Experimental Eye Research 125 (2014) 142–155

Contents lists available at ScienceDirect

Experimental Eye Research

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

A novel light-dependent activation of DAGK and PKC in bovine photoreceptor nuclei☆

Paola M. Natalini, Melina V. Mateos, Mónica G. Ilincheta de Boschero*, Norma M. Giusto

Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), Universidad Nacional del Sur (UNS) and Consejo Nacional de Investigaciones Científicas y Técnicas, 8000 Bahía Blanca, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 10 March 2014
Accepted in revised form 7 June 2014
Available online 17 June 2014

Keywords:
diacylglycerol kinase
protein kinase C
bovine photoreceptor nuclei
light-dependent distribution
retina
phosphatidic acid
diacylglycerol
lipid second messengers

ABSTRACT

In this work, we describe a selective light-dependent distribution of the lipid kinase 1,2-diacylglycerol kinase (EC 2.7.1.107, DAGK) and the phosphorylated protein kinase C alpha (pPKCα) in a nuclear fraction of photoreceptor cells from bovine retinas. A nuclear fraction enriched in small nuclei from photoreceptor cells (PNF), was obtained when a modified nuclear isolation protocol developed by our laboratory was used. We measured and compared DAGK activity as phosphatidic acid (PA) formation in PNF obtained from retinas exposed to light and in retinas kept in darkness using [γ-^{32}P]ATP or [^{3}H]DAG. In the absence of exogenous substrates and detergents, no changes in DAGK activity were observed. However, when DAGK activity assays were performed in the presence of exogenous substrates, such as stearoyl arachidonoyl glycerol (SAG) or dioleoyl glycerol (DOG), and different detergents (used to make different DAGK isoforms evident), we observed significant light effects on DAGK activity, suggesting the presence of several DAGK isoforms in PNF. Under conditions favoring DAGKζ activity (DOG, Triton X-100, dioleoyl phosphatidylserine and R59022) we observed an increase in PA formation in PNF from retinas exposed to light with respect to those exposed to darkness. In contrast, under conditions favoring DAGKε (SAG, octylglucoside and R59022) we observed a decrease in its activity. These results suggest different physiological roles of the above-mentioned DAGK isoforms. Western blot analysis showed that whereas light stimulation of bovine retinas increases DAGKζ nuclear content, it decreases DAGKε and DAGKβ content in PNF. The role of PIP_2-phospholipase C in light-stimulated DAGK activity was demonstrated using U73122. Light was also observed to induce enhanced pPKCα content in PNF. The selective distribution of DAGKζ and ε in PNF could be a light-dependent mechanism that in vertebrate retina promotes selective DAG removal and PKC regulation.

1. Introduction

Diacylglycerol kinase (DAGK) catalyzes the phosphorylation of diacylglycerol (DAG), a glycerolipid containing two acyl chains, to convert it into phosphatidic acid (PA). DAG is not only a major intermediate product in the synthesis of several kinds of lipids but also a bioactive molecule. It is well known that DAG acts as a second messenger that modulates the activity of protein kinase C (PKC) and that its activity plays a central role in the control of proliferation and differentiation of different cells types. By lowering DAG levels, DAGKs may downregulate membrane localization of PKCs isoforms and/or may terminate transient receptor-induced PKC activation.

Previous research has shown that DAG also modulates the activity of several proteins, thus potentially affecting other cellular functions, such as cytoskeleton reorganization, cellular growth and carcinogenesis (Cai et al., 2009; Topham and Epand, 2009). On the other hand, several reports have demonstrated that PA also has

Abbreviations: DAGK, diacylglycerol kinase; PKC, protein kinase C; pPKCα, phosphorylated protein kinase C alpha; PLC, phospholipase C; PNF, photoreceptor nuclear fraction; RNF, retina nuclear fraction; DAG, diacylglycerol; PA, phosphatidic acid; DOG, 1.2-dioleoyl-sn-glycerol; SAG, 1-stearoyl-2-arachidonoyl-sn-glycerol; DOPS, dioleoyl phosphatidylserine; OG, octylglucoside; NaDC, sodium deoxycholate; Triton X-100, polyoxyethylene octyl phenyl ether; TLC, thin-layer chromatography; TEM, transmission electron microscopy; WB, western blot.

☆ Contract Grant Sponsors: Secretaría General de Ciencia y Tecnología, Universidad Nacional del Sur (PGI 24/B176), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 112-201101-00437).

* Corresponding author. Instituto de Investigaciones Bioquímicas de Bahía Blanca, CONICET-Bahía Blanca and Universidad Nacional del Sur, Edificio E1, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina. Tel.: +54 291 4861201; fax: +54 291 4861200.

E-mail address: milinch@criba.edu.ar (M.G. Ilincheta de Boschero).

http://dx.doi.org/10.1016/j.exer.2014.06.007
0014-4835/

signaling properties, for example, it is PA involved in vesicle trafficking and it can both bind and regulate the activity of numerous enzymes, including PAK1, Ras-GAP, PKC-ζ, (Abramovici et al., 2009; Limatola et al., 1994; Siddhanta and Shields, 1998; Zhang and Du, 2009). In addition, further studies have demonstrated that PA functions as a selective inhibitor of protein-serine-treonine phosphatase 1 (PP1) (Kishikawa et al., 1999). Interestingly, PA has also been reported to be a protein-tyrosine phosphatase (PTP) and SHP1 activator (Frank et al., 1999), thus suggesting that through PA generation DAGK plays a protein phosphorylation-promoting role.

Ten mammalian isoforms of DAGK identified to date have been classified into five subtypes based on shared structural motifs. Class I includes the α (Sakane et al., 1990; Schaap et al., 1990), β (Goto and Kondo, 1993), and γ (Goto et al., 1994; Kai et al., 1994); class II the δ (Sakane et al., 1996), η (Klauck et al., 1996), and κ (Imai et al., 2005); class III the ε (Tang et al., 1996); class IV the ζ (Bunting et al., 1996; Goto and Kondo, 1996) and ι (Ding et al., 1998); class V the θ (Houssa et al., 1997). Furthermore, alternative splicing variants have been reported in several of such isozymes as DAGKβ (Caricasole et al., 2002), -γ (Kai et al., 1994), -δ (Sakane et al., 2002), -η (Murakami et al., 2003), -ι (Ito et al., 2004), and -ζ (Ding et al., 1997).

There is now solid evidence that lipid-dependent signaling pathways operate within the nucleus (Cocco et al., 2001; D'Santos et al., 2000; D'Santos et al., 1998; Martelli et al., 2004). A key second messenger which is generated at the nuclear level along these pathways is DAG. Data reported by independent laboratories have shown that nuclear DAG can derive from either phosphoinositides or phosphatidylcholine (D'Santos et al., 1999; Divecha et al., 2000). Several DAGK isozymes are present in this organelle where they may be involved in regulating the amount of intranuclear DAG (Evangelisti et al., 2006). In some cell culture studies, the activity of nuclear DAGKs has been demonstrated to be very critical for the control of PKC-dependent cell proliferation (Divecha, 1998; Topham et al., 1998).

PKC isoforms are catalytically activated by several lipid cofactors, including DAG. It is known that the phosphorylation state of PKC is necessary for the catalytic activity as well as interactions with binding partners which are of fundamental importance for PKC subcellular localization (Newton, 2009). After PKC "maturation" it is thought to reside in the cytoplasm in an inactive conformation and to translocate to the plasma membrane or cytoplasmic organelles upon cell activation by different stimuli. Evidence collected over the last years has shown PKC to be capable of translocating to the nucleus. Furthermore, PKC isoforms can reside within the nucleus. Studies from independent laboratories have led to the identification of several nuclear proteins which act as PKC substrates as well as to the characterization of some nuclear PKC-binding proteins (Martelli et al., 2006).

DAGKζ, a type IV DAGK, contains a unique region homologous to the phosphorylation site domain (PSD) of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein, a prominent substrate for PKC in cells (Blackshear, 1993; Bunting et al., 1996). This MARCKS motif is the predominant nuclear localization signal of DAGKζ, and its phosphorylation by PKC isoforms in culture cells reduces the nuclear localization of DAGKζ, which modifies nuclear DAG accumulation (Topham et al., 1998). Thus, it has been suggested that cells regulate the concentration of PKC-activating nuclear DAG by controling nuclear localization of DAGKζ. In addition, PKCα phosphorylates DAGKζ in cells, and this phosphorylation inhibits its kinase activity leading to high levels of DAG, thereby affecting cell growth (Luo et al., 2003a).

It is known that vertebrate retinal photoreceptor cells have an active phosphoinositide (PI) metabolism and that several steps in the PI cycle are stimulated by light (Giusto et al., 2000). Previous research also reported that a DAGK activity identified as a DAGKγ isozyme is photoassociated and activated by light in rods from bovine and rat retina (Huang et al., 2000). A 64 kDa isozyme with very high homology to human DAGKε has been shown to be present in rat retina (Kohyama-Koganeya et al., 1997). Although until 2012 DAGKγ was the only isoform detected in light activated rods (Huang et al., 2000) we recently reported a light-dependent different distribution of DAGKε in bovine and rat photoreceptor cells (Natalini et al., 2013).

Our previous immunohistochemistry studies revealed a light-dependent localization of DAGK isoforms in different layers of rat retinas (Natalini et al., 2013). Interestingly, the outer nuclear layer, comprising small nucleus from photoreceptor cells, is one of these immunostained layers. The aim of the present work was therefore to explore DAGK presence and activity as well as the effect of light in a purified nuclear fraction of photoreceptor cells (PNF) from bovine retinas. PA synthesis through DAGK activity as a function of nuclear proteins, assay time, ATP and DAG increasing concentrations was measured. The present work describes for the first time a light-dependent different content of DAGKε and DAGKζ in bovine PNF. The role of a light-dependent phospholipase C (PLC) activation in the regulation of nuclear DAGK activity was also demonstrated by experimental data employing U73122. A light-induced phosphorylation of PKCα was also found, thus suggesting a novel light-dependent mechanism in nuclear signaling.

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 during their transfer to the laboratory.

Polyclonal antibody raised against CRX was generously supplied by Dr. C. Craft (University of Southern California, Los Angeles, USA). Polyclonal antibodies raised against DAGKε and ζ were a generous gift from Dr. Matthew K. Topham (Huntsman Cancer Institute, University of Utah, USA) and from Dr. Nicolas Bazan (Neuroscience Center of Excellence at Louisiana State University Health Sciences Center, New Orleans, USA). Mouse monoclonal anti-rhodopsin (Rho4D2) antibody was generously supplied by Robert Molday (University of British Columbia, Vancouver, BC, Canada). Rabbit polyclonal antibodies anti-Phospho-PKCα/βII (Thr638/641) (#9375), and anti-Phospho-PKCδ (Thr505) (#9374) were from Cell Signaling (Beverly, MA, USA). Mouse monoclonal anti- PKCα (#610107), anti- PKCδ (#610397) and anti-LAP-2β (#611000) were from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal anti-calnexin antibody (#11397) was from Santa Cruz biotechnology. Supplies such as U73122, Dioleoyl- and 1 stearoyl, 2 arachidonoyl-glycerol (DOG and SAG), from Avanti Polar Lipids, were also generously provided by Dr. Bazan. The radioactive substrates [γ-^{32}P]ATP (10 Ci/mmol), [2^{3}H]-Glycerol (200 Ci/mmol) and the Preblended Dry Fluor 2a70 for scintillation cocktail were obtained from Research Products International Corp, USA All the other chemicals used in the present research were from Sigma Aldrich (St. Luis, MO).

2.2. Light-dark protocol applied to bovine retinas and retina nuclear fraction (RNF) or photoreceptor nuclear fraction (PNF) preparations

In order to evaluate the effect of light on a photoreceptor cell nuclear population, bovine eyes were kept in darkness for 2.5 h (darkness adaptation period). The cornea, lens and aqueous humor were subsequently removed from the eyeballs under dim

red light. The eyecups were placed on ice in appropriate containers and filled with oxygenated (95% O_2, 5% CO_2) Ames medium with 0.5% glucose (119.5 mM NaCl, 3.6 mM KCl, 0.1 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1.15 mM $CaCl_2$, 22.6 mM $NaHCO_3$, pH = 7.33). Optic cups were either kept in darkness for 30 min or exposed to light (288 cd/m^3). Subsequent procedures for nuclei preparation were carried out under room light or under dim red light at 2–4 °C. Retinas were dissected from the eyes after darkness or light exposure and homogenized in a 1:2 (wt by vol) ratio in 0.25 M sucrose TKM medium (50 mM Tris–HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ and 1 mM EGTA in the presence of 0.1 mM PMSF, 1 ug/ml aprotinin, 1 ug/ml pepstatin, 1 mM DTT and 2 ug/ml leupeptin. The nuclear fraction isolation protocol for brain tissue (Gaveglio et al., 2010) was adapted to the retina. Homogenization (30% weight/vol) was carried out in a Potter Eveljhem homogenizer using 10 up-and-down strokes of a teflon pestle driven by a motor at 700 rpm. The homogenate was filtered through four layers of cheesecloth and two volumes of 2.3 M sucrose in TKM were added to reach a sucrose concentration of 1.6 M (10% weight/vol). The filtered homogenate (1.6 M sucrose) was placed onto a 2.3 M sucrose layer and centrifuged at 134074 g for 70 min using a Beckman SW41 rotor in a Beckman Optima LK-90 ultracentrifuge. The purified nuclear population was obtained in the pellet (Traditional Method). Nuclear preparation purity was checked by DAPI staining and was named RNF (retina nuclear fraction).

In order to purify small photoreceptor nuclei, which comprise a high proportion of nuclear pellet, a different sucrose gradient was performed. Homogenate (1.6 M sucrose) was placed onto a 1.40 ml 2.2 M sucrose TKM and 1.40 ml 2.4 M sucrose TKM gradient and centrifuged at 106883 for 70 min using a Beckman SW41 rotor in a Beckman Optima LK-90 ultracentrifuge. After centrifugation two nuclear preparations were obtained, at the 2.2 M-2.4 M sucrose interface and in the pellet. The purified nuclear pellet, rich in small nuclei of photoreceptor cells, was named PNF (photoreceptor nuclear fraction). PNF was resuspended in a 50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA and 1 mM DTT (BAFI). Ice-cold buffer was placed in contact with the pellet for 30 min before a loosely up and down pipette suspension.

2.3. Determination of DAGK activity

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

2.3.1. DAGK assay using [γ-^{32}P]ATP

When DAGK activity was determined by measuring radioactive phosphate incorporation into DAG in PNF, 3 μCi [γ-^{32}P] ATP and either endogenous DAG, or exogenous DOG or SAG, were used as substrates. The enzyme reaction was carried out in a medium containing 50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA and 1 mM DTT in a volume of 200 μl using 100 μg of PNF proteins per assay. The reaction was carried out at 37 °C for 10 min and stopped by the addition of chloroform/methanol/1 N HCl (2:1:0.2, by vol). Blanks were prepared identically, except that membranes were boiled for 5 min before use. Lipids were extracted according to Folch et al. (1957) and five additional washes of the lipid extract for [γ-^{32}P]ATP elimination were carried out using theoretical upper phase (chloroform/methanol/water, 3:48:47 by vol). Lipids were subsequently separated by one-dimensional thin-layer chromatography (TLC) using 1% potassium oxalate in silica gel H plates (Merck) in a mobile phase consisting of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7.5, by vol) (Ilincheta de Boschero and Giusto, 1992). PA spots were visualized by exposure of the plate to iodine vapors, identified with appropriate lipid standards and scraped off for counting by liquid scintillation spectroscopy. Proteins were determined following Lowry (1976). DAG acyl chain composition and content as well as ATP content in assays are indicated in the Figures.

2.3.1.1. Kinetic characterization and effect of detergents on nuclear DAGK activity. Protein, time and substrate concentration dependence of nuclear DAGK activity was determined in PNF using [γ-^{32}P]ATP and exogenous DOG. An appropriate volume of DOG stock solution was evaporated under a stream of nitrogen in a glass test tube. It was subsequently re-suspended in the assay buffer alone. The lipid was sonicated in a test tube placed in a Cup Horn system (Branson Digital Sonifier, model 450). Prior to incubation, nuclear suspension was sonicated during 15 s with radioactive ATP and the lipid suspension in a water bath (Branson Sonifier). Lipid substrate preference and effect of detergents over nuclear DAGK activity was determined in PNF using [γ-^{32}P]ATP and exogenous DAGs alone or in the presence of detergents (1 mM Na deoxycholate (NaDC), 15 mM Triton X-100 or 50 mM octylglucoside (OG)) and were evaluated over DOG or SAG, substrates of DAGK, using a micellar detergent-lipid assay. An appropriate volume of DOG or SAG stock solutions was evaporated under a stream of nitrogen in a glass test tube and re-suspended alone or with detergents in the assay buffer. Lipid-detergent suspension was also sonicated, aliquoted, and enzyme reaction was started after 15 s sonication with radioactive ATP as above. Enzyme reaction, lipid isolation and quantification of radiolabeled PA were performed as described above (2.3.1).

2.3.1.2. Assay for preferential DAGKζ and DAGKε measurement using [γ-^{32}P]ATP

2.3.1.2.1. DAGKζ preferential measurement. A DAGK assay with [γ-^{32}P]ATP, 500 μM DOG as exogenous substrate and 15 mM Triton X-100 was performed. PNF pellets from dark adapted or light exposed retinas (100 μg protein) were incubated in the presence of 4 mM phosphatidylserine (DOPS condition) and 10 μM R59022 (Type 1 DAGK preferential inhibition) for 10 min before [γ-^{32}P]ATP addition.

2.3.1.2.2. DAGKε preferential measurement. DAGK assay with [γ-^{32}P]ATP was performed in the presence of 500 μM SAG and 50 mM OG. PNF pellets from dark adapted or light exposed retinas (100 μg protein) were incubated in the absence or in the presence of 10 μM R59022 for 10 min before [γ-^{32}P]ATP addition.

The R59022 vehicle, DMSO, was present in the control and in all experimental conditions in both preferential assays at a final concentration of 0.1%. Enzyme reaction, lipid isolation and quantification of radiolabelled PA were performed as described above (2.3.1).

2.3.1.3. Role of retina PLC activation in DAGK activity from PNF. To study the role of PLC in light-induced nuclear DAGK activation, PIP_2-PLC inhibitor U73122 was used. After 2.5 h dark adaptation of the eyes, the cornea, lens and aqueous humor were subsequently removed from the eyeballs under dim red light. The eyecups were placed on ice in appropriate containers and filled with 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), under dim red light. After 15 min incubation, half of the eyecups from each condition was maintained under dim red light while the other half was exposed to light condition for 30 min. The PNF was obtained as previously described (2.2.). DAGK assay with [γ-^{32}P]ATP was performed in the absence of detergents and in the presence of endogenous DAG or 100 μM SAG. Neomicyn, a polycation that binds and sequesters PIP_2, was also used as a PIP_2 regulator (James et al., 2004). Condition for retina exposition to Neomicyn was as previously described for U73122 (dark–light protocol) but in the absence of DMSO. Enzyme reaction, lipid

isolation and quantification of radiolabelled PA were performed as described above (2.3.1).

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

Radioactive glycerolipids were 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 Folch (Folch et al., 1957). [^{3}H] DAG plus cholesterol were isolated by mono-dimensional TLC in silica gel G plates with a solvent system of hexane:ethyl ether:-acetic acid (60:40:2.3 by vol). Lipids were eluted as described Arvidson (Arvidson, 1968) and [^{3}H]DAG was purified by one-dimensional TLC on silica gel G developed with chloroform:methanol:acetic acid (98:2:1, by vol). The substrate was eluted as described 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 (30000 dpm/sample) was evaporated under a stream of nitrogen in a glass test tube and subsequently re-suspended in a Triton X-100 containing medium to reach a final assay concentration of 13 μM [^{3}H]-DAG and 15 mM detergent. Prior to incubation, nuclear fraction membranes (100 μg protein) were sonicated for 15 s with ATP (2 mM final concentration) and the detergent-lipid suspension in a water bath (Branson Sonifier).

Reactions were stopped by adding chloroform/methanol (2:1 by volume) and lipids were extracted following Folch (Folch et al., 1957). [^{3}H]PA was isolated by mono-dimensional TLC in silica gel G plates and with a solvent system of hexane:ethyl ether:acetic acid (60:40:2,3 by vol.). 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. Proteins were determined following Lowry (Lowry et al., 1951).

2.3.2.1. Assay with [^{3}H]DAG for a preferential DAGKζ measurement in PNF from retinas exposed to light. A DAGK assay with [^{3}H]DAG was performed in the presence of 15 mM Triton X-100. PNF pellets from dark adapted or light exposed retinas (100 μg protein) were incubated in the presence of 4 mM phosphatidylserine (DOPS condition) for 10 min before [^{3}H]DAG addition. Buffer conditions were as previously described (2.3.1). All subsequent procedures were performed as previously described (2.3.2.).

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (WB) assays

Membrane suspension of RNF or PNF containing nuclear proteins were denatured with Laemmli sample buffer at 100 °C for 5 min (Laemmli, 1970) and 25 μl aliquot (40 μg protein) of each sample were resolved in a 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) which were blocked with 10% BSA in TTBS buffer [20 mM Tris- HCl (pH 7.4), 100 mM NaCl and 0.1% (w/v) Tween 20] at room temperature for 2 h. Membranes were subsequently incubated with primary antibodies [anti-DAGKζ (1:1000, v:v), anti-DAGKε (1:1000, v:v) and anti-Rho4D2 (1:1000, v:v)] over night at 4 °C; [anti- pPKCα/βII (1:2000, v:v), anti- pPKCδ (1:1000, v:v), anti-CRX (1:2000,v:v), anti-PKCα (1:1000,v:v), anti-PKCδ(1:1000, v:v), anti-Calnexin (1:1000, v:v) and anti-LAP-2β (1:2000,v:v)] at room temperature for 2 h. After four washes with TTBS membranes were exposed to the appropriate HRP-conjugated secondary antibody (anti-rabbit or anti-mouse) for 2 h at room temperature.

Membranes were washed again three times with TTBS and immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences) using standard x-ray film (Kodak X-Omat AR). Quantification analyses of the immunoreactive bands were carried out using ImageJ 1.38 software (Schneider et al., 2012). The molecular weight of the bands was determined using the spectra multicolor broad range protein ladder (Thermo scientific).

2.5. Lipid peroxidation assay in retina homogenates

Lipid peroxidation was measured in retina homogenates from dark adapted or light exposed retinas using the thiobarbituric acid assay as previously described (Uranga et al., 2007)). After 30 min exposition to light or to darkness (control condition), 1 ml of 30% trichloroacetic acid (TCA) was added to 0.5 ml of retina homogenates (200 μg protein). Then, 0.1 ml of 5 N HCl and 1 ml of 0.75% thiobarbituric acid were added. Reaction tubes were heated for 15 min at 100 °C in a water bath and samples were subsequently centrifuged at 1000 × *g* for 10 min.

Thiobarbituric acid reactive substances (TBARS) were measured in the supernatant at 535 nm and results were expressed as units of absorbance at 535 nm per mg of proteins.

2.6. Fluorescence confocal microscopy

Fluorescence studies were performed to analyze nuclear purity of nuclear fractions. The nuclear suspension from bovine retinas was fixed with paraformaldehyde (2%) at room temperature for 30 min. After 1 h incubation the samples were centrifuged at 4500 g for 20 min. The pellets were resuspended in buffer to finish the fixation period. A drop from nuclear fraction was subsequently placed on a slide and was left for 15–30 min to allow drying. Then, samples were permeabilized with 0.1% of Triton X-100 at room temperature for 15 min. Nuclei were subsequently washed three times at room temperature for 5 min with BAFI solution and blocked with 5% BSA. Rabbit polyclonal anti-CRX and anti-DAGKε antibodies diluted in 5% BSA (1:100) were added to PNF and RNF samples respectively, and the mixture was incubated overnight at 4 °C. After four washes with BAFI, nuclei 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 BAFI, were suspended in 50 μl of 30% sucrose. An aliquot of the suspension was analyzed by fluorescence confocal microscopy at 488 nm wavelength. Primary antibodies were omitted for the negative control and no specific staining was detected in these suspensions. The samples were observed under a TCS-SP2 confocal microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) equipped with an acousto-optical beam splitter and a × 63 (1.2 numerical aperture) water immersion objective.

In order to show photoreceptor nuclei enrichment in PNF with respect to RNF, fluorescence staining of isolated nuclei was performed as follows. A similar suspension of RNF and PNF pellets from bovine retinas were resuspended and fixed as previously described (2.2). Slides were incubated with an aliquot of propidium iodide (PI) for 25 min. After three washes with a high ionic strength Hepes-buffered solution (BAFI) (50 mM HEPES pH = 7.4, 10 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA and 1 mM DTT), the samples were observed under a TCS-SP2 confocal microscope. Different focal planes were also observed in an attempt to detect any possible nuclear damage (DNA fragmentation).

2.7. Statistical analysis

Statistical analysis was performed using ANOVA followed by Bonferroni's test to compare means or student's *t*-test using the GraphPad Prism software. *p*-values lower than 0.05 were considered statistically significant (indicated with asterisks). Data represent the mean value ± SD of three independent experiments. The WB assays shown are representative of three analyses carried out on samples from three independent experiments.

3. Results

3.1. Isolation of photoreceptor cells nuclei from bovine retina

It is known that phototransduction occurs in the outer segments of photoreceptor cells. Our interest was therefore to determine if changes in DAGK activity and localization occur at the nuclear level of these cells as a consequence of light stimulus. To this end, we first decided to obtain a nuclear fraction enriched in photoreceptor nuclei.

A retina nuclear fraction (RNF) could be briefly obtained with slight modifications of the liver nuclear fraction isolation procedures previously described (Gaveglio et al., 2011; Matunis, 2006). The key of this new isolation procedure nuclei sedimentation through a sucrose gradient containing the appropriate density (2.3 M) to allow flotation of the outer and inner segment, endoplasmic reticulum and mitochondria while the nuclei remained in the pellet. As nuclei move down through the sucrose solution, endoplasmic reticulum (ER) moves upward and is stripped from the nuclei without significantly affecting the outer nuclear membrane.

Nuclei from different cell types in mammalian retinas show important differences in size, being the nuclei of photoreceptors in the outer nuclear layer (ONL) the smallest ones. Taking into account these important size differences we proposed a new method based on the inclusion of an additional layer of high density sucrose solution (2.4 M) to isolate a nuclear fraction enrichment in photoreceptor nuclei (PNF).

The nuclear purity of both fractions was evaluated. Results from confocal microscopy with specific nuclear markers such as DAPI and Topro (data not shown) and also Propidium Iodide (Fig. 1a)) were used to study the purity of the nuclear fraction. Confocal microscopy images using a nuclear probe (Propidium Iodide) are convincing evidences of the purity of the nuclear preparations. Immunofluorescence studies using a-CRX, a specific transcription factor of photoreceptor cells were also performed (Fig. 1S). Taking into account that DAGKε has been extensively reported to be localized in plasma and ER membranes (Decaffmeyer et al., 2008; Shulga et al., 2010), a-DAGKε has been used to ensure the absence of ER vesicles in RNF preparations (Fig. 2S). Fluorescence images and their merged images (Nomarski) from both experiences are convincing evidences of the purity of both nuclear preparations since no membrane vesicles positive for DAGKε staining were seen in RNF. In fact, no membrane vesicles were seen in Nomarski images. (Figs. 1S and 2S, Supplementary material).

Furthermore, in order to corroborate even further the purity of the PNF fraction, we performed WB assays in total retina homogenate and PNF using a- Calnexin antibody, a type I transmembrane protein that interacts with intermediates of glycoproteins, used as ER marker (Zhang et al., 2007) and a-Rho4D2 in order to detect rhodopsin, the major protein marker of photoreceptor membranes. Fig. 3S showed that both proteins were present in the retina homogenate but absent in the PNF fraction.

It is known that in mammalian retina the content of photoreceptor cells greatly exceeds the content of neural cells. As was described by Masland, rods are in abundance one and two magnitude orders higher than amacrine/bipolar and ganglion/horizontal cells, respectively (Jeon et al., 1998). It is therefore not surprising that in RNF (obtained with the Traditional method), photoreceptor nuclei represent about 85% of the nuclear pellet. The New Method allowed us to isolate a nuclear population where photoreceptor nuclei were even more enriched than with the Traditional Method, representing about 95–99% of the nuclear pellet.

In order to compare the enrichment in photoreceptor nuclei obtained with the new protocol with respect to the traditional one, fluorescence staining with propidium iodide was performed in isolated nuclei from both methods as described in materials and method. In Fig. 1 representative fluorescence images are shown. Fig. 1a), (second panel) indicates that with the New Method, a high proportion of nuclei shows the typical nuclear photoreceptor shape, size and dense chromatin content. In contrast, the first panel of Fig. 1a)) shows that nuclei obtained with the Traditional Method have a complex composition with a very different shape, size and chromatin distribution (indicated with white arrows).

Results from TEM also showed an improved photoreceptor nuclei purification when the New Method was used (Fig. 1b)). In the first panel of Fig. 1b)) (Traditional Method), two different nuclear types (in shape, size and chromatin distribution) can be observed. One of these nuclear types showed the typical non-regular heterochromatin extended distribution of rod nuclei from diurnal mammals (Solovei et al., 2009). RNF also contained nuclei from other retinal cells with an homogenous chromatin distribution (indicated with black arrows). In the second panel of Fig. 1b)), an TEM representative image from the pellet of New Method showed that there is mainly one type of nuclei in the preparation, with the size, shape and chromatin distribution of rod nuclei. Occasional vesicles can be seen in both preparations. The figure shows that the vast majority of the organelles are, indeed, nuclei.

It is known that CRX is a specific transcription factor of photoreceptor cells, the expression of Crx, an otx-like homeobox gene isolated from mouse retina, being restricted to developing and mature photoreceptor cells (Furukawa et al., 1997). Western blot (WB) assays were also performed to determine CRX content in nuclear pellets obtained from both protocols. LAP-2β (a general nuclear marker) was used as loading control and a representative result is shown in Fig. 1c)). A one-fold increase in CRX/LAP-2β relative content was observed in PNF with respect to RNF. Our results demonstrate that the modified method provides a nuclear fraction highly enriched in photoreceptor cell nuclei.

3.2. DAGK activity in the photoreceptor nuclear fraction

In order to determine if DAGK is present in the nuclear fraction of photoreceptor cells, our first aim was to establish the best conditions to measure and characterize the enzyme activity. These first experiments were performed under room-light standard conditions. Nuclear DAGK activity was measured through radioactive phosphate incorporation into PA using [γ-^{32}P]ATP and endogenous DAG present in the nuclear fraction suspension or in the presence of DOG or SAG. Although lyso phosphatidic acid (LPA) could also be isolated by the TLC system employed, only PA was found to be radiolabeled. With endogenous DAG, PA synthesized in PNF was 52.8 ± 17.5 pmol/mg/min. When 500 μM DOG or SAG were present a twenty-fold increase in PA synthesis was obtained (989.5 ± 2.24 and 864.8 ± 112.2 pmol/mg/min, respectively) (data no shown). These results demonstrate for the first time a DAGK activity in photoreceptor nuclei.

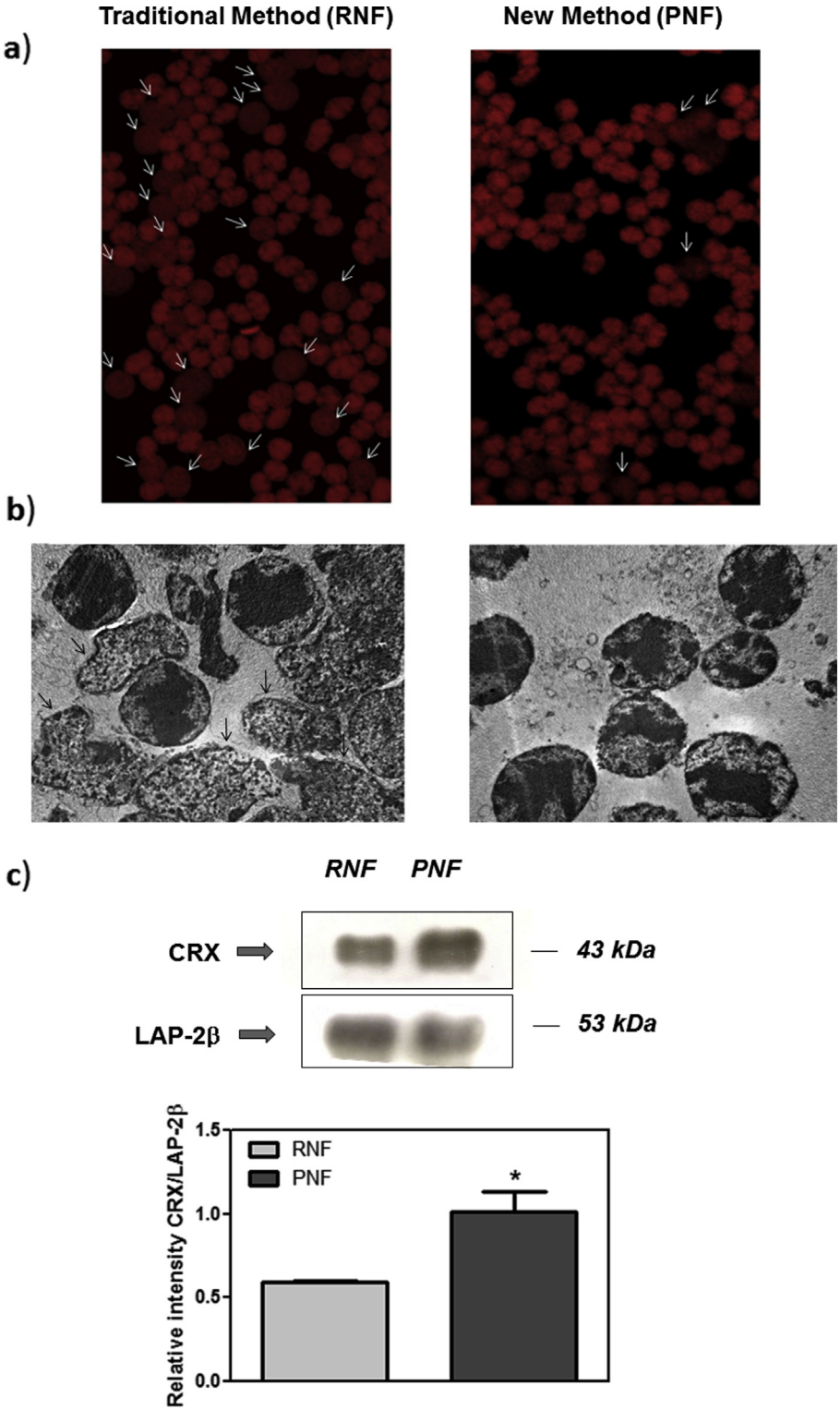

Fig. 1. Comparative analysis of photoreceptor nuclei enrichment in retina nuclear fraction (RNF) and photoreceptor nuclear fraction (PNF). RNF and PNF from bovine retinas were obtained following either a traditional or a new method as described in Materials and Methods. a) Nuclear staining and fluorescense detection: nuclei were treated and incubated with propidium iodide as detailed in Materials and Methods and were subsequently analyzed by fluorescence microscopy. Representative images at 543 nm are shown. White arrows indicate non-photoreceptor nuclei. b) TEM of nuclei from RNF and PNF. Black arrows indicate non-photoreceptor nuclei. c) WB detection of CRX in RNF and PNF. WB to detect CRX and LAP-2β were performed as described in Materials and Methods and immunoreactive bands were detected by enhanced chemiluminescence. Numbers on the right indicate molecular weight. The bar graph shows the densitometry values of CRX/LAP-2β expressed as ratio of the control. Asterisks indicate significant differences with respect to the dark condition ($^{*}p < 0.05$).

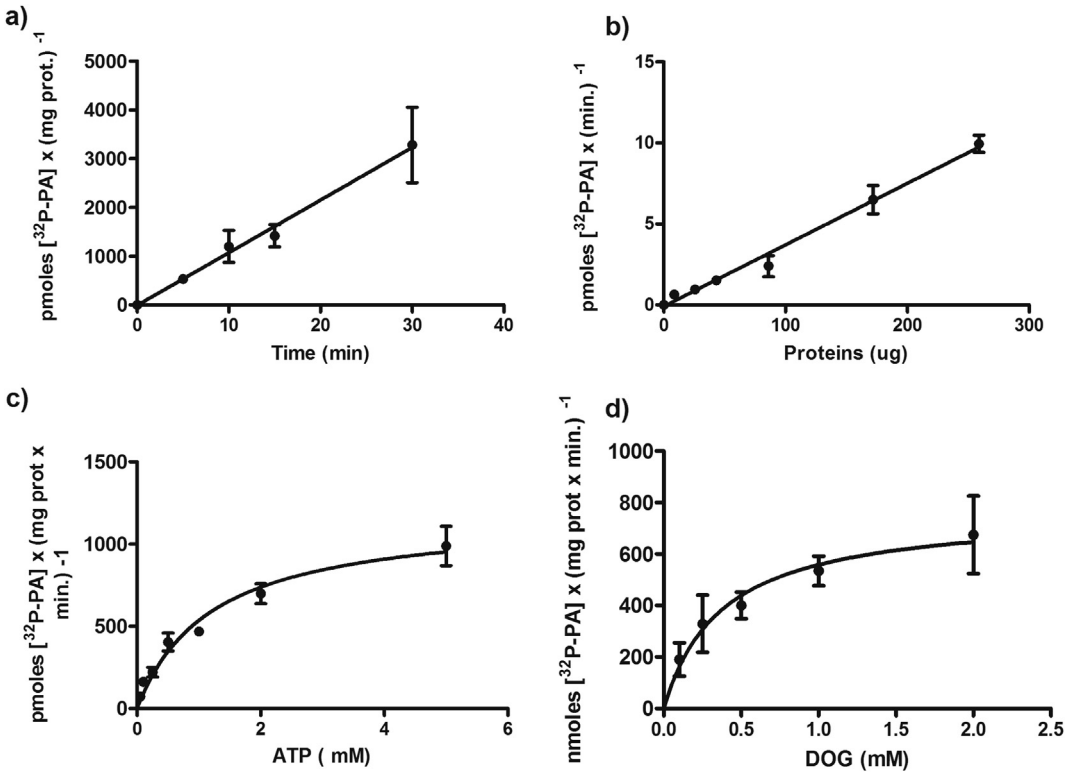

Fig. 2. Kinetics of DAGK-mediated phosphorylation of 1,2-dioleoyglycerol (DOG) in PNF from bovine retina. PA formation was measured in PNF isolated from bovine retina under room light condition. For a) and b), enzyme reaction was carried out in the presence of 500 μM DOG and 250 μM [γ-^{32}P]ATP (3 μCi of [γ-^{32}P]ATP per assay) as described in Materials and Methods. a) PA formation was measured as a function of incubation time and results were expressed as pmoles x (mg prot)$^{-1}$. b) PA formation was measured as a function of protein content and results were expressed as pmoles x (min.)$^{-1}$. For c) and d), PA formation was measured after 10 min incubation using either an increasing concentration of ATP and 500 μM DOG (c) or an increasing concentration of DOG and 4 mM ATP (d). In both cases, results were expressed as pmoles × (mg prot × min.)$^{-1}$. Data shown are averages of three independent experiments. Error bars indicate standard error.

To evaluate DAGK kinetics in photoreceptor nuclei the enzyme activity was measured in the presence of DOG and as a function of protein concentration and time. A linear response was obtained when DAGK activity (100 ug of nuclear proteins) was measured between 5 and 30 min (Fig. 2a)) and between 10 and 250 ug of proteins (10 min reaction time) (Fig. 2b)). Under our experimental conditions nuclear PA synthesis was observed to increase as a function of ATP concentrations showing a steady state at 2 mM ATP (Fig. 2c)). At 2 mM ATP, a Michaelis–Menten typical graph was obtained when DOG concentrations were increased. Saturability condition for DOG appeared to be 1 mM (Fig. 2d)).

The effect of detergents, such as sodium deoxycholate (NaDC), Triton X-100 and octylglucoside (OG) was analyzed when DOG or SAG were employed as DAGK substrates, using a micellar detergent-lipid assay. Percentual ratios with respect to the non-detergent condition (effect of detergents on substrate utilization) are shown in Fig. 3a)). When non-ionic detergents, such as OG or Triton X-100 were used, PA formation from DOG was found to

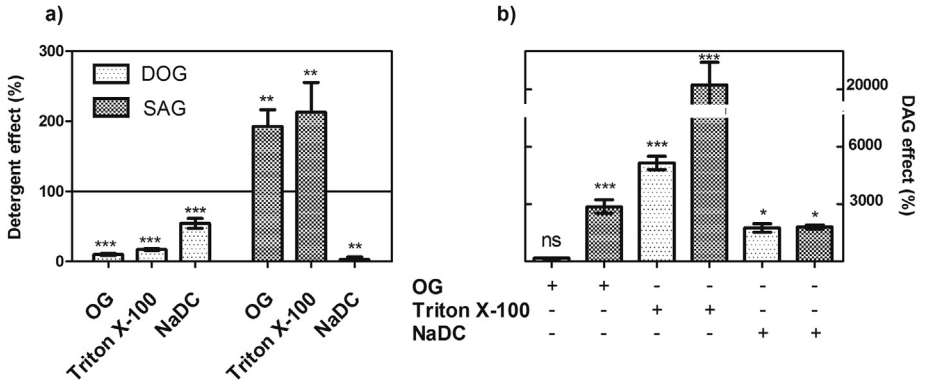

Fig. 3. DOG and SAG utilization and effect of detergents in PNF DAGK activity. PA formation was measured in PNF isolated from bovine retina under room light condition. Enzyme reaction was carried out in the presence of endogenous DAG or 500 μM DOG or SAG, with or without detergents, and in the presence of 250 μM [γ-^{32}P]ATP (3 μCi of [γ-^{32}P]ATP per assay) as described in Materials and Methods. 15 mM Triton-X100 or 50 mM OG or 1 mM NaDC were used as detergents. In a), data represent the "**Detergent Effect**" and results are expressed as percentual changes with respect to the control condition (detergent free). In b), data represent the "**DAG effect**" and results are expressed as percentual changes with respect to the control condition (endogenous DAG). Asterisks indicate significant differences with respect to the control condition (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

decrease by 90 and 83%, respectively, with respect to the non-detergent condition. When the ionic detergent NaDC was used, it was observed that PA formation from DOG was only decreased by 45% (Fig. 3a)). Interestingly, when SAG was used as exogenous substrate, OG and Triton X-100 were observed to strongly increase PA formation in a similar way (93% and 113%, respectively). In contrast, the presence of NaDC caused a decrease of 92% in PA production from SAG (Fig. 3a)). Percentual ratios with respect to the condition without exogenous DAG but in the presence of detergents (effect of lipids) are shown in Fig. 3b). In the presence of OG or Triton X-100, SAG was preferred as DAGK substrate. However, in the presence of NaDC, DOG or SAG were equally used by DAGK (Fig. 3b)). In addition, both substrates were avidly used by DAGK in the presence of Triton X-100. Detergents strongly affect DAGK activity over DOG or SAG, thus suggesting that more than one DAGK isoform is present in PNF.

Previous research has demonstrated that DAGKε has a preferential activity over SAG and that this substrate selectivity is also observed in the presence of OG or Triton X-100 (Thirugnanam et al., 2001). In PNF, our results are indicative of the highest DAGK activity in the presence of OG and Triton X-100 when SAG was used as exogenous substrate (Fig. 3b)), thus suggesting that DAGKε could be present in bovine PNF.

3.3. Evaluation of light-induced damage in bovine retina

Once we evidenced DAGK activity in PNF, we further investigated if our light exposure protocol (280 cd/m^3 for 30 min) induces possible retina damage. To this end, lipid peroxidation was measured as TBARS formation in total retina homogenates. Furthermore, DNA fragmentation was evaluated in isolated PNF using propidium iodine (PI) staining (Fig. 4Sa) and DAPI staining (data not shown). Different focal plains were analyzed by confocal microscopy in order to discard DNA fragmentation. As shown in Fig. 4Sa, supplementary material, no DNA fragmentation was observed under our experimental conditions. TBARS formation was barely detected both in retina homogenates from the control condition and from light-exposed retinas (Fig. 4Sb). It can thus be concluded that under our experimental conditions no light-induced damage was observed.

3.4. Light-effect on DAGK activity in nuclei from photoreceptor cells

In order to explore light effect on nuclear DAGK activity, experiments using a micellar detergent assay containing Triton X-100, [^{3}H]DAG and ATP were conducted as detailed in materials and methods. Results obtained in PNF were also compared with those found in RNF.

DAGK activity in PNF from light stimulated retinas was 275% higher than that in PNF from dark-adapted retinas (Fig. 4a)). DAGK activity under these experimental conditions was higher in PNF than in RNF (200%) and interestingly no significant effect of light was observed in RNF (Fig. 4a)), thus suggesting that the effect of light observed in PNF (in the presence of Triton X-100, [^{3}H]DAG and ATP) is a selective photoreceptor nuclear response (Fig. 4a)).

DAGK activity was also analyzed in PNF from either light or dark exposed retinas using [γ-^{32}P]ATP as radiolabelled substrate and endogenous DAG, or exogenous DOG or SAG as co-substrates, in the absence of detergents (Fig. 4b)). As shown in Fig. 4b)), in the absence of exogenous DAG and detergents, no changes in DAGK activity was observed between PNF from retinas exposed to light and those obtained from dark adapted retinas. In contrast, light was found to induce a significant decrease in DAGK activity when exogenous DOG or SAG were present in the assay (Fig. 4b)). These

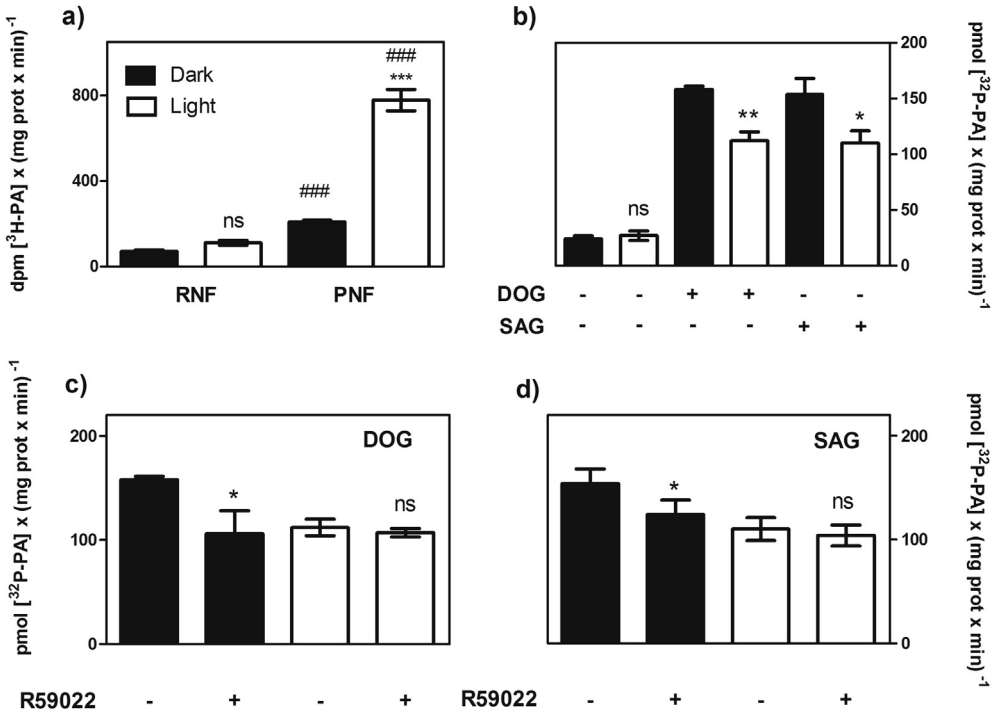

Fig. 4. Light-effect on nuclear DAGK activity evaluated with different DAGK assays. a) DAGK activity was evaluated in RNF and PNF obtained from light or dark exposed retinas using [^{3}H]DAG in the presence of Triton -X100 and ATP. b) PA formation in PNF from light or dark exposed retinas was measured using [γ-^{32}P]ATP and endogenous DAG, or exogenous DOG or SAG as co-substrates, in absence of detergents. In c) and d) DAGK assay was measured as described in b, after 10 min preincubation with 10 μM R59022 or vehicle (0.1% DMSO). DAGK assays were performed as described in Materials and Methods and results are expressed as dpm [^{3}H-PA] × (mg prot × min)$^{-1}$ (a) or pmol [^{32}P-PA] × (mg prot × min)$^{-1}$ (b, c and d). Data shown are averages of three independent experiments. Error bars indicate standard error. In a), asteriks and numerals indicate significant differences between light and dark condition and between PNF and RNF, respectively (***$p < 0.001$, ###$p < 0.001$). In b), asterisks indicate significant differences with respect to the dark condition (*$p < 0.05$, **$p < 0.01$). In c) and d), asterisks indicate significant differences with respect to control conditions (without R59022), (*$p < 0.05$).

differences in substrate utilization in the presence or absence of detergents suggest that more than one DAGK isoform is present in PNF and that they could be differentially modulated by light.

Whereas type I DAGK isoforms have no selectivity for any acyl composition of DAG, DAGKε (type III) is known to be selective for SAG. In contrast, DAGKζ (type IV) has been found to prefer DOG to SAG (Thirugnanam et al., 2001). Our results in the presence of exogenous DOG and SAG suggest the presence of DAGKζ and DAGKε isoforms in PNF. In order to indirectly analyze type I DAGKs contribution, R59022 (a type I DAGK selective inhibitor) was used (van Blitterswijk and Houssa, 2000). As shown in Fig. 4c) and d), when DOG or SAG were used as exogenous substrates, R59022 was observed to decrease DAGK activity by 33% and 20% in the dark condition, respectively. However, under light condition R59022 was not found to change DAGK activity, when both exogenous substrates were used (Fig. 4c) and d)). Results obtained with R59022 suggest that type I DAGKs are activated in PNF only under dark conditions.

Data obtained from the evaluation of the effect of light in experiments carried out without detergents and with exogenous DOG and SAG ([γ-^{32}P]ATP as co-substrate), and from [^{3}H]DAG in the presence of Triton X-100, suggest that changes in DAGK isoforms localization in PNF as a consequence of light exposure cannot be discarded.

3.5. Effect of light on the localization of DAGKε, DAGKζ and type I isoforms in nuclei from photoreceptor cells

In order to evaluate the effect of light exposure on the localization of different DAGK isoforms WB assays were carried out. DAGKε, ζ, α, β and γ levels were determined in PNF from dark-adapted bovine retinas or from light exposed bovine retinas as detailed in materials and methods. Whereas light exposure was observed to increase the content of DAGKζ in PNF by 50% with respect to the dark condition, a decrease in the content of DAGKε and β (by 30% and by 38%, respectively) was observed (Fig. 5(a) and (b)). CRX was used as loading control. Although α and γ isoforms were present in PNF, no light-induced differences in PNF content were detected for these isoforms (data not shown).

At the moment we have only immunodetected all class I DAGK (α, β and γ isoforms), class III (ε isoform) and class IV (ζ isoform). However, we do not discard that other isoforms could be present.

DAGKε and ζ have contrasting conditions for their maximal activity and on the basis of differential activation or inhibition it has been suggested that they have different physiological roles (Thirugnanam et al., 2001). The light-dependent differential localization of these isoforms in the PNF is indeed an interesting finding, so selective experimental conditions to favor the activity of DAGKε or DAGKζ were used.

3.6. Effect of light under experimental conditions that favor DAGKζ or DAGKε activity in nuclei from photoreceptor cells

PA formation was studied under different assay conditions to preferentially evaluate DAGKζ or DAGKε activity in PNF from dark or light exposed retinas.

It is known that the specificity of DAGKε for SAG as well as the specificity of DAGKζ for DOG are relative and not absolute (Thirugnanam et al., 2001). However, the effects of di-oleoyl phosphatidylserine (DOPS) on both isoforms are opposite, DAGKζ being activated by DOPS and DAGKε being inhibited by this phospholipid (Thirugnanam et al., 2001). In nuclei obtained from light exposed retinas, under experimental conditions that favor DAGKζ activity (DOG as exogenous substrate, [γ-^{32}P]ATP and Triton X-100), we observed a higher PA formation with respect to the dark condition in the presence of DOPS (DAGKε inhibitor) and R59022 (type I DAGK inhibitor), all these being the optimal conditions to assay DAGKζ activity (Fig. 6a)). The effect of DOPS was also observed when [^{3}H]DAG was used as exogenous substrate (Fig. 6b)).

In addition, to favor DAGKε activity, a micellar detergent lipid assay with OG as detergent, SAG as exogenous substrate and [γ-^{32}P]ATP was performed. Under these conditions, which are used to preferentially measure DAGKε, light exposure was observed to increase PA formation by 40% with respect to the dark condition. However, this increase was also abolished by R59022, a type 1 selective inhibitor of DAGK. These results suggest that light-induced enhanced PA formation is not mediated by DAGKε but by type I DAGKs, instead.

3.7. Light-dependent phosphorylated PKCα (pPKCα) localization and DAGK effects on pPKCα in PNF

It has been previously reported that in cell culture PKCα and DAGKζ are co-regulated (Luo et al., 2003a, 2003b). Whereas by phosphorylating MARCKS motif, PKCα attenuates DAGKζ activity,

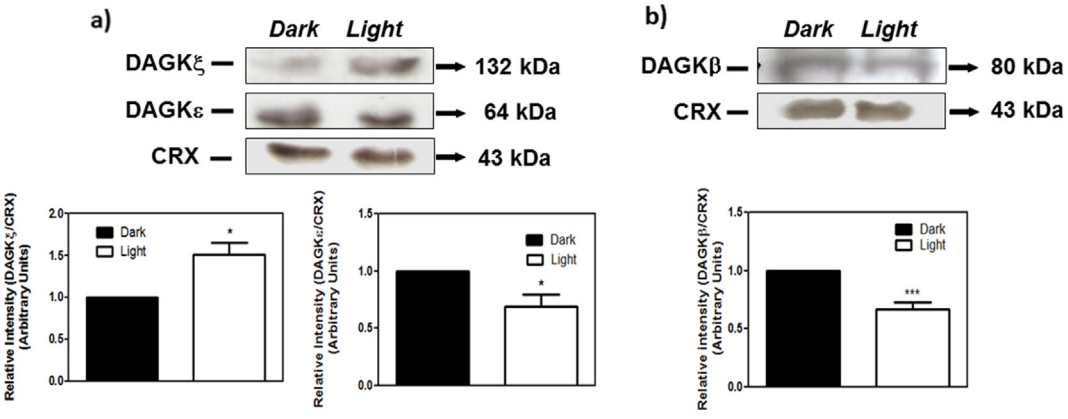

Fig. 5. Effect of light on the nuclear localization of DAGKε, DAGKζ and DAGK β in photoreceptor cells. WB assays were performed to detect DAGKε and ζ (a) or DAGKβ (b). PNF proteins (40 μg) were resolved in a 10% SDS-PAGE and transferred to a PDVF membrane. Membranes were blocked and incubated with primary and secondary antibodies as detailed in Materials and Methods. Immunoreactive bands were detected by enhanced chemiluminiscence. Numbers on the right indicate molecular weights and the data shown are representative results of three independent experiments. The bar graph shows the densitometry values of each DAGK/CRX expressed as ratio of the control (Dark condition). Asterisks indicate significant differences with respect to the control ($^{*}p < 0.05$, $^{***}p < 0.001$).

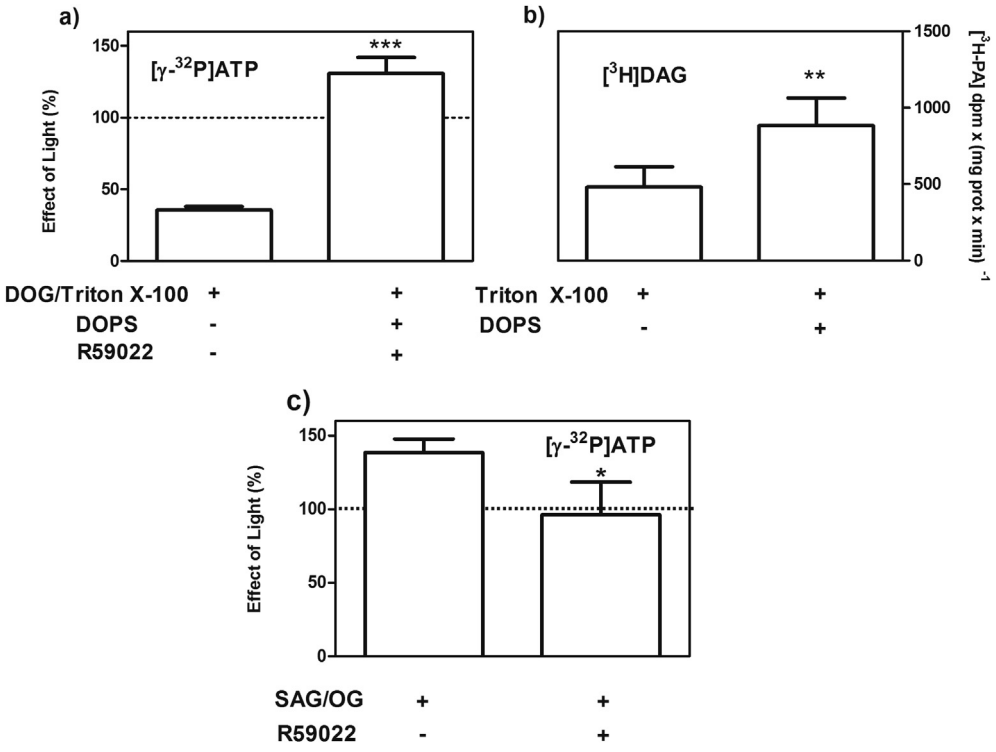

Fig. 6. Assays under conditions used to preferentially measure DAGKζ and DAGKε activities. To favor DAGKζ, assays using a) [γ-^{32}P]ATP and 500 μM DOG as exogenous substrate (DOG/Triton-X100) or b) [^{3}H]DAG and Triton X100 were performed. In a), PNF from dark adapted or light exposed retinas (100 μg protein) were preincubated in the presence of 4 mM phosphatidylserine (DOPS condition) and with 10 μM R59022 (Type 1 DAGK preferential inhibition) for 10 min before [γ-^{32}P]ATP addition, and DAGK activity was subsequently measured as described in Material and Methods. To preferentially measure DAGKε, assays using [γ-^{32}P]ATP in the presence of 50 mM OG and 500 μM SAG were carried out. PNF from dark adapted or light exposed retinas (100 μg protein) were incubated in the presence of 10 μM R59022 or 0.1% DMSO (control condition) for 10 min before [γ-^{32}P]ATP addition (c). DAGK activity was measured as described in Material and Methods. In a) and c), the "**Effect of light**" is shown. Results are expressed as percentual changes with respect to the control condition (dark condition). In b), data are expressed as dpm [^{3}H-PA] × (mg prot × min)$^{-1}$. Error bars indicate standard error. Asterisks indicate significant differences with respect to the dark condition (*$p < 0.05$, **$p < 0.01$, ***$P < 0.001$).

this phosphorylation also attenuates their co-immunoprecipitation and co-localization, thus suggesting that DAGKζ phosphorylation by PKCα negatively regulates the interaction of these kinases. On the other hand, because light was found to induce an increased localization and activation of DAGKζ in PNF (Figs. 5a and 6a, respectively), we decided to analyze PKCα content and its light-induced phosphorylation in photoreceptor nuclei.

Phosphorylated PKCα (pPKCα) and total PKCα were detected by WB in PNF from dark-adapted retinas and from retinas exposed to light. CRX detection was used as loading control (Fig. 7a)). The mature phosphorylated form of PKCα was found to be significantly increased in PNF from retinas exposed to light with respect to nuclei from dark-adapted retinas (275%) with no significant changes in total PKCα content in PNF (Fig. 7a)).

All DAGK isoforms regulate DAG content and conventional and novel PKCs, such as PKCα and PKCδ respectively, are DAG-regulated isoforms differing in the presence of a Ca^{++} sensitive or insensitive domain, respectively. We thus also analyzed the presence of pPKCδ and total PKCδ by WB in PNF from dark-adapted or light exposed retinas. PKCδ was not found in PNF (data not shown).

In order to evaluate the role of DAGK in the regulation of PKCα phosphorylation in the nuclei, experiments with R59022 were performed. When nuclei obtained from light-exposed retinas were incubated in the presence of R59022, a significant decrease in pPKCα was observed (Fig. 7b)). This result demonstrates that PKCα phosphorylation depends on DAGK activity.

Light-dependent changes in the content of pPKCα in PNF could also be connected with a potential modulation of DAGK activities. To study a possible DAGK modulation by PKC, DAGK activity was measured (using [γ-^{32}P]ATP and SAG as substrate in the absence of detergents) in nuclei pre-incubated with the PKC activator (PMA) or with BIM (a PKC inhibitor). Neither PKC activation nor PKC inhibition were observed to affect DAGK activity in PNF from dark-adapted or light-exposed retinas (data not shown).

3.8. PIP_2-Phospholipase C role in PNF DAGK activity

Phospholipase Cγ1 (PLCγ1) has been previously detected in the signaling complex of PKCα and DAGKζ (Luo et al., 2003b). Based on this, we decided to analyze whether or not DAGK activity is modulated by PLC under dark or light conditions. To this end, retinas were preincubated for 15 min with U73122 (PIP_2-PLC inhibitor) as detailed in 2.3.1.2. DAGK assay in PNF was carried out in the absence of exogenous DAG in order to evaluate the effect of DAG generated endogenously, possibly through PLC activation. In addition, the DAGK assay was also performed in the presence of SAG (Fig. 8).

Whereas under the dark condition U73122 was not found to affect DAGK activity, in PNF from retinas exposed to light a significant decrease (by 90%) was observed (Fig. 8). These results indicate that, when no exogenous DAG is added, basal DAGK activity depends on PLC only under light conditions. Interestingly, U73122 was also found to reduce PA formation (by 70%) in light exposed photoreceptor nuclei when DAGK assay was performed in the presence of SAG (Fig. 8). These results demonstrate that when DAG is added exogenously the inhibition of PLC partialy reduces DAGK activity, thus suggesting that DAG is not the only PLC-derived messenger involved in this regulation.

It has been reported that PIP_2 inhibits DAGKε (Thirugnanam et al., 2001; Walsh et al., 1995). Taking into account that PIP_2-PLC

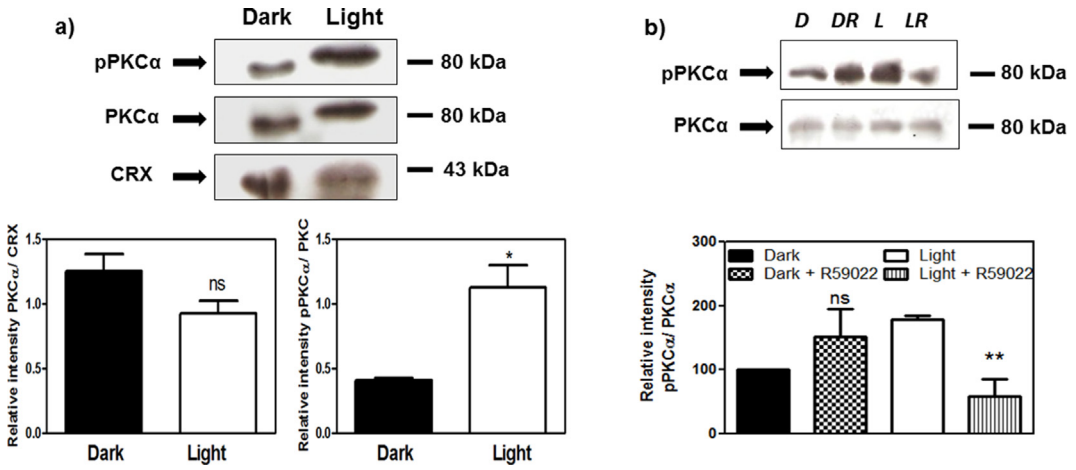

Fig. 7. Light-dependent localization of phosphorylated PKCα and effect of a DAGK inhibitor (R59022). a) WB assays from PNF proteins obtained from light-exposed or dark-exposed retinas were performed as described in Fig. 5. Numbers on the right indicate molecular weights and the data shown are representative results of three independent experiments. The bar graphs show the densitometry values of each pPKCα/PKCα or PKCα/CRX expressed as relative intensities. Asterisks indicate significant differences with respect to the dark condition ($^{*}p < 0.05$). b) PNFs obtained from light-exposed or dark-exposed retinas were preincubated in the presence of R59022 (10 μM) or vehicle (0.1% DMSO, control conditions). WB assays were performed as described in Fig. 5. Numbers on the right indicate molecular weights and the data shown are representative results of three independent experiments. The bar graph shows the densitometry values of each pPKCα/PKCα expressed as a ratio of the control (Dark condition). Asterisks indicate significant differences with respect to the condition without R59022 ($^{**}p < 0.01$).

inhibition causes PIP_2 increase, the effect of Neomycin, a polycation that binds and sequesters PIP_2 (James et al., 2004) was studied. Independently of light or dark conditions, preincubation with Neomycin was observed to strongly increase PA formation in PNF (Fig. 9). These data suggest that in the presence of Neomycin the increased DAGK activity could be due to an enhanced DAGKε activity induced by PIP_2 sequestration. These data also suggest that DAGK inhibition by U73122, in the absence or in the presence of exogenous SAG (Fig. 8), could be partially due to a PIP_2 modulation.

4. Discussion

Our findings are not only the first evidence of the presence of DAGK isoforms and PKCα in the nucleus of bovine photoreceptor cells but also demonstrate that the activity and localization of DAGKs as well as PKCα phosphorylation are regulated by light.

We have recently reported that DAGKε is differently localized in bovine and rat photoreceptors cells in response to light. DAGKε was demonstrated to be concentrated in the inner segment of the photoreceptor layer under dark condition. In contrast, under light stimuli, DAGKε was observed to be higher in the outer segment than the inner segment (Natalini et al., 2013). Representative images from immunohistochemistry assays showed that DAGKε appears to be also concentrated in nuclei of the photoreceptor layer (ONL) under dark conditions. In contrast, under light stimuli, DAGKε appears to be partially depleted in ONL (Natalini et al., 2013).

With the present study we wanted to determine if as a result of phototransduction, changes occur in the DAGK activity at the nuclear level of photoreceptor cells.

Nuclear and subnuclear localization and functions of several types of DAGKs have been described in different cellular types (Martelli et al., 2006). However, this is the first report of their

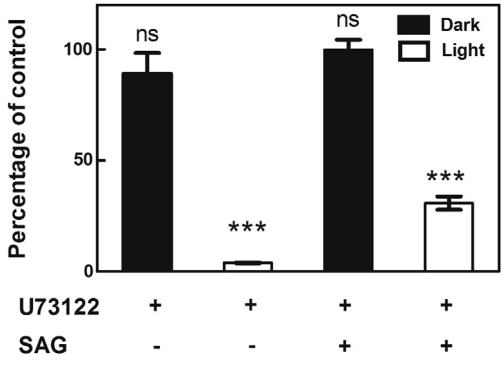

Fig. 8. Role of PIP_2-PLC on DAGK activity in PNF from bovine retinas. To evaluate PIP_2-PLC role on DAGK activity in PNF, bovine retinas were preincubated with 10 μM U73122 (PIP_2-PLC inhibitor) or with the vehicle (0.1% DMSO, control conditions) in oxygenated (95% O_2, 5% CO_2) Ames medium for 15 min under dim red light. Eyecups were subsequently exposed to light or kept in darkness and the PNF were isolated as described in Material and Methods. DAGK assay was carried out using [γ-^{32}P]ATP in the absence of detergents and in the presence of endogenous DAG or 0.1 mM SAG. Data are expressed as percentage of control (without U73122). Error bars indicate standard error. Asterisks indicate significant differences between light and dark condition ($^{***}p < 0.001$).

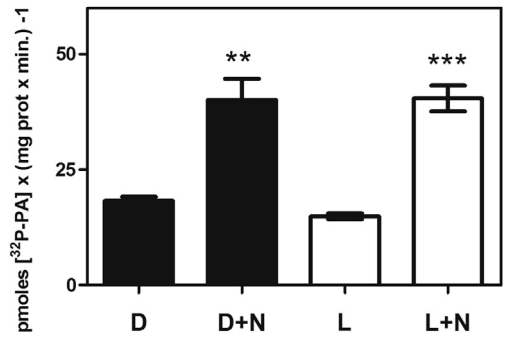

Fig. 9. Effect of Neomycin as a PIP_2 binder on DAGK activity in PNF from bovine retinas. To evaluate nuclear DAGK activity in the presence of Neomycin (PIP_2 sequester), bovine retinas were preincubated with 0.5 mM neomycin or with the vehicle (water) in oxygenated (95% O_2, 5% CO_2) Ames medium for 15 min under dim red light. Eyecups were subsequently exposed to light or kept in darkness and the PNF were isolated as described in Material and Methods. DAGK assay was carried out using [γ-^{32}P]ATP in the absence of detergents and exogenous substrates. Results are expressed as pmol [^{32}P-PA] × (mg prot × min)$^{-1}$. Error bars indicate standard error. Asterisks indicate significant differences between light and dark condition ($^{***}p < 0.001$, $^{**}p < 0.01$).

presence in a nuclear fraction of photoreceptor cells from bovine retina.

By using the new purification protocol described in this work, we obtained a nuclear fraction highly enriched in photoreceptor nuclei (PNF), demonstrated by propidium iodide staining, WB and TEM (Fig. 1). Although RNF and PNF are purified nuclear fractions (Figs. 1S, 2S and 3S), results indicate that our PNF isolation protocol renders a significantly improved photoreceptor nuclear enrichment.

Using two different radioactive precursors, DAGK activity was found in isolated PNF. As described in Fig. 2, PA synthesis from exogenous DOG and using [γ-^{32}P]ATP was linear as a function of time and protein content. DOG and ATP saturating concentrations for a DAGK activity were also determined.

Results obtained with ionic and non-ionic detergents and different lipid substrates (Fig. 3) revealed that more than one DAGK isotype is active in photoreceptor nuclei. Results obtained in the presence of SAG, OG and type I DAGK inhibitor (R59022) indicated DAGKε activity. In addition, DOPS stimulation of DAGK activity in the presence of Triton X-100 (Fig. 6a)) was also indicative of DAGKζ presence in PNF. Moreover, WB assays were conclusive about the presence of both isoforms as well as DAGKβ in PNF (Fig. 5). In addition, DAGKα and DAGKγ were also observed by WB in PNF (data not shown). Our results showed a selective light-modulated nuclear localization of DAGKs. Whereas DAGKε and β nuclear localization was reduced in PNF from light exposed retinas with respect to the dark condition, DAGKζ nuclear localization increased after light exposure (Fig. 5).

As was previously mentioned, in immunohistochemistry assays DAGKε was observed to be partially depleted in ONL when dark-adapted rats were exposed to light (Natalini et al., 2013). Taking into account that light/dark conditions cannot be controlled precisely in the bovine model, our previous data in the rat *in vivo* model strengthen our present results.

The results obtained by WB regarding DAGKζ were supported by DAGK activity assays under conditions that favor the activity of this isoform. Under the best conditions to measure DAGKζ activity (DOG, Triton X-100, EGTA, R59022 and DOPS) a higher PA formation was observed in PNF from light-exposed retinas (Fig. 6a)).

Although it is unlikely to be measuring a type I DAGK under conditions that favor the activity of DAGKζ, a minor contribution of other DAGK isoform (stimulated by PS and not inhibited by EGTA and R59022) cannot be completely discarded. Sequence based analysis indicated that DAGKβ, a type I DAGK, could be stimulated by PS, however no experimental data of this stimulation was reported to date.

In addition, R59022 (a type I DAGK inhibitor) was observed to reduce PA formation in PNF from dark-adapted retinas (assayed in the presence of DOG or SAG) although it did not inhibit DAGK activity when PNF was from light exposed retinas (Fig. 4c) and d)). This finding is in accordance to the reduced DAGKβ nuclear localization observed under light condition (Fig. 5).

Light-dependent changes in the content and activity of different isoforms of DAGK in PNF could be connected with a potential nuclear PKC modulation. Increasing evidence has suggested a role of PKC in nuclear functions, which could be indicative of a pathway that connects signals generated at the plasma membrane with the nucleus (Eyster et al., 1993; Rosenberger et al., 1995). Almost every PKC isoform has been reported to be present in the nucleus in several cellular systems under certain conditions, either as a consequence of translocation from cytoplasm or as a resident enzyme.

Our results demonstrate a light-dependent increased content of DAGKζ and pPKCα in PNF from light exposed retinas with respect to dark adapted retinas (Figs. 5 and 7).

It has been reported that in culture cells the pattern of tissue and cellular expression of PKCα closely parallels that of DAGKζ and that these two proteins could interact, thus suggesting that they reside in the same signaling complex (Luo et al., 2003b). Although protein–protein association cannot be disregarded, the low content of DAGKζ in PNF from dark adapted retinas and the similar content of total PKCα under dark and light conditions suggest that these enzymes are not present in a signaling complex.

In addition, a light-dependent decreased content of nuclear DAGKε and β was found.

It is known that DAGKζ and ε have differential substrate avidity and they are differently modulated by phospholipids and calcium ions (Thirugnanam et al., 2001). Differences in nuclear localization of these DAGK isoforms could thus regulate the concentration of nuclear DAG and PA. It was previously reported that PA functions as an inhibitor of the protein phosphatase 1 (PP1) (not for PP2A or PP2B) and that it may reverse or counteract the PP1-mediated effects of ceramide, such as apoptosis and retinoblastoma gene product dephosphorylation (Kishikawa et al., 1999). Nuclear activation and phosphorylation of PKCα could therefore be light-regulated through DAG and PA. Nuclear DAG could activate PKCα while PA could keep PKCα phosphorylation by inhibiting PP1 activity. Nuclear DAGKs activation appears to be crucial to modulate DAG and PA levels.

According to our hypothesis, in light-exposed retinas pPKCα nuclear content was strongly decreased when nuclear DAGK was inhibited with R59022, a condition that decreases PA formation.

Important light-dependent changes in nuclear localization of DAGK isoforms have been described in this work (Fig. 5). Nuclear DAGK activity over endogenous DAG, which appears to be similar between PNF from dark-adapted or light exposed retinas, could thus be misinterpreted. Because DAGKζ content was increased in PNF from light-exposed retinas whereas DAGKε and β content were decreased, no changes in DAGK activity over endogenous DAG were observed. However, light-induced changes in DAGK activity could be observed when the enzyme assay was performed in the presence of detergents and exogenous substrates (Fig. 6).

Experiments carried out in the presence of the PIP_2-PLC inhibitor U73122 demonstrated that PLC regulates nuclear DAGK activity under light conditions (both, when endogenous DAG or SAG were used as substrates), thus suggesting a close relationship between both enzymatic activities (Fig. 8).

Therefore, apart from the changes in nuclear DAGKs content, light-induced PLC activation also modulates nuclear DAGK activity. It is interesting to note that a light-induced PIP_2-PLC activation increases DAG content but also decreases PIP_2 content. Interestingly a selective PIP_2 regulation on DAGK activity has been described for DAGKε and DAGKζ isoforms. DAGKε, the type III enzyme, is inhibited by both PIP_2 and phosphatidylserine, whereas DAGKζ is activated by both lipids (Thirugnanam et al., 2001). Although nuclear DAGKε content was found to be decreased in PNF after light exposure, PIP_2 depletion through a light-induced activation of PLC could increase the activity of DAGKε. In contrast, although DAGKζ content was found to be increased, PIP_2 depletion through a light-induced PLC activity could operate as a negative regulator. Likewise, results obtained with Neomycin (a high affinity binder of PIP_2) confirmed that nuclear DAGK activity could also be stimulated by PIP_2 sequestration, independently of light or dark conditions (Fig. 9).

Our present findings reveal that phototransduction events elicited in the outer segments of photoreceptor cells, and the signaling mechanisms triggered by light stimulation, activate signal transduction pathways at the nuclear level of photoreceptor cells. The exposure of retinas to light not only induces a partial depletion of nuclear DAGKε and β but also promotes the activation of the remaining ε isoform possibly to ensure SAG remotion and generation of SAG-derived PA. In parallel, an increased content of DAGKζ

(an isoform with contrasting modulation with respect to DAGKε) reveals light-dependent specific conditions for nuclear DAG transformation. In line with this, nuclear pPKCα, which could be involved in several nuclear functions, such as the control of DNA replication, mRNA synthesis and processing, nucleo-cytoplasmic transport and chromatin structure (Martelli et al., 2003), could be modulated by this differentially regulated nuclear DAGKs. Taken together, our findings reveal for the first time a novel light-dependent regulation of DAGKs and PKCα in nuclei of photoreceptor cells.

Acknowledgments

Authors thank Dr. M.K. Topham, Huntsman Cancer Institute, University of Utah, USA, and Dr. F. Sakane, Chiba University Graduate School Science, Japan, who generously provided antibodies for DAGKs; Dr. N.G. Bazán, Neuroscience Center of Excellence at Louisiana State University Health Sciences Center, New Orleans, USA, who kindly provided antibodies for DAGK and supplies such as U73122, DOG and SAG; Dr. C. Craft, University of Southern California, Los Angeles, USA, who generously provided CRX antibody; Dr. Beatriz Caputo, Universidad Nacional de Córdoba, Cordoba, Argentina, who kindly provided calnexin antibody and Dr. Robert Molday, University of British Columbia, Vancouver, BC, Canada, who generously supplied Rho4D2 antibody.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2014.06.007.

References

Abramovici, H., Mojtabaie, P., Parks, R.J., Zhong, X.P., Koretzky, G.A., Topham, M.K., Gee, S.H., 2009. Diacylglycerol kinase zeta regulates actin cytoskeleton reorganization through dissociation of Rac1 from RhoGDI. Mol. Biol. Cell. 20, 2049–2059.
Arvidson, G.A., 1968. Structural and metabolic heterogeneity of rat liver glycerophosphatides. Eur. J. Biochem 4, 478–486.
Blackshear, P.J., 1993. The MARCKS family of cellular protein kinase C substrates. J. Biol. Chem. 268, 1501–1504.
Bunting, M., Tang, W., Zimmerman, G.A., McIntyre, T.M., Prescott, S.M., 1996. Molecular cloning and characterization of a novel human diacylglycerol kinase zeta. J. Biol. Chem. 271, 10230–10236.
Cai, J., Abramovici, H., Gee, S.H., Topham, M.K., 2009. Diacylglycerol kinases as sources of phosphatidic acid. Biochim. Biophys. Acta 1791, 942–948.
Caricasole, A., Bettini, E., Sala, C., Roncarati, R., Kobayashi, N., Caldara, F., Goto, K., Terstappen, G.C., 2002. Molecular cloning and characterization of the human diacylglycerol kinase beta (DGKbeta) gene: alternative splicing generates DGKbeta isotypes with different properties. J. Biol. Chem. 277, 4790–4796.
Cocco, L., Martelli, A.M., Gilmour, R.S., Rhee, S.G., Manzoli, F.A., 2001. Nuclear phospholipase C and signaling. Biochim. Biophys. Acta 1530, 1–14.
D'Santos, C.S., Clarke, J.H., Divecha, N., 1998. Phospholipid signalling in the nucleus. Een DAG uit het leven van de inositide signalering in de nucleus. Biochim. Biophys. Acta 1436, 201–232.
D'Santos, C.S., Clarke, J.H., Irvine, R.F., Divecha, N., 1999. Nuclei contain two differentially regulated pools of diacylglycerol. Curr. Biol. 9, 437–440.
D'Santos, C., Clarke, J.H., Roefs, M., Halstead, J.R., Divecha, N., 2000. Nuclear inositides. Eur. J. Histochem 44, 51–60.
Decaffmeyer, M., Shulga, Y.V., Dicu, A.O., Thomas, A., Truant, R., Topham, M.K., Brasseur, R., Epand, R.M., 2008. Determination of the topology of the hydrophobic segment of mammalian diacylglycerol kinase epsilon in a cell membrane and its relationship to predictions from modeling. J. Mol. Biol. 383, 797–809.
Ding, L., Bunting, M., Topham, M.K., McIntyre, T.M., Zimmerman, G.A., Prescott, S.M., 1997. Alternative splicing of the human diacylglycerol kinase zeta gene in muscle. Proc. Natl. Acad. Sci. U. S. A. 94, 5519–5524.
Ding, L., Traer, E., McIntyre, T.M., Zimmerman, G.A., Prescott, S.M., 1998. The cloning and characterization of a novel human diacylglycerol kinase, DGKiota. J. Biol. Chem. 273, 32746–32752.
Divecha, N., 1998. Signal transduction. Marked for nuclear export? Nature 394, 619–620.
Divecha, N., Clarke, J.H., Roefs, M., Halstead, J.R., D'Santos, C., 2000. Nuclear inositides: inconsistent consistencies. Cell. Mol. Life Sci. 57, 379–393.
Evangelisti, C., Bortul, R., Tabellini, G., Papa, V., Cocco, L., Martelli, A.M., 2006. Nuclear expression of diacylglycerol kinases: possible involvement in DNA replication. Eur. J. Histochem. 50, 9–13.
Eyster, K.M., Teixeira, F., Zakar, T., Olson, D.M., 1993. Protein kinase-C stimulatory activity in human amnion cytosol. J. Clin. Endocrinol. Metab. 76, 424–428.
Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.
Frank, C., Keilhack, H., Opitz, F., Zschornig, O., Bohmer, F.D., 1999. Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. Biochemistry 38, 11993–12002.
Furukawa, T., Morrow, E.M., Cepko, C.L., 1997. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91, 531–541.
Gaveglio, V.L., Pasquare, S.J., Giusto, N.M., 2011. Metabolic pathways for the degradation of phosphatidic acid in isolated nuclei from cerebellar cells. Arch. Biochem. Biophys 507, 271–280.
Giusto, N.M., Pasquare, S.J., Salvador, G.A., Castagnet, P.I., Roque, M.E., Ilincheta de Boschero, M.G., 2000. Lipid metabolism in vertebrate retinal rod outer segments. Prog. Lipid Res. 39, 315–391.
Goto, K., Kondo, H., 1993. Molecular cloning and expression of a 90-kDa diacylglycerol kinase that predominantly localizes in neurons. Proc. Natl. Acad. Sci. U. S. A. 90, 7598–7602.
Goto, K., Funayama, M., Kondo, H., 1994. Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum. Proc. Natl. Acad. Sci. U. S. A. 91, 13042–13046.
Goto, K., Kondo, H., 1996. A 104-kDa diacylglycerol kinase containing ankyrin-like repeats localizes in the cell nucleus. Proc. Natl. Acad. Sci. U. S. A. 93, 11196–11201.
Houssa, B., Schaap, D., van der Wal, J., Goto, K., Kondo, H., Yamakawa, A., Shibata, M., Takenawa, T., van Blitterswijk, W.J., 1997. Cloning of a novel human diacylglycerol kinase (DGKtheta) containing three cysteine-rich domains, a proline-rich region, and a pleckstrin homology domain with an overlapping Ras-associating domain. J. Biol. Chem. 272, 10422–10428.
Huang, Z., Ghalayini, A., Guo, X.X., Alvarez, K.M., Anderson, R.E., 2000. Light-mediated activation of diacylglycerol kinase in rat and bovine rod outer segments. J. Neurochem. 75, 355–362.
Ilincheta de Boschero, M.G., Giusto, N.M., 1992. Phosphatidic acid and polyphosphoinositide metabolism in rod outer segments. Differential role of soluble and peripheral proteins. Biochim. Biophys. Acta 1127, 105–115.
Imai, S., Kai, M., Yasuda, S., Kanoh, H., Sakane, F., 2005. Identification and characterization of a novel human type II diacylglycerol kinase, DGK kappa. J. Biol. Chem. 280, 39870–39881.
Ito, T., Hozumi, Y., Sakane, F., Saino-Saito, S., Kanoh, H., Aoyagi, M., Kondo, H., Goto, K., 2004. Cloning and characterization of diacylglycerol kinase iota splice variants in rat brain. J. Biol. Chem. 279, 23317–23326.
James, D.J., Salaun, C., Brandie, F.M., Connell, J.M., Chamberlain, L.H., 2004. Neomycin prevents the wortmannin inhibition of insulin-stimulated Glut4 translocation and glucose transport in 3T3-L1 adipocytes. J. Biol. Chem. 279, 20567–20570.
Jeon, C.J., Strettoi, E., Masland, R.H., 1998. The major cell populations of the mouse retina. J. Neurosci. 18, 8936–8946.
Kai, M., Sakane, F., Imai, S., Wada, I., Kanoh, H., 1994. Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in human retina with a truncated and inactive enzyme expression in most other human cells. J. Biol. Chem. 269, 18492–18498.
Kishikawa, K., Chalfant, C.E., Perry, D.K., Bielawska, A., Hannun, Y.A., 1999. Phosphatidic acid is a potent and selective inhibitor of protein phosphatase 1 and an inhibitor of ceramide-mediated responses. J. Biol. Chem. 274, 21335–21341.
Klauck, T.M., Xu, X., Mousseau, B., Jaken, S., 1996. Cloning and characterization of a glucocorticoid-induced diacylglycerol kinase. J.Biol.Chem. 271, 19781–19788.
Kohyama-Koganeya, A., Watanabe, M., Hotta, Y., 1997. Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in rat retina. FEBS Lett. 409, 258–264.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Limatola, C., Schaap, D., Moolenaar, W.H., van Blitterswijk, W.J., 1994. Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. Biochem. J. 304 (Pt 3), 1001–1008.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Luo, B., Prescott, S.M., Topham, M.K., 2003a. Association of diacylglycerol kinase zeta with protein kinase C alpha: spatial regulation of diacylglycerol signaling. J. Cell Biol. 160, 929–937.
Luo, B., Prescott, S.M., Topham, M.K., 2003b. Protein kinase C alpha phosphorylates and negatively regulates diacylglycerol kinase zeta. J. Biol. Chem. 278, 39542–39547.
Martelli, A.M., Faenza, I., Billi, A.M., Fala, F., Cocco, L., Manzoli, L., 2003. Nuclear protein kinase C isoforms: key players in multiple cell functions? Histol. Histopathol. 18, 1301–1312.
Martelli, A.M., Fala, F., Faenza, I., Billi, A.M., Cappellini, A., Manzoli, L., Cocco, L., 2004. Metabolism and signaling activities of nuclear lipids. Cell. Mol. Life Sci. 61, 1143–1156.
Martelli, A.M., Evangelisti, C., Nyakern, M., Manzoli, F.A., 2006. Nuclear protein kinase C. Biochim. Biophys. Acta 1761, 542–551.
Matunis, M.J., 2006. Isolation and fractionation of rat liver nuclear envelopes and nuclear pore complexes. Methods 39, 277–283.

Murakami, T., Sakane, F., Imai, S., Houkin, K., Kanoh, H., 2003. Identification and characterization of two splice variants of human diacylglycerol kinase eta. J. Biol. Chem. 278, 34364–34372.
Natalini, P.M., Zulian, S.E., Ilincheta de Boschero, M.G., Giusto, N.M., 2013. Diacylglycerol kinase epsilon in bovine and rat photoreceptor cells. Light-dependent distribution in photoreceptor cells. Exp. Eye Res. 112, 139–150.
Newton, A.C., 2009. Lipid activation of protein kinases. J. Lipid Res. 50 (Suppl.), S266–S271.
Pasquare de Garcia, S.J., Giusto, N.M., 1986. Phosphatidate phosphatase activity in isolated rod outer segment from bovine retina. Biochim. Biophys. Acta 875, 195–202.
Rosenberger, U., Shakibaei, M., Buchner, K., 1995. Localization of non-conventional protein kinase C isoforms in bovine brain cell nuclei. Biochem. J. 305 (Pt 1), 269–275.
Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C., Tanabe, T., 1990. Porcine diacylglycerol kinase sequence has zinc finger and E-F hand motifs. Nature 344, 345–348.
Sakane, F., Kai, M., Wada, I., Imai, S., Kanoh, H., 1996. The C-terminal part of diacylglycerol kinase alpha lacking zinc fingers serves as a catalytic domain. Biochem. J. 318 (Pt 2), 583–590.
Sakane, F., Imai, S., Yamada, K., Murakami, T., Tsushima, S., Kanoh, H., 2002. Alternative splicing of the human diacylglycerol kinase delta gene generates two isoforms differing in their expression patterns and in regulatory functions. J. Biol. Chem. 277, 43519–43526.
Schaap, D., de, W.J., van der Wal, J., Vandekerckhove, J., van, D.J., Gussow, D., Ploegh, H.L., van Blitterswijk, W.J., van der Bend, R.L., 1990. Purification, cDNA-cloning and expression of human diacylglycerol kinase. FEBS Lett. 275, 151–158.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.
Siddhanta, A., Shields, D., 1998. Secretory vesicle budding from the trans-Golgi network is mediated by phosphatidic acid levels. J. Biol. Chem. 273, 17995–17998.
Solovei, I., Kreysing, M., Lanctot, C., Kosem, S., Peichl, L., Cremer, T., Guck, J., Joffe, B., 2009. Nuclear architecture of rod photoreceptor cells adapts to vision in mammalian evolution. Cell 137, 356–368.
Shulga, Y.V., Myers, D.S., Ivanova, P.T., Milne, S.B., Brown, H.A., Topham, M.K., Epand, R.M., 2010. Molecular species of phosphatidylinositol-cycle intermediates in the endoplasmic reticulum and plasma membrane. Biochemistry 49, 312–317.
Tang, W., Bunting, M., Zimmerman, G.A., McIntyre, T.M., Prescott, S.M., 1996. Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. J. Biol. Chem. 271, 10237–10241.
Thirugnanam, S., Topham, M.K., Epand, R.M., 2001. Physiological implications of the contrasting modulation of the activities of the epsilon- and zeta-isoforms of diacylglycerol kinase. Biochemistry 40, 10607–10613.
Topham, M.K., Bunting, M., Zimmerman, G.A., McIntyre, T.M., Blackshear, P.J., Prescott, S.M., 1998. Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta. Nature 394, 697–700.
Topham, M.K., Epand, R.M., 2009. Mammalian diacylglycerol kinases: molecular interactions and biological functions of selected isoforms. Biochim. Biophys. Acta 1790, 416–424.
Uranga, R.M., Mateos, M.V., Giusto, N.M., Salvador, G.A., 2007. Activation of phosphoinositide-3 kinase/Akt pathway by FeSO4 in rat cerebral cortex synaptic endings. J. Neurosci. Res. 85, 2924–2932.
van Blitterswijk, W.J., Houssa, B., 2000. Properties and functions of diacylglycerol kinases. Cell. Signal 12, 595–605.
Walsh, J.P., Suen, R., Glomset, J.A., 1995. Arachidonoyl-diacylglycerol kinase. Specific in vitro inhibition by polyphosphoinositides suggests a mechanism for regulation of phosphatidylinositol biosynthesis. J. Biol. Chem. 270, 28647–28653.
Zhang, X., Szabo, E., Michalak, M., Opas, M., 2007. Endoplasmic reticulum stress during the embryonic development of the central nervous system in the mouse. Int. J. Dev. Neurosci. 25, 455–463.
Zhang, Y., Du, G., 2009. Phosphatidic acid signaling regulation of Ras superfamily of small guanosine triphosphatases. Biochim. Biophys. Acta 1791, 850–855.