposure to primary antibodies, no signal from FITC-conjugated antibody was detected.

Differences in DAGKε immunoreactivity between isolated BDROS and BBROS were analyzed using ImageJ software (Fig. 3C). Quantification analyses of fluorescent intensity using ImageJ 1.38 software revealed a significant two-fold increase in DAGKε presence in BBROS with respect to BDROS ($p < 0.05$).

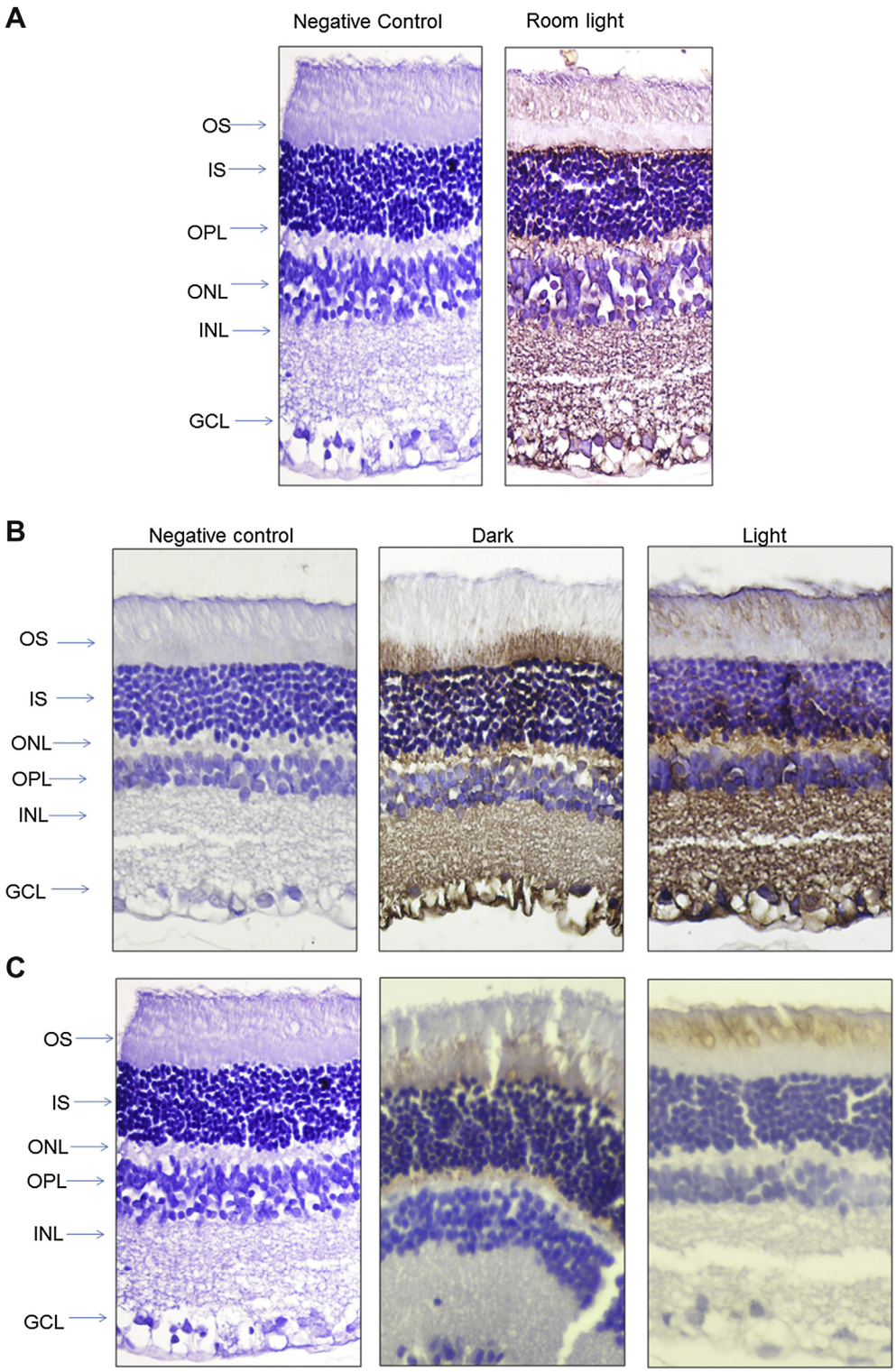

Fig. 4. Immunohistochemical localization of DAGKε and visual arrestin in the retina photoreceptor layer from dark-adapted rats exposed to light. Retina cross-sections from rats exposed naturally to room light conditions (A) or from dark-adapted rats exposed to light conditions (B, C) were obtained as described in Materials and Methods. Detection was performed using Rabbit polyclonal anti-DAGKε (1:100) (A, B) and Rabbit polyclonal anti-visual arrestin (1:750) (C). Immunoreactions were detected with secondary antibody; biotinylated anti-rabbit IgG (Vector) diluted 1:400. Negative control was carried out omitting the primary antibody.

As a control of this methodology for detecting protein content differences in bovine ROS, DAGKγ immunofluorescence was also determined and quantified. Photoassociation of this isoform and its enrichment in BBROS compared with BDROS was reported (Huang et al., 2000). The presence of DAGKγ in purified ROS could then be observed applying fluorescence microscopy to permeabilized bovine purified ROS treated with FITC-secondary antibody (goat anti-rabbit FITC-conjugated antibody) after the exposure of bovine ROS to anti-DAGKγ antibody from rabbit. As shown in Fig. S2A, a higher FITC signal was observed in BBROS than in BDROS, thus

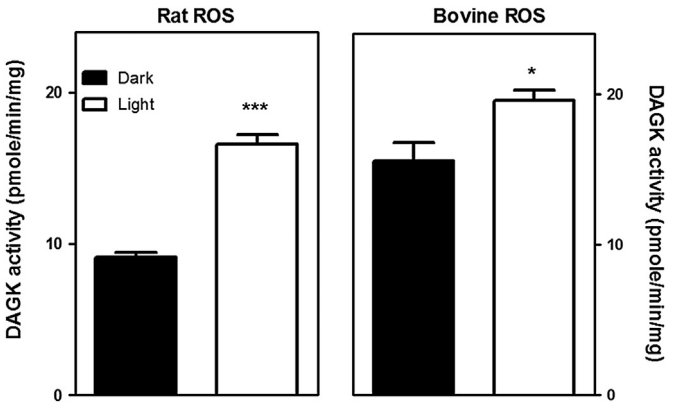

Fig. 5. Increased DAGK activity in isolated ROS from retinas obtained after light exposure of dark-adapted eyecups (bovine) or dark-adapted animals (rat). DAGK activity was measured in rat and bovine ROS from retinas obtained after light exposure (30 min) of dark-adapted eyecups (bovine) or dark-adapted animals (rat). Dark controls (eyecups or animals) were kept in darkness during the light exposure time. ROS were incubated with radioactive [γ^{32}P]-ATP (3 μCi) using SAG (500 μM) as exogenous substrate. The reaction was performed at 37 °C for 5 min as described in Materials and Methods. Results are expressed as pmol PA × (mg protein × min)$^{-1}$(mean ± SD). An unpaired *t*-test was performed to determine the difference between light/dark means (two tail *p* value election). Asterisks indicate significant differences with respect to the dark condition. $^{*}p < 0.05$; $^{***}p < 0.001$.

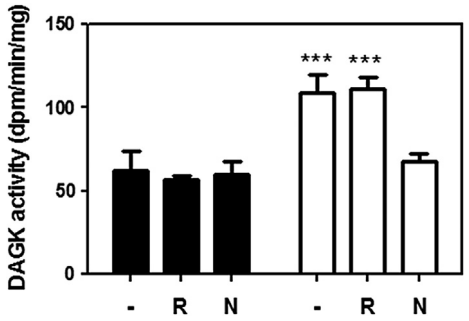

Fig. 7. Light-dependent DAGK activation was impaired when dark-adapted bovine retinas were exposed to Neomycin. BDROS and BBROS (300 μg protein) were incubated in the presence of 10 μM R59022 (R) or 0.2 mM neomycin (N) for 10 min before [^{3}H] DAG addition. As DMSO is the R59022 vehicle, it was present in control and all experimental conditions at a final concentration of 0.1%. Radioactive DAG was resuspended in OG at a final assay concentration of 50 mM. Buffer conditions were as previously described in Materials and Methods, except that 1 mM EGTA was present in the assay. The reaction was performed at 37 °C for 10 min as described in Materials and Methods. Results are expressed as dpm PA (mg protein × min)$^{-1}$ (mean ± SD). Two-way ANOVA with the Bonferroni post-test was used to determine the difference between light and dark conditions and inhibitors treatment (R or N) and the possible interactions of between each. Interaction between factors is considered extremely significant (*p* value 0.0003). Asterisks indicate significant differences with respect to the dark condition (Bonferroni post test). $^{***}p < 0.001$.

confirming previously published data. Differences in DAGKγ immunoreactivity (ImageJ) indicate a three-fold increase in BBROS with respect to BDROS (Fig. S2B; $p < 0.01$).

3.3. Localization of DAGKε in the photoreceptor layer of rat retinas

To determine the subcellular localization of DAGKε, paraffin-embedded rat retina sections were probed with anti-DAGKε using immunohistochemistry. As shown in Fig. 4A, DAGKε in rat retina naturally exposed to room light is seen to be present in all retinal layers and is distributed along the photoreceptor cell. However, in the light–dark *in vivo* model, in which 12 h dark-adapted rats were killed either under dim red light or after a 30 min exposure to room light of 1600 lux intensity, DAGKε appears to be concentrated in the

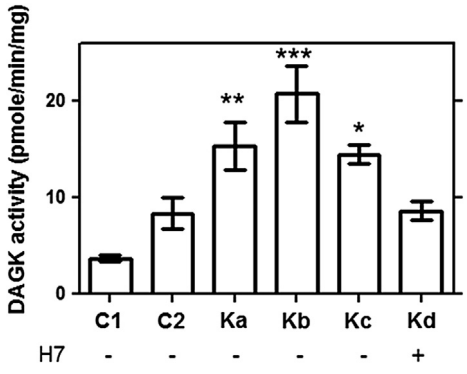

Fig. 6. PKC-dependent phosphorylation of ROS regulates DAGK activity. ROS obtained from bovine retinas at room light (LROS) were incubated under different phosphorylation conditions. The reaction was performed at 37 °C for 30 min before DAGK measurement. Controls were made in the absence of exogenous ATP and in a buffer condition for alkaline phosphatase activation without fluoride and with 3.5 units/ml of alkaline phosphatase (C1), or in 60 mM Tris–HCl buffer, 10 mM KF, 0.5 mM DTT, 7.5 mM magnesium acetate and 0.5 mM EGTA (C2 condition). In the presence of 2.5 mM ATP, phosphorylation conditions were: 0.8 μg 1,2-diolein (DOG), 20 μg PS and 0.5 mM $CaCl_2$ (Ka); 50 μM dioctanoylglycerol (di-C8) or 1 μM phorbol dibutyrate (PDBu) (Kb and Kc condition respectively) or 1 μM H7 (PKC inhibitor) incubated for 10 min before the PDBu activation condition (Kd condition). After this phosphorylation protocol ROS membranes were prepared for DAGK measurement as described in Materials and Methods. Results are expressed as pmol PA × (mg protein × min)$^{-1}$ (mean ± SD). One-way ANOVA with Dunnett's post-test was performed to determine the difference between control (C2) and PKC activators and inhibitor (H7) treatments Asterisks indicate significant differences with respect to the C2 condition. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$.

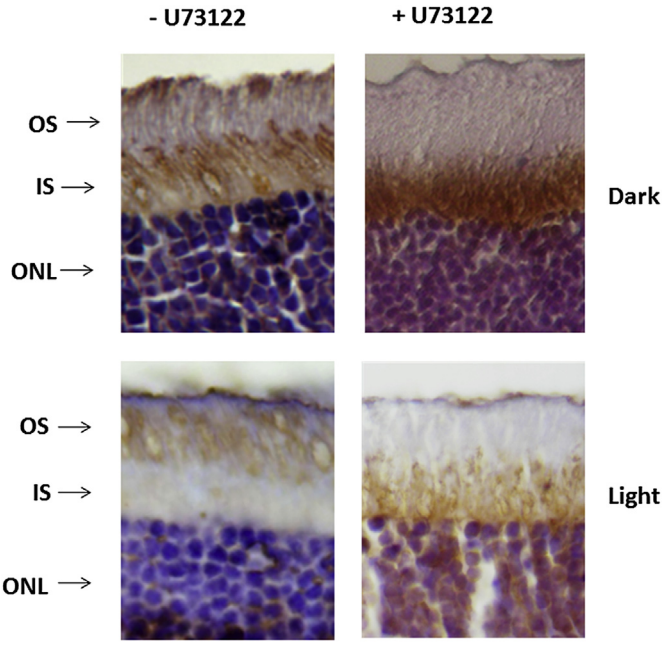

Fig. 8. Immunohistochemical localization of DAGKε in retina photoreceptor layer from dark adapted rats exposed to light. Effect of U73122. After 12 h dark adaptation rat eyes were enucleated and the cornea, lens and humors were subsequently removed from the eyeballs under dim red light. The eyecups were placed in appropriate containers on ice-cold and were immersed in oxygenated (95% O_2, 5% CO_2) Ames medium with 0.5% glucose, with 0.1% DMSO (DMSO controls) or with 10 μM U73122 in 0.1% DMSO (PLC inhibition condition), in dim red light. After 15 min incubation, half the material was maintained under dim red light (Dark) while the other half was exposed to light for 30 min (Light) as described in Materials and Methods. Rabbit polyclonal anti-DAGKε (1:100) was used. Immunoreactions were detected with secondary antibody; biotinylated anti-rabbit IgG (Vector) diluted 1:400. The negative control was carried out omitting the primary antibody.

inner segment of the photoreceptor layer under dark conditions. In contrast, under light stimuli, DAGKε was higher in the outer segment than the inner segment (Fig. 4B), indicating the light-dependent distribution of DAGKε in photoreceptor cells.

Since it is known that Arr is differentially distributed along photoreceptor cells under light or dark conditions, Arr localization was also used as a control of the immunohistochemistry carried out under the same conditions as those for the analysis of DAGKε. Similarly to DAGKε distribution, visual Arr was found to be present in the inner segment under dark conditions but in ROS under light conditions (Fig. 4C). This observation is in agreement with previous reports (Sokolov et al., 2002).

3.4. DAGK activity in ROS from bovine and rat retinas

As stated above, DAGKε appears to be higher in ROS from bleached bovine retinas and from rat retinas exposed to light than in those under dark conditions. Taking into account that DAGKγ is also present, we measured PA formation through DAGK activity under conditions which inhibit type I isoforms such as DAGKγ and favor DAGKε. Type I DAGKs, including DAGKα, -β and -γ, have EF-hand motifs and two cysteine-rich regions in the regulatory domain (Goto et al., 1994; Sakane et al., 1996). The calcium-sensitive domain is related to plasma membrane association and activation of the enzyme. EGTA, which acts to trap Ca^{++} endogenously, was used in our assay to inhibit type I DAGK activity. Previous research has demonstrated that light-dependent PLCγ1 is photoassociated with ROS membranes (Ghalayini et al., 1998). An increase in DAG content in BBROS is therefore expected to induce DAGK activation. In order to measure DAGK activity independently of PLC stimulated activity, and to favor DAGKε activity, a micellar assay was carried out using exogenous SAG. Experiments were performed with isolated bovine ROS and rat ROS obtained from bleached retinas (dark-adapted bovine eyecups or dark-adapted rats, both exposed to light). As shown in Fig. 5 (left panel, rat ROS) DAGK activity in RBROS was twice as high as that in RDROS. Slightly but still significantly higher DAGK activity was also found in BBROS with respect to BDROS (Fig. 5, right panel). As stated earlier, 2 h of darkness may be insufficient time for light-dependent mechanisms to be fully deactivated in excised bovine eyes. A significant difference between BBROS and BDROS was obtained (p value 0.04) and an extremely significant difference between RBROS and RDROS (p value 0.0004).

3.5. PKC-dependent phosphorylation and DAGK activity in ROS obtained from bovine retinas under room light

DAGK redistribution into photoreceptor cells could also be related with ROS PKC regulation under light conditions. DAGK activity in LROS was subsequently assayed after activation of PKC-dependent protein phosphorylation conditions. As a control of ROS protein phosphorylation, two conditions were assayed in the absence of exogenous ATP and fluoride and in the presence of EGTA. Under the C1 condition, alkaline phosphatase was also present in the pre-incubation of ROS membranes prior to the DAGK assay. PA formation was higher under the C2 condition than under C1, revealing that protein phosphorylation positively regulates DAGK activity. Higher DAGK activity was observed with respect to control C2 (Fig. 6) in ROS incubated with DOG, PS and calcium ions (Ka condition), conventional PKC activators and those pre-phosphorylated with PKC activators such as di-C8 or PDBu (Kb and Kc conditions, respectively). Under the Kd condition, H7 (PKC inhibitor) was present for 10 min after PKC activation with PDBu; PA formation was not stimulated by PDBu, the values being the same as those under the C2 condition. Significant differences between activators (DOG, PDBu or di-C8) were obtained. A non-significant difference with respect to the C2 condition was obtained when PDBu was used after H7 pre-incubation. These data suggest that protein phosphorylation by a PKC-dependent mechanism positively regulates DAGK activity. This PKC-dependent mechanism that stimulates DAGK activity in LROS appeared to be absent when DROS were assayed after activation of PKC-dependent protein phosphorylation conditions. Equivalent conditions (Ka, Kb and Kc) in DROS did not show significant differences with respect to C2 (data not shown).

3.6. Light-dependent PA synthesis and DAGKε distribution in ROS. Effect of PIP2-PLC inhibitors

It has recently been reported that light-dependent Arr translocation is initiated by a G-protein-coupled cascade through PLC and PKC signaling (Orisme et al., 2010). Light-dependent DAGKε redistribution was observed in our IH studies in slices of dark-adapted rat retinas and of rat retinas exposed to light for 30 min (*in vivo* dark–light model) (Results, 3.3). In addition, protein phosphorylation by a PKC-dependent mechanism positively regulates DAGK activity in LROS (Results, 3.5.).

In order to analyze the relationship between PLC activity and DAGK activity, isolated BDROS and BBROS were incubated in the presence of neomycin for 10 min before [^{3}H]DAG addition. In this experimental approach, substrate is provided exogenously and utilization of DAG from the light-dependent activation of PLC (Ghalayini et al., 1998) is avoided.

In agreement with data obtained using [γ-^{32}P]ATP and SAG as substrate (Fig. 5, right panel), PA formation in BBROS was significantly higher than in BDROS in the controls (Fig. 7) ($p < 0.001$). R59022 failed to inhibit DAGK activity in BDROS and BBROS (Fig. 7). This latter finding for BBROS using R59022 is in agreement with data obtained using [γ-^{32}P]ATP and SAG as substrate (Fig. 1, OG).

Neomycin-induced inhibition of PLC activity in BBROS was observed to produce a significant decrease in [^{3}H]PA formation (N, white bar, Fig. 7). In contrast, no decrease was observed in BDROS pre-incubated with neomycin. The statistical analysis (two-way ANOVA) indicates a significant correlation between treatments and light/dark conditions ($p < 0.001$), suggesting that the light-dependent activation of PLC positively regulates DAGK activity or modifies DAGK distribution in the photoreceptor cell.

To explore the latter possibility, a rat model was used. Rat eyes were excised from dark-adapted rats and *in situ* light exposure (or dark condition) was performed as described in 2.5. Pre-incubation with U73122 in 0.1% DMSO as vehicle was used prior to light exposure. As a control, eyecups were incubated with 0.1% DMSO under the dark condition, prior to light exposure. Paraffin-embedded rat retina sections of different conditions were probed with anti-DAGKε and anti-Arr using immunohistochemistry. As shown in Fig. 8, DAGKε signal was observed in the inner segment when U73122 was present prior to light exposure (lower right panel). A similar pattern was observed in controls with U73122 under the dark condition (upper right panel). Controls with DMSO (as U73122 vehicle) in the left panels showed a similar result to that shown in Fig. 4B. In contrast, the Arr signal was observed in the outer segment after light exposure under conditions of PLC inhibition (data not shown).

This result suggests that DAGKε distribution in the photoreceptor cell is dependent on PIP2-PLC activation by light.

4. Discussion

The main finding of our study is that DAGKε is differently localized in bovine and rat cell photoreceptors in response to light.

Although DAGKε was reported in rat retina (Kohyama-Koganeya et al., 1997), WB studies failed to detect this isoform in isolated ROS (Huang et al., 2000). We obtained the first evidence of DAGKε in bovine outer segments when DAGK activity was measured in isolated ROS (light condition) by means of a detergent-lipid micellar assay. In the presence of OG as detergent and exogenous SAG or DOG as substrate, SAG preference over DOG and the fact that R59022 did not induce inhibition of PA formation from SAG (Fig. 1, left) are both indicative of the presence of DAGKε. Furthermore, when Na-deoxycholate -a recommended detergent for measuring soluble DAGK activities- was used in the assay, utilization of the two diacylglycerols was similar (Fig. 1, right). Combined with the fact that under this detergent condition R59022 inhibited DAGK action on DOG and SAG, this finding also confirms the presence of type I DAGK activity in isolated bovine ROS.

WB revealed the presence of DAGKε in bovine ROS and higher DAGKε content in BBROS than in those prepared from dark-adapted retinas (BDROS) (in Fig. 2, A). The *in vivo* model of dark-adapted rats exposed to light adds conclusive evidence in support of a different DAGKε content in OS under light–dark conditions (Fig. 2B).

IF studies on isolated BBROS and BDROS also revealed significant differences in DAGKε detection and fluorescence quantification (Fig. 3). A two-fold increase in DAGKε content in BBROS with respect to BDROS is apparent with this methodology. Interestingly, IF studies also revealed a three-fold increase in DAGKγ in BBROS with respect to BDROS (Fig. S2). This is in agreement with previous findings derived from WB studies in isolated B-ROS with respect to D-ROS in which light-induced DAGKγ light-activation and photoassociation were observed, thus suggesting a soluble or membrane localization of this isoform under dark or light conditions, respectively (Huang et al., 2000). Nonetheless, DAGKε is known to be a membrane-associated protein and enzyme protein loss under the dark condition similar to that suggested for soluble DAGKγ, seems unlikely.

Our IH studies in rat retina clearly showed a change in DAGKε intracellular localization upon illumination. Previous studies in rat retina (Kohyama-Koganeya et al., 1997) showed a positive DAGKε signal throughout different retinal layers under light conditions with no previous dark-adaptation period, a finding also observed in our results obtained using retinas from rats naturally exposed to room light conditions (Fig. 4A). In contrast, after a 12-h dark-adaptation period, DAGKε immunoreactivity was detected in the IS of photoreceptor cells (Fig. 4B, Dark). Interestingly, after 30 min of bright light exposure, DAGKε immunoreactivity was observed to decrease in IS but to increase in OS (Fig. 4B, Light).

Light-dependent activation of PIP2-PLCγ1 (Ghalayini et al., 1998) could be responsible for an endogenous SAG increase in bleached ROS. The use of SAG as exogenous substrate guarantees that light exerts its effect specifically on PA synthesis through DAGK activity (Fig. 5A and B).

Previous studies demonstrated that PIP2-PLCγ1 and DAGKγ from ROS are soluble proteins that are photoactivated and photoassociated with disc membranes (Ghalayini et al., 1998; Huang et al., 2000). Similar results were obtained when DAGK activity was measured in ROS treated with different ionic strength buffers (Ilincheta de Boschero and Giusto, 1992). Although these reports show evidence of DAGK photoassociation with membranes and photoactivation, the present study reveals that light also produces a different distribution of at least DAGKε.

A similar distribution pattern between Arr and DAGKε was also observed (Fig. 4). In fact, it has been suggested that Arr's ability to keep Rho inactive during the day is critical to the protection of rods from light-induced cell death (Slepak and Hurley, 2008). In vertebrate photoreceptor layers, light-activated components of the phosphoinositide pathway, such as PIP2-PLCγ1, DAGKγ, PIPKIIα (Ghalayini et al., 1998; Huang et al., 2000, 2011) and DAGKε (this paper), appear to be similarly localized as Arr. Could some of these enzymes be associated with a light-triggered protection mechanism of ROS?

It has recently been reported that Arr translocation is initiated by a G-protein-coupled cascade through PLC and PKC signaling (Orisme et al., 2010). It was suggested that under light conditions, Arr binding to Rho* recruits soluble PLC and leads to the activation of PIP2 hydrolysis, both of which induce the formation of DAG in these membranes (Ghalayini and Anderson, 1992).

Neomycin-induced DAGK activity inhibition using [^{3}H]DAG as substrate in BBROS revealed that light-activated PLC seems to be related to DAGK activation (Fig. 7). Substrate was exogenously provided in this approach and PLC activation by light could be a positive regulator of DAGK activity or/and modify DAGK localization in OS. Interestingly, when retinas were incubated 15 min with U73122 prior to light exposure, DAGKε was only found in IS (Fig. 8). Inhibition of PIP2-PLC was, in fact, critical to DAGKε distribution in OS.

However, U73122 treatment prior to 30 min light exposure failed to inhibit Arr migration to OS (Fig. 8). It is interesting to note that U73122 was reported to reduce Arr migration to OS by 40% when mouse organotypic retina cultures were exposed for 4 h prior to the light condition (Orisme et al., 2010). In our model, DAGKε appears to be more sensitive than Arr in its localization response to light-dependent PLC activation.

Light-induced OS localization of DAGKε and light-activation of PA formation could be related to the negative regulation of PKC by ROS.

It was reported that DAGKε overexpression in cell culture, through the selective removal of polyunsaturated DAG by this isoform, is accompanied by regulation of the cellular distribution of PKCα and ε (Pettitt and Wakelam, 1999). A slow PKC-regulated phosphorylation mechanism of Rho* has been suggested to contribute to rhodopsin deactivation (Adams et al., 2003). Activation of conventional PKC may therefore regulate visual transduction by decreasing the photoresponse. In addition, PKC signaling has also been reported to be involved in Arr translocation (Orisme et al., 2010).

In our study, PKC-dependent DAGK activation could be observed (Fig. 6). When ROS from retinas obtained under room light conditions (LROS) were pre-phosphorylated with ATP in the presence of PS-DOG or PDBU or di-C8, DAGK activity was observed to increase significantly. In contrast, when LROS were pre-incubated with H7, a non-selective PKC inhibitor, no increase in DAGK activity was detected. However, this PKC-dependent mechanism that stimulates DAGK activity appeared to be only present when LROS were assayed. Although under this condition a higher DAGKε content than in DROS was found, further research is required to clarify its involvement. In addition to the light-regulated localization of DAGKε in OS, our interesting finding of PKC-dependent DAGK regulation in ROS membranes suggests that DAGK may play a SAG-dependent role in PKC regulation.

Additionally, DAGKε translocation to the outer segment and 18:0–20:4 PA formation can both be associated with phosphoinositide resynthesis. The translocation of PIPKIIα providing the enzymatic machinery for PIP2 formation (Huang et al., 2011) is in agreement with our suggestion.

Furthermore, in addition to having the ability to phosphorylate saturated DAGs, DAGKε is highly efficient in 2-arachidonoyl DAGs phosphorylation (Thirugnanam et al., 2001). Its efficient arachidonoyl-DAG utilization is of particular interest because it suggests that DAGKε has a role in maintaining the fatty acid composition of phosphatidylinositols. PIP2 is highly enriched in arachidonic acid (Prescott and Majerus, 1981) but such enrichment does not result from the acyl chain remodeling of this lipid (Postle

et al., 2004). Based on the substrate preferences (18:0–20:4 PA) of brain CDP-DAG synthase, key to PI synthesis, and on the light regulation of phosphoinositide-related enzymes in vertebrate photoreceptor cells, it was suggested that CDP-DAG synthase has possible functional significance in vertebrate photoreceptors (Saito et al., 1997) In these cells, enhanced labeling of PI could only be observed when bovine retinas were light-adapted prior to ROS preparation (Ghalayini and Anderson, 1995), indicating that activation of PI synthesis requires an intact rod. Our study could therefore be a step further toward understanding the possible functional significance of light-induced activation of PI synthesis in vertebrate photoreceptors.

Furthermore, a significant reduction in arachidonate-containing lipids for several phospholipid classes in DAGKε-KO cells in culture from embryonic DAGKε-KO mice has been reported (Topham and Epand, 2009). An even higher increase in arachidonoyl content between PA and PI in WT cells compared with DAGKε-KO cells has also been observed. Both DAGKε (Walsh et al., 1995) and rat brain CDP-DAG synthase (Saito et al., 1997) are strongly inhibited by PIP2 in vitro, providing a potential negative-feedback mechanism for regulating phosphoinositide generation.

It has been suggested that the ability to keep Rho inactive during the day is a critical function of Arr (Slepak and Hurley, 2008). The question therefore arises as to the functional consequence of DAGKε redistribution.

We propose that the functional importance of DAGKε redistribution lies in the regulation of PKC activity for the removal of DAGs, mainly SAG from light-activated PIP2-PLC of disc membranes in which DAGKε is highly concentrated under light conditions. Furthermore, this localization also generates 1-stearoyl, 2-arachidonoyl phosphatidic acid, which is suitable for the eventual synthesis of a phosphatidylinositol intermediate in OS. Our findings suggest a novel light-dependent mechanism in OS second lipid messenger regulation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2013.04.012.

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