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Research article

Insulin-related signaling pathways elicited by light in photoreceptor nuclei from bovine retina

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ABSTRACT

Retina light stimulation triggers phototransduction events as well as different signaling mechanisms in outer segments (sensorial portion) of photoreceptor cells. We have recently reported a novel light-dependent activation of diacylglycerol kinase (DAGK) and protein kinase C (PKC) at the nuclear level of photoreceptor cells. The aim of the present study was to analyze whether *ex-vivo* light exposure of bovine retinas also modulates insulin-related signaling pathways in nuclei from photoreceptor cells. To this end, a nuclear fraction enriched in small nuclei from photoreceptor cells (PNF) was obtained using a modified nuclear isolation protocol. In PNF obtained from bovine retinas exposed to light or darkness, the presence of insulin receptor (IR) and phosphorylated insulin receptor (pIR), the activation of Akt, p38 and extracellular signal-regulated kinase (ERK1/2) and the local action of insulin on lipid kinases were studied.

Immunofluorescence (IF) and Western blot (WB) studies revealed the presence of IR in photoreceptor nuclei. In PNF a light-dependent increase in IR total content was observed. The presence of activated IR (pIR) was also observed in PNF by WB, being its content higher in PNF from light than in to darkness. Light exposure also produced a significant increase in the content of p-Akt (3 fold) and p-p38 (60%) without changes in total Akt and p38. In addition, an increase in the content of total ERK1/2 (2 fold) was found without changes in p-ERK/total ERK ratio, indicating that light induces translocation of p-ERK to the nucleus.

Polyphosphoinositide kinase and diacylglycerol kinase (DAGK) activities were measured in isolated nuclei from light-activated or darkness-adapted retinas through the formation of polyphosphoinositides (PPIs) and phosphatidic acid (PA) using nuclear lipid substrates and [γ-^{32}P]ATP as radioactive substrate. A light-dependent increase in PPIs and PA formation was detected when isolated nuclei were exposed to 0.8 μM insulin plus 0.2 mM vanadate.

WB studies revealed that retina's exposure to insulin under light condition increased nuclear IR content. In addition, PNF exposure to insulin increased ERK1/2 phosphorylation with no changes in total ERK1/2.

Our results demonstrate the presence and the functional state of IR in the nucleus from photoreceptor cells. They also show that molecular signaling components linked to tyrosine kinase receptors and MAPK pathways, such as Akt and ERK1/2, respectively, are present in photoreceptor nuclei and are regulated by insulin and light.

1. Introduction

Phototransduction occurs in the outer segments of photoreceptor cells. Other signaling mechanisms, such as phosphoinositide cycle, phosphoinositide 3-kinase (PI3K) pathway and insulin receptor (IR) activation, have been reported to be activated by light in this portion of the photoreceptor cell (Ghalayini et al., 1998; Ghalayini and Anderson, 1995; Guo et al., 1997; Huang et al., 2000; Ilincheta de Boschero and Giusto, 1992; Rajala et al., 2007,

Abbreviations: DAGK, diacylglycerol kinase; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated extracellular signal-regulated kinase; p-Akt, phosphorylated Akt; p-p38, phosphorylated p38; LAP2-β, lamina-associated polypeptide 2 beta isoform; CRX, cone-rod-homeobox transcription factor; PI-PLC, phosphatidylinositol specific phospholipase C; IR, insulin receptor; pIR, phosphorylated insulin receptor; PNF, photoreceptor nuclear fraction; RNF, retinal nuclear fraction; DAG, diacylglycerol; PA, phosphatidic acid; PPI, polyphosphoinositides.

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2002). However, little is known about the possible consequences of light stimuli at the nuclear level of photoreceptor cells.

Recent findings from our laboratory have reported that light stimulation activates signal transduction pathways at the nuclear level of photoreceptor cells (Natalini et al., 2014). The exposure of retinas to light induces a partial depletion of two diacylglycerol kinase (DAGK) isoforms (DAGKε and β) and promotes the increase of DAGKζ in photoreceptor nuclei. A light-dependent phosphoinositide-specific phospholipase C (PI-PLC) activation has been found to be related to PA formation at the nuclear level. Further findings from our laboratory showed that light increases phosphorylated protein kinase C α (p-PKCα) in the nuclear fraction and suggested that this protein kinase could be modulated by DAGK activity in the nucleus (Natalini et al., 2014). The modulation of either nuclear PKCα or its possible regulator DAGK could be regulated by upstream light-activated photoreceptor components also present in the nuclear fraction.

Previous studies showed that light induces tyrosine phosphorylation of retinal IR and that this activation leads to the binding of PI3K to ROS membranes (Rajala et al., 2002) and the subsequent activation of Akt (Li et al., 2008; Rajala et al., 2010). They also demonstrated that photobleaching of rhodopsin regulates the phosphorylation state of IR, IGF-1R, and insulin-related receptor (IRR), all of which belong to the same receptor-tyrosine kinase (RTK) family.

It is also known that 3-Phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop of a number of protein serine/threonine kinases of the AGC kinase superfamily, including Akt/PKB and PKC isoforms. Interestingly, it has been recently reported that phosphoinositide-dependent phosphorylation of PDK1 regulates its nuclear translocation (Scheid et al., 2005). Further research revealed that insulin-like growth factor (IGF)-I produces a transient elevation of intranuclear DAG levels and an increase in the content of PKC in the nucleus of Swiss 3T3 cells, intranuclear DAGK being involved in terminating PKC-mediated signaling events (Martelli et al., 2000).

Therefore, based on our findings showing light-dependent changes in nuclear DAGK and in p-PKC, we explored if these light-dependent changes are produced in relation to IR activation by light. In view of this, the aim of the present work was to explore the effects of light on insulin-related signaling pathways, such as mitogen-activated protein kinase (MAPK) and PI3K pathways, as well as the effects of insulin on DAGK activity and phosphoinositide phosphorylation in nuclei from photoreceptor cells. To this end, the presence of IR and pIR in photoreceptor nuclei was studied and their content in nuclei from retinas exposed to light or darkness was compared.

This work provides the first lines of evidence that confirm the presence of components of signaling pathways linked to G-protein coupled receptors and TRKs, such as Akt and ERK1/2, in photoreceptor nuclei and their regulation by light. Both Akt and ERK1/2 are activated in photoreceptor nuclei from retinas exposed to light with respect to those kept in darkness. Findings from the present study confirm the presence of IR in photoreceptor nuclei and show that its content is modulated by light and by the exposure of bovine retinas to insulin. In addition, our results demonstrate that light modulates IR activation. They also reveal that *in situ* insulin treatment induces a significant increase in DAGK and in phosphoinositide kinase activities in photoreceptor nuclei from retinas exposed to light.

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). Mouse monoclonal anti-LAP-2β (#611000) from BD Biosciences (San Jose, CA, USA) was generously supplied by Dr. Ana Ves Losada (Instituto de Investigaciones Bioquímicas de La Plata, Argentina). Rabbit polyclonal antibodies anti-p-Akt (#9275), anti-Akt (#9272), anti-p-ERK1/2 (#9101), anti-ERK1/2 (#9102), and rabbit monoclonal and anti-insulin receptor β (#3025) were purchased from Cell Signalling (Beverly, MA, USA). Rabbit polyclonal anti-p-insulin receptor β (#44806G) was purchased from Life Technology. Anti-p-p38 (sc-101759) and polyclonal horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG and polyclonal HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 546 (goat anti-rabbit IgG) A11035, and TO-PRO®-3 Stain, from Life Technologies Corporation, were generously provided by Dr. Luis E. Politi (Instituto de Investigaciones Bioquímicas de Bahía Blanca, Argentina).

Supplies, such as U73122 and LY294002 from Sigma Aldrich Corporation, USA, were generously provided by Dr. N. Bazán (Neuroscience Center of Excellence at Louisiana State University Health Sciences Center, New Orleans, USA). Latrunculin was purchased from Molecular Probes Inc. (Eugene, OR). Radioactive substrates [γ-^{32}P]ATP (10 Ci/mmol) and Preblended Dry Fluor 2a70 for scintillation cocktail were obtained from Research Products International Corp, USA. Insulin and all the other chemicals used in the present research were purchased from Sigma Aldrich (St. Luis, MO, USA).

2.2. Light–darkness protocol applied to bovine retinas and isolated nuclear fractions. Isolation of retinal nuclear fraction (RNF) and photoreceptor nuclear fraction (PNF)

In order to analyze the effect of light on a photoreceptor cell nuclear population, bovine eyes were treated as previously described (Natalini et al., 2014). Briefly, after a 2.5 h darkness adaptation period, the cornea, lens and aqueous humor were removed under dim red light. Eyecups were placed on ice and filled with oxygenated (95% O_2, 5% CO_2) Ames medium (2 mg/ml 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). Then, eyecups were either kept in darkness or exposed to light (288 cd/m^2) for 30 min. For nuclear fraction isolation retinas were dissected from the eyes after darkness or light exposure and homogenized (30% weight/vol) with 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 protease inhibitors). To isolate a retinal nuclear fraction (RNF) the homogenate was filtered and two volumes of 2.3 M sucrose in TKM were added to reach a sucrose concentration of 1.6 M. The homogenate in 1.6 M sucrose solution (8.4 ml) was placed onto a 2.3 M sucrose layer (2.7 ml) and centrifuged at 130,000 *g* for 70 min using a Beckman SW41 rotor in a Beckman Optima LK-90 ultracentrifuge. RNF was obtained in the pellet of the gradient (Natalini et al., 2014).

In order to purify a photoreceptor nuclear fraction (PNF) retinal homogenate (8.4 ml, 1.6 M sucrose) was placed onto a 2.2 M (1.4 ml) sucrose and 2.4 M (1.4 ml) sucrose TKM gradient and centrifuged at 106,000 *g* for 70 min using a Beckman SW41 rotor in a Beckman Optima LK-90 ultracentrifuge and PNF was obtained in the pellet of the gradient (Natalini et al., 2014). Both nuclear fractions were briefly washed with TKM buffer in order to eliminate the high density sucrose solution and ice-cold buffer (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA and 1 mM DTT) was placed in contact with the pellet for 30 min before resuspension. The