Light Activation of the Phosphoinositide Cycle in Intrinsically Photosensitive Chicken Retinal Ganglion Cells

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Purpose. In vertebrates, intrinsically photosensitive retinal ganglion cells (ipRGCs) acting as nonvisual photoreceptors transmit environmental illumination information to the brain, regulating diverse non–image-forming tasks. The phototransduction cascade in chicken ipRGCs has been shown to resemble that of rhabdomeric photoreceptors and involves phospholipase C (PLC) activation. The current work was an investigation of the participation of the phosphoinositide (PIP) cycle in this mechanism and of whether changes in activities of inositol 1,4,5-trisphosphate (IP_3) and PIP kinase are triggered by light.

Methods. Primary cultures of Thy-1 immunopurified chicken embryonic RGCs were exposed to bright light pulses or kept in the dark, to assess intracellular Ca^{2+} mobilization by Fluo-3 AM fluorescence microscopy, IP_3 levels, and enzymatic activities of diacylglycerol, phosphatidylinositol, and phosphatidylinositol phosphate kinases (DAGK, PIK, and PIPK, respectively), by radioactive assays. The presence of different melanopsins (Opn4m and Opn4x) and other photopigments was determined by RT-PCR and immunochemistry.

Results. Cultured RGCs expressing different nonvisual photopigments displayed a significant and rapid increase in IP_3 levels (1.3-fold) and Ca^{2+} mobilization by light, which was reversed by administration of the PLC inhibitor U73122 (5 μM). Brief light pulses also caused a very rapid and transient activation of DAGK, PIK, and PIPK compared with that in the dark control.

Conclusions. The results indicate for the first time that light stimulation of chicken RGC cultures activates the PIP cycle, causing an increase in intracellular levels of IP_3, changes in levels of phosphatidic acid, PIP, and PIP_2; and mobilization of Ca^{2+}. (*Invest Ophthalmol Vis Sci.* 2010;51:5491–5498) DOI:10.1167/iovs.10-5643

In vertebrates, intrinsically photosensitive retinal ganglion cells (ipRGCs)[1,2] are responsible for transducing information about ambient lighting conditions to brain areas involved in non–image-forming tasks (e.g., entrainment of the circadian clock and pupillary light reflexes).[3–6] These ipRGCs may have evolved from a common ancestor with invertebrate rhabdomeric photoreceptors and coexist with ciliary photoreceptors (rods and cones) in the vertebrate retina.[7–15] Melanopsin (Opn4), the major photopigment present in ipRGCs of different vertebrates,[7] has been implicated in the photosensitivity of ipRGCs[2,4,11–13,15,16]; this photopigment is closely related to the invertebrate Gq-coupled visual pigment. Two quite separate Opn4 genes have evolved in vertebrates: *Opn4m* and *Opn4x*, which are mammalian and nonmammalian vertebrate orthologs, respectively.[17–20] In the chicken, the expression of Opn4 genes has been reported in diverse retinal cell layers, including RGCs of developing and mature retinas of wild-type animals and the inner retina of blind birds.[6,17–23] These two genes encode at least five isoforms,[17–20] two of which show blue light sensitivity.[18] Moreover, other putative nonopsin photopigments, or cryptochromes (Crys), have been reported to be expressed in the chicken retina.[15,24–27]

Results of electrophysiological and pharmacologic studies with ipRGCs and melanopsin-expressing cultured cells suggest that the biochemical events of phototransduction involve a canonical phosphoinositide (PIP) cascade similar to that of the invertebrate Gq-coupled visual pigment.[9,12,13,15,28] After light stimulation, the photocascade in *Drosophila* photoreceptors involves the activation of a phospholipase Cβ (PLCβ), causing the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG),[29,30] a process that ultimately activates at least two classes of the large TRP ion channel superfamily.[29,31,32] In ipRGCs, light activates signaling through Gq/11-class G proteins involving PLC activity, elevation of cytoplasmic Ca^{2+} levels, and induction of membrane depolarization.[9,10,12,13,15,16,33,34] In earlier work, we have shown the presence of the Gq mRNA in chicken RGC cultures and that treatment with different PLC inhibitors abolishes the light-suppressive effect on ^{3}H-melatonin synthesis.[15] Subsequently, in whole-cell recordings of dissociated mouse retinal cell cultures, specific blockers of PLC and Gq/11 class G proteins abolish the light responses.[34] Nevertheless, since the complete phototransduction mechanism taking place in these cells is still not known, it remains unclear whether $PI(4,5)P_2$ and DAG are associated with the light responses of ipRGCs. Moreover, there is no direct report in the literature on the activation of PIP enzyme activities in any vertebrate cell in response to light, whereas work in *Drosophila* rhabdomeric phototransduction supports PIP involvement by using mutants

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of the PIP cycle enzymes.[35,36] In the current work, we evaluated light-induced activation of PLC activity, of the PIP cycle enzymes, and of changes in intracellular Ca^{2+} responses in chicken RGCs. We used immunopurified RGC cultures from embryonic day (E)8 chickens exposed to light or maintained in the dark and evaluated the formation of the PLC activity product IP_3; the mobilization of intracellular Ca^{2+}; and the enzyme activities of the PIP cycle, such as DAG kinase (DAGK), phosphatidylinositol kinase (PIK), and phosphatidylinositol phosphate kinase (PIPK).

Materials and Methods

All reagents were analytical grade. α-Tubulin (α-Tub) was detected by the mouse monoclonal DM1A antibody (Sigma-Aldrich, St. Louis, MO). The secondary antibodies were AlexaFluor 488 goat anti-rabbit, AlexaFluor 488 goat anti-rat IgG, and AlexaFluor 546 goat anti-mouse IgG (Invitrogen-Molecular Probes, Eugene, OR). Aqueous mounting medium (FluorSave) was from Calbiochem (San Diego, CA). Propidium iodide, protease inhibitor, PLC inhibitor, and other biochemical reagents were from Sigma-Aldrich;-pluronic acid F-127 and Fluo-3 AM were from Invitrogen-Molecular Probes; and serum free supplement (B-27 Supplement 50X) was from Invitrogen-Gibco (Grand Island, NY). The primary antibody against the chicken Opn4x was raised in rabbit with the specific melanopsin x peptide 1 RQKRDLLPDSYSCSEE,[23] the kind gift of Martin Zatz and Arjun Natesan (NIH, Bethesda, MD) and of Ignacio Provencio and Ana M. Castrucci (University of Virginia Charlottesville, VA); the anti-chicken Opn4m was raised in rat and generated with the specific Opn4m peptide: CKHGNRELQKQYHR (Bio-Synthesis Inc., Lewisville, TX). This antiserum recognizes only the chicken Opn4m and was tested by immunochemistry with positive immunostaining in chicken RGCs of the developing and mature retinas and in primary RGC cultures. No staining was visualized in control cells (CHO cells and primary cultures of rat hippocampal neurons) (Verra DM, unpublished data, 2010).

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local animal care committee (School of Chemistry, Universidad Nacional de Córdoba-Córdoba, Exp. 15-99-39796).

Chicken Thy-1, Antisera Preparation, and Purification

Preparation of anti-chicken Thy-1 sera was performed by Bio-Synthesis, Inc., with the NH2-KNITVIKDKLEKC-OH peptide sequence conjugated with KLH to immunize two rabbits. After 6 weeks, serum was obtained from the rabbits and tested by ELISA. A total of 100 to 150 mL of crude serum with five boosts and four bleeds was purified by affinity column purification.

Primary Cultures of Embryonic RGCs

RGCs were purified from E8 neural retinas dissected in ice-cold Ca^{2+}-Mg^{2+}-free Tyrode's buffer containing 25 mM glucose, as described elsewhere,[37] with modifications.[38] Briefly, after trypsin dissociation, the cell suspension from 30 to 60 retinas was poured into Petri dishes pretreated with 2.5 μg/mL protein A, followed by incubation at 37°C for 30 minutes with an anti-chicken Thy-1 polyclonal antibody (pAb). After the cells were washed exhaustively, identical aliquots of the remaining bound RGCs were harvested in Dulbecco's modified Eagle's medium (DMEM) containing serum free supplement (B27; dilution: 1:500 vol/vol; Invitrogen-Gibco) and seeded in Petri dishes previously treated with 10 μg/mL polylysine and 5 μg /mL laminin; some of with 12-mm glass coverslips. The RGC cultures were incubated at 37°C under constant 5% CO_2-air flow in a humid atmosphere. All control cultures were maintained in complete darkness, with no ambient light exposure at any time.

Immunochemistry

Tissue was treated according to Bobu et al.[39] Eyes were fixed overnight in 4% paraformaldehyde at 4°C, transferred to an ascending series of sucrose solutions (10%, 20%, and 30%, each for 2 hours), and embedded (Tissue-Tek; Sakura Fintek, Tokyo, Japan); 12-μm-thick cryostat sections were prepared and stored at −20°C until ready for use. The sections were permeabilized with Triton X-100 (0.1% in PBS for 5 minutes) and then saturated with PBS containing 0.1% BSA, 0.1% Tween-20, and 0.1% sodium azide (buffer D) for 1 hour. Retinal sections and RGC cultures were fixed for 30 minutes (4% paraformaldehyde in phosphate-buffered saline; PBS), washed in PBS, and incubated with monoclonal anti α-tubulin (DM1A dilution 1:1000; Sigma) at 4°C for 24 hours, anti-chicken Opn4x (dilution. 1:2000) at 4°C for 48 hours, or anti-chicken Opn4m (dilution 1:500) at 4°C for 48 hours. They were then rinsed in PBS and incubated with AlexaFluor 488 goat anti-rabbit, AlexaFluor 488 goat anti-rat IgG, or AlexaFluor 546 goat anti-mouse IgG (dilution 1:1000) for 1 hour at room temperature. In some experiments, the samples were incubated with propidium iodide (0.05 mg/mL) and visualized by confocal microscopy (FV1000; Olympus, Lake Success, NY).

IP_3 Assessment

RGC cultures were metabolically labeled with 2 $\mu Ci \cdot mL^{-1}$ of myo-[2-^{3}H(N)] inositol (PerkinElmer-Life and Analytical Sciences, Waltham, MA) during 48 hours. The cells were then stimulated with cool, white fluorescent light (1200 lux) during different times, according to conditions used to depolarize the ipRGCs in mammals,[16] in the presence of 10 mM LiCl. The lipids were recovered by TCA extraction methods[40–42] and the IPs were recovered from the protein/membrane pellets.[43] The IPs were then separated on resin columns (Dowex AG1-X8; Bio-Rad Laboratories, Hercules, CA) and eluted with increasing concentrations of ammonium formate and formic acid, according to published methods.[40,41] The IP content was determined in a scintillation counter.

PIP Labeling of RCG Cultures

After experimental treatment, PIP kinase activities (PIK and PIPK) were assayed by adding 100 μL of 2× assay buffer (500 μM ATP with 4 μCi [γ-^{32}P]ATP, 10 mM $MgCl_2$, and 20 mM HEPES buffer [pH 7.5]) to 100 μL of cellular lysates (200 μg protein). The reaction was performed for 5 minutes at 37°C and stopped by the addition of 5 mL of chloroform/methanol (2:1, vol/vol). Blanks were prepared identically, with membranes boiled for 5 minutes before use. Lipid extraction and separation were performed as published,[44] with slight modification. The lipids were separated by thin-layer chromatography (TLC) on oxalate-coated plates (silica gel 60), with a solvent of chloroform/methanol/acetone/glacial acetic acid/water (40:13:15:12:7.5, vol/vol). After development, the lipids were visualized with iodine vapor. The spots were scraped from the plate and counted for radioactivity by liquid scintillation counting. PIPK products were identified on the basis of their relative mobility on TLC plates versus commercial standards.

DAGK Activity in RGC Lysates

DAGK activity was also determined in the RGC lysates by measuring radioactive phosphate incorporation into phosphatidic acid (PA) using [^{32}P]ATP and endogenous DAG as substrates. The standard assay contained 50 mM HEPES (pH 7.4), 20 mM NaF, 10 mM $MgCl_2$, 1 mM DTT, and 500 μM ATP in a volume of 200 μL. When a micelle-based assay system was used, sodium deoxycholate (1 mM), Triton X-100 (15 mM), or octyl-β-glucopyranoside (50 mM) was used as a detergent. The reactions were performed at 37°C for 5 minutes and stopped by adding chloroform/methanol/1 N HCl (2:1:0.2 by volume), after which the lipids were extracted.[45] Lipid extracts were washed five times with theoretical upper phase to eliminate [^{32}P]ATP. PA was separated by TLC on 1% potassium oxalate in silica gel H and developed with

chloroform/acetone/methanol/acetic acid/ water (40:15:13:12:7.5, by vol). Lipids visualized by exposure of the chromatograms to iodine vapors were scraped into vial for counting by liquid scintillation.

Calcium Imaging by Fluo-3 AM Fluorescence Microscopy

Cells were grown in an eight-well recording chamber (Laboratory-Tek; Nunc, Rochester, NY) to 40% to 50% confluence and then incubated in colorless DMEM (Invitrogen-Gibco) containing 0.1% of pluronic acid F-127 and 5 μM Fluo-3 AM (Invitrogen-Molecular Probes), a Ca^{2+} indicator dye, for 30 minutes at 25°C. The fluorescence imaging technique was performed as published,[33,46] with modifications by exciting the Fluo-3 AM at 515 nm (25.8 μW laser intensity) with a laser coupled to a confocal microscope (FluoView-300; Olympus). The emitted fluorescence was captured every 2 seconds, with an oil-immersion objective (PlanApo N 60×Uplan SApo, NA 1.42; Olympus). The 12-bit, 4×4 binned fluorescence images for each micrograph were used to quantify the fluorescence levels in the cells (MetaMorph, ver. 4.5; Universal Imaging, Downingtown, PA). The mean fluorescence intensity in each cell was background-corrected by subtracting the mean fluorescence of an area with no cells. The mean intensity over a particular area of cells in a selected field was measured in each captured image series. Changes in fluorescence levels were quantified as the ratio between each relative intensity level measured after a light stimulus of 1200 lux (F) and the mean of intensities of 50 serial micrographs before stimulation (F_0). Values of F/F_0 are not linearly related to changes in $[Ca^{2+}]_i$ but are intended to provide a qualitative indication of variations in $[Ca^{2+}]_i$. No significant vehicle effects or changes in focus were detected. Responses were considered significant when the ratio at the peak differed from the baseline levels by at least 20%.

RNA Isolation and cDNA Synthesis. Total RNA was extracted from RGC cultures according to a published method[47] (TRIzol; Invitrogen) and treated with DNase (Promega, Madison, WI). cDNA was synthesized with M-MLV (Promega) by using oligo(dT). Each RT-PCR quantitation experiment was performed in triplicate in three independent experiments.

PCR Primers. The following *Gallus gallus* sequences were used: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; accession K01458), *Opn4x* (accession AY036061), *Opn4m* (accession NM_204625), *Gallus gallus* red cone-opsin (accession NM_205440), cryptochrome 1 (*Cry1*; accession NM_204245), and cryptochrome 2 (*Cry2*; accession NM_204244). Oligonucleotides were designed by using a program that generates 200- to 600-base-pair products with minimal hairpin or loop formation (NTI Advance 10; Vector Laboratories, Burlingame, CA). The oligonucleotide sequences used for RT-PCR were as follows: *GAPDH* forward: 5′-AGGCGAGATGGTGAAAGTCG-3′, reverse: 5′-TCTGCCCATTTGATGTTGCT-3′; *Cry1* forward: 5′-AGAGAGTGTCCAGAAGGCTGCAAA-3′, reverse: 5′-ACTGTTGCAAGAAGACCCAGTCCT-3′; *Cry2* forward: 5′-GCCAAGTGCATCATTGGAGTGG-3′, reverse: 5′-CTTCAGTGCACAGCTCTTCTGCTC-3′; *Opn4m* forward: 5′-TCTCGCCGTAGAACATCC-3′, reverse: 5′-GAAGTGTTTCAGAGCAAGGTAGGA-3′; *Opn4x* forward: 5′-TGTAGAGCTTGACACTGTAGAACCA-3′, reverse: 5′-TGACCGCCCTAGCACCTT-3′; and red cone opsin forward: 5′-AAGGCCAAGACTTCTACGTGC3′, reverse: 5′-CGAGATCTGGTTGATGACGCT-3′.

Statistics

Statistics involved one- or two-way analysis of variance (ANOVA) with the Duncan post hoc test or Student's *t*-test, as appropriate (significance at $P < 0.05$).

Results

Characterization of Primary Cultures of RGCs

To study the phototransduction mechanisms taking place in ipRGCs, we used primary cultures of immunopurified chicken RGCs at E8, which have been described as intrinsically photosensitive.[15] These highly pure cultures of RGCs allow the direct study of light responses in the absence of cone and rod photoreceptors and show the early expression of different potential photopigment mRNAs such as *Opn4x*, *Opn4m*, *Cry1*, and *Cry2*, but not of the red cone opsin (Fig. 1A). Figure 1B showing immunopurified cells by Thy1-antibody immunopanning,[15,37,38] indicates that a percentage (≥20%) of total cells stained with α-tubulin (Fig. 1Bb) or propidium iodide (Fig. 1Be) at E8 and maintained for 3 to 5 days in the cultures, expressed both melanopsin proteins: Opn4x (Fig. 1Ba; 11% ± 2% of total cells, $n = 1557$ cells) and the Opn4m (Fig. 1Bd; 22% ± 2% of total cells, $n = 2500$ cells). Moreover, a developing retinal section from a chick embryo at the same stage (E8) clearly showed positive immunoreactivity for both the Opn4x and Opn4m proteins, which are mainly localized in the forming layer of RGCs (Figs. 1Bc, 1Bf).

Based on the photopigment complexity observed, we further investigated the biochemical events occurring downstream of the photon capture, in immunopurified RGC cultures.

Changes in Intracellular Ca^{2+} Levels of RGCs after Light Exposure

Our previous observations showed that the inhibitory effect of light on melatonin synthesis in chicken ipRGCs was reversed by the administration of the Ca^{2+} chelator BAPTA-AM (10 mM) or lanthanum (La^{3+}; 1 mM), a known TRPC/TRPL-channel blocker.[15] To further confirm the direct participation of Ca^{2+} in the photic responses of chicken ipRGCs, we measured Ca^{2+} levels by using Fluo-3 AM confocal microscopy of individual cultured RGCs, before and after 30 seconds of bright light stimulation (Fig. 2, arrows). The results indicated that ~20% of the cells in each culture displayed a significant increase in somatic Ca^{2+} levels after light stimulation (black line) with differential responses as illustrated for individual cells (Figs. 2A, 2B, 2D, white arrowheads), whereas more than 75% of the cells did not respond to the photic input (Figs. 2A, 2B, gray line; 2D, white arrows).

Most striking, we observed different types of photic responses by Ca^{2+} imaging in the cell population tested (Fig. 2C), which can be classified as sustained (Fig. 2A, black line) and transient (Fig. 2B, black line) responses. Sustained responses were at an amplitude of ~50% with respect to basal levels and a time to peak of ~250 seconds. By contrast, transient responses exhibit an amplitude of ~30% with a time to peak of ≤30 seconds. Moreover, when the PLC inhibitor U73122 (5 μM) was applied to the cultures before light stimulation, no Ca^{2+} variations were seen in any of the tested cells (data not shown).

IP_3 Production by Light

Cultures previously incubated with myo-^{3}H inositol for 48 hours were light-stimulated during 90 seconds, to further investigate PLC activity directly in RGCs. IP_3 production was then evaluated as a major product of PLC activity in bright light and in control cells maintained in the dark. We found a significant increase (30%) in levels of labeled IP_3 in cultures treated with 10 mM LiCl, the well-known inositol monophosphate phosphatase (IPP) inhibitor, and exposed to light compared with the control cells kept in the dark (Fig. 3A; $P < 0.04$). Moreover, when we assessed the effect of the specific PLC inhibitor (5 μM) on the cultures, we found no increase in levels of radiolabeled IP_3 after light stimulation, compared with the dark condition or vehicle-treated cultures exposed to light (Fig. 3A). The rapid activation of PLC by light caused a fast hydrolysis of $PI(4,5)P_2$ and other PIPs, together with the concomi-

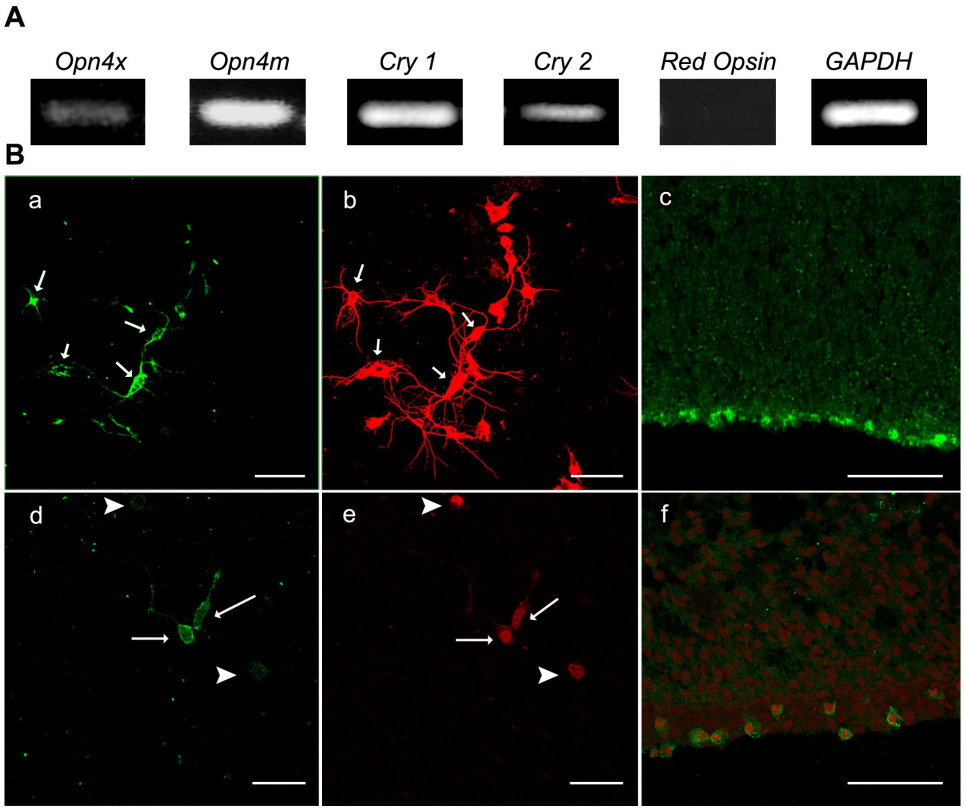


FIGURE 1. Expression of different nonvisual photopigments in immunopurified RGC cultures and retinal sections from E8 chicks. (**A**) RT-PCR of RGC cultures showed detectable mRNA levels of *Opn4x*, *Opn4m*, *Cry1*, and *Cry2* but not of red cone opsin. (**B**) Primary cultures of embryonic RGCs maintained for 3 to 5 days were immunolabeled for α-tubulin (**Bb**) or propidium iodide (**Be**) and Opn4x-like (**Ba**) or Opn4m-like (**Bd**) proteins with specific primary antibodies and visualized by confocal microscopy. *Arrows*: Opn4 (+) cells located both in a few cells in the primary cultures of RGCs (**Ba**, **Bd**) and the RGC layer (**Bc**, **Bf**); *arrowheads*: cells nonimmunoreactive for Opn4m (**Bd**, **Be**). Chick embryonic retinal sections dissected at E8 were immunolabeled for Opn4x-like (**Bc**) or Opn4m-like (**Bf**) protein and propidium iodide and visualized by confocal microscopy with positive immunostaining located in the GCL of the developing retina. Scale bar: (**Ba**, **Bb**, **Bd**, **Be**) 10 μm; (**Bc**, **Bf**) 40 μm.

tant and transient formation of IP_3 and other inositol phosphates. A complication in monitoring changes in PIPs alone is the cells' ability to resynthesize phosphatidylinositol (PI) rapidly, and therefore also PIP and $PI(4,5)P_2$. For this reason, we inhibited IPP with LiCl (10 mM), thus allowing the accumulation of IP_3 products over a longer stimulation time.[48] To see

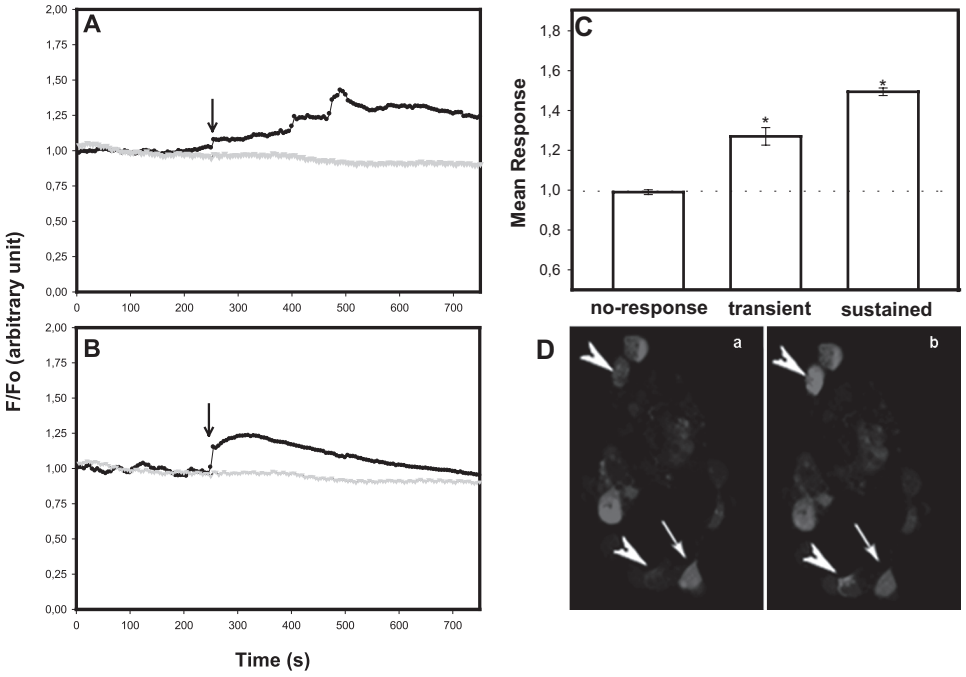


FIGURE 2. Light effect on $[Ca^{2+}]_i$ levels in RGC cultures. RGC cultures were maintained in constant darkness. On day 5, the cultures were loaded with Fluo-3 AM and stimulated with bright white light for 30 seconds. (**A**, **B**) Graphic representations showing the light's effect on differential Ca^{2+} responses by fluorescence imaging in individual cultured RGCs. (**A**, **B**) Sustained (**A**, *black line*), transient (**B**, *black line*), and no (**A**, **B**, *gray line*) Ca^{2+} responses after light stimulation. Changes in fluorescence levels were quantified as the ratio between each relative intensity level measured after a light stimulus of 1200 lux (F) and the mean of intensities in 50 serial images before stimulation (F_0). Values of F/F_0 were not linearly related to changes in $[Ca^{2+}]_i$ but provided a qualitative indication of variations in $[Ca^{2+}]_i$ (n = 125 cells tested). Sustained and transient responses present an amplitude ($\Delta F/F_0$) of 1.51 ± 0.02 and 1.27 ± 0.04, respectively, over the arbitrary value of 1.00 assigned to nonresponsive cells (n = 11–13 from three independent experiments) and a time to peak of 250 and 30 seconds, respectively. Data are expressed as the mean ± SD. Sustained responses ($P \leq 0.0001$; t-value 9.7) and transient responses ($P \leq 0.001$, t-value 11.6) compared with the responses of the nonphotosensitive cells ($\Delta F/F_0$ = 1.08 ± 0.09) by Student's t-test. (**C**) Mean normalized results of the photic effect on the fluorescence increased over basal levels (no response) in RGC cultures, showing transient and sustained light-evoked responses. Histograms represent the mean ± SE (n = 11–15 for each group). $^*P \leq 0.001$. (**D**) Fluo-3 AM imaging showing the fluorescence before (**Da**) and after (**Db**) light stimulation by confocal microscopy. *Arrowheads*: detectable variations in fluorescence levels; *arrows*: no significant fluorescence changes after photic stimulation. Scale bar, 40 μm.

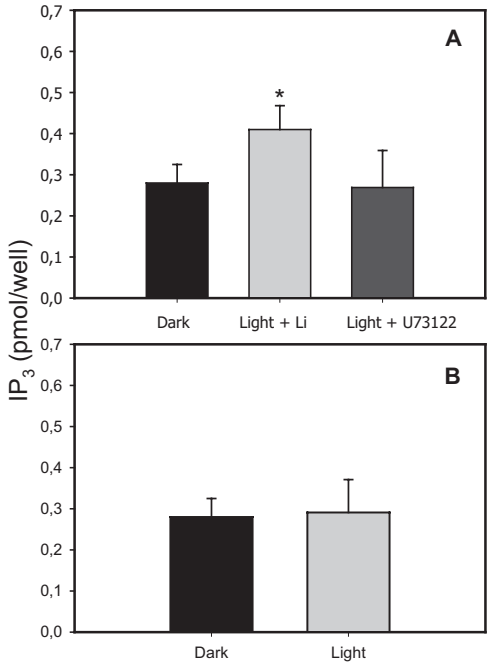


FIGURE 3. Effects of light on formation of ^{3}H-IP$_3$ in RGC cultures. RGC cultures were fed with 2 μCi · mL^{-1} myo-[2-^{3}H(N)]inositol for 48 hours in the dark and assayed for formation of [^{3}H]-inositol phosphates. Data are expressed as the mean ± SEM (n = 3–4/group). The data shown are representative of observations in three independent experiments. (**A**) Effect of light on ^{3}H-IP$_3$ levels in the presence of 10 mM LiCl (Li, *light gray bar*) or 10 mM Li+5 μM U73122, the PLC inhibitor (*dark gray bar*). *$P < 0.04$ compared with the dark control (*black bar*). (**B**) Effect of the light on ^{3}H-IP$_3$ levels in the absence of 10 mM Li inhibitor (*light gray bar*) and in the dark control (*black bar*).

whether IP products produced by light were metabolized after light stimulation, we performed a series of experiments in the absence of LiCl (10 mM). No light/dark differences were observed (Fig. 3B), indicating the rapid cellular metabolism of IPs.

The phototransduction cascade is one of the fastest known G protein-coupled signaling cascades.[29,31] Thus, we speculate that the PLC activity in RGC phototransduction may be a rapid gating mechanism. To evaluate this hypothesis, we assessed PLC activity during the shortest possible stimulation period ranging from 5 to 90 seconds of light exposure by measuring IP$_3$ production after chromatography separation. The results showed higher production of IP$_3$, even after only 5 to 30 seconds of light-induced stimulation, indicating that the 30% increment observed is generated during the first second of stimulation (data not shown).

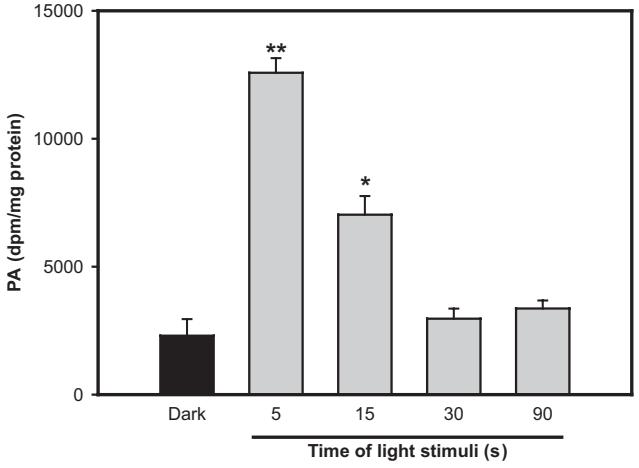


FIGURE 4. Light effect on DAGK activity in RGC cultures. The cultures were maintained in the dark, and, on day 5, the cells were stimulated with bright white light for 5, 15, 30, or 90 seconds, whereas the control cells remained in the dark. The cells were then collected to measure DAGK activity. Data are expressed as the mean ± SEM (n = 3–4/group) from three independent experiments. *$P < 0.05$, **$P < 0.007$ compared with basal levels of activity in the dark control.

DAGK and PIK/PIPK Activities in RGC Cultures

The spatial restriction and steady state levels of specific PIPs are determined primarily by the concerted action of PIPKs and phosphatases, with tightly controlled localizations. PI(4,5)P$_2$ is produced locally at the plasma membrane either by the phosphorylation of PI(4)P (by type I PIPKs) or PI(5)P (by type II PIPKs).[49,50] DAGK is the enzyme that converts DAG to PA, which is used to resynthesize PI(4,5)P$_2$.

We investigated DAGK activity in light-stimulated cultures of chicken RGCs and controls maintained in the dark. Cells were light-stimulated during different times and enzyme activity was determined in RGC homogenates by assessing the

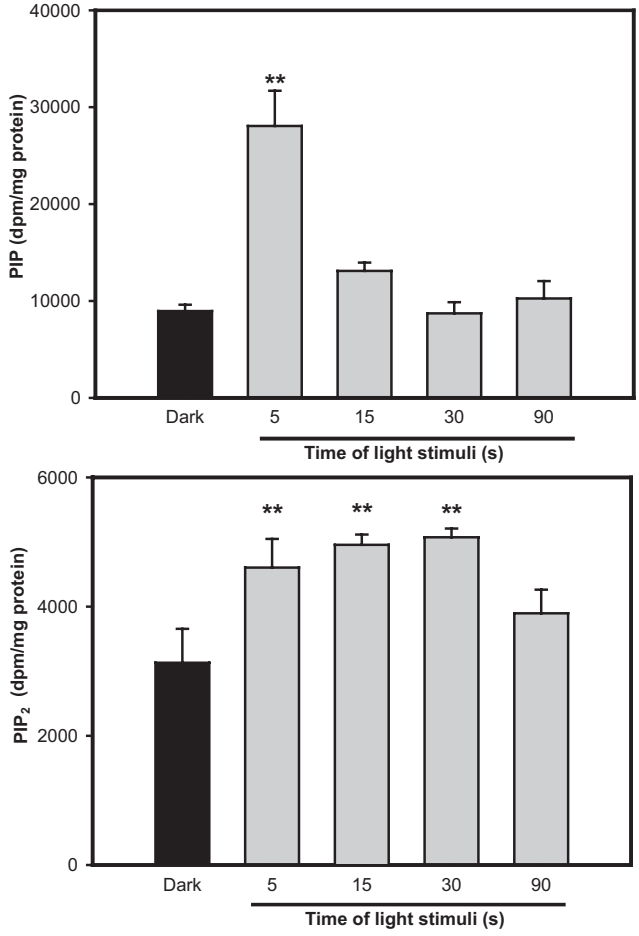


FIGURE 5. Light effect on PI and PIPK activities in RGC cultures. RGC cultures were maintained in the dark and, on day 5, the cells were stimulated with bright white light for 5, 15, 30, or 90 seconds, whereas the control cells remained in the dark. The cells were then collected to assess PIK and PIPK activities. Data are expressed as the mean ± SEM (n = 3–4/group) from three independent experiments. *Top*: light's effect on PIK activity. **$P < 0.007$ compared with the dark control. *Bottom*: light's effect on PIPK activity **$P < 0.007$ compared with the dark control.

incorporation of radioactive phosphate into PA using [^{32}P]ATP and endogenous DAG. As shown in Figure 4, we found a significant increase in [^{32}P]PA formation after only 5 seconds of light stimulation, and the level remained elevated at least up to 15 seconds of light exposure. When DAGK was assayed in RGC cultures (dark and 10 seconds of light) in a micelle-based assay with Na deoxycholate as the detergent, the [^{32}P]PA levels were 138% higher than in the standard assay without detergent, and a similar light-stimulated activity pattern was observed (data not shown). However, when 10 mM Triton X-100 was present, [^{32}P]PA formation was strongly inhibited (data not shown).

To study the effect of light on PI(4,5)P_2 levels, we assayed PI and PIPK activities in chick RGC cultures exposed to light for different lengths of time (5–90 seconds) or maintained in the dark. The kinase activities were determined in the cellular homogenates and evaluated by assessing the incorporation of ^{32}P from [^{32}P]ATP into PIP or PI(4,5)P_2 in the presence of the endogenous substrates.

The results depicted in Figure 5 show a significant increase in [^{32}P]PIP labeling at 5 seconds of light stimulation, indicating that PIK was very rapidly and transiently activated during a brief light pulse. In addition, [^{32}P]PI(4,5)P_2 levels peaked after 5 seconds of light stimulation. The increased [^{32}P]PI(4,5)P_2 levels were maintained during up to 30 seconds of exposure to light, unlike in the dark control.

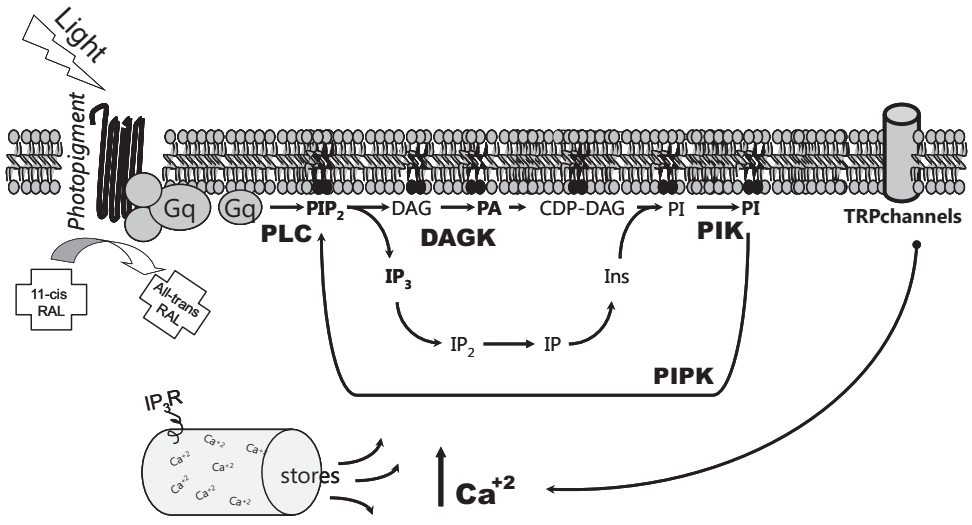
Discussion

In this work, primary cultures of immunopurified chicken RGCs at very early embryonic stages (E8) responded to light through a photoactivated biochemical cascade involving the activation of the enzyme PLC with a concomitant increase in the level of IP_3, activation of the PIP cycle, and mobilization of intracellular Ca^{2+}. This is the first study to directly relate the phototransduction cascade and the PIP cycle in ipRGCs of the vertebrate retina.

In a previous study, we showed that the phototransduction cascade in cultures of chicken RGCs could activate a PLC enzyme effector.[15] These results are in agreement with previous data reported in *Xenopus* melanophores,[9,12] showing a phosphoinositide signaling pathway similar to that in invertebrate phototransduction. The vertebrate ipRGCs form part of a complex retinal structure with different layers and cellular types. We used Thy-1 immunopurified embryonic chicken RGCs[15,37,38] that were free of other retinal cell types and at a very early developmental stage at which only RGCs are postmitotic and mostly mature.[51] As shown in Figure 1, RGCs in primary cultures and in developing retina at the same stage (E8) exhibited expression of the Opn4x and Opn4m photopigments. Furthermore, after 5 days, primary cultures contained ~20% of cells displaying positive immunoreactivity for Opn4 photopigments, as well as the expression of other putative photopigments (Cry1 and Cry2; Fig. 1), indicating that a considerable number of Thy-1-positive cells in RGC cultures may be photosensitive.

One striking characteristic of the photosensitivity in vertebrate ipRGCs is that which is related to changes in intracellular Ca^{2+} levels after light exposure, ultimately causing cell depolarization.[33,52] The chicken RGC cultures showed very rapid and distinct increases in Ca^{2+} in response to a 30-second light stimulus (Fig. 2). Two types of photic responses were observed: One was sustained over at least 8 to 10 minutes after stimulation, and the other was a transient that disappeared or diminished at longer times after stimulation. These distinct Ca^{2+} responses most likely reflect different subsets of ipRGCs, as previously described in mammals.[33,53,54] Most notably, responses were abolished in all cells tested after blockade of PLC activation by the specific inhibitor U73122. This result further supports the idea of a PIP cascade operating in the ipRGCs. In addition, the activation of Ca^{2+}-permeable, light-sensitive channels, such as the TRP and the TRPL channels, may cause the $[Ca^{2+}]_i$ increase since the treatments with a Ca^{2+} chelator (BAPTA) or TRP channel blockers (La^{3+}) were able to reverse the photoresponses observed.[14,15,34] However, IP_3 receptors triggering a Ca^{2+} release from intracellular stores may play a modulatory role, but are apparently not essential for ipRGC phototransduction.[34]

In this work, we directly investigated the light activation of PLC activity in RGC cultures by assessing one of its enzymatic products, ^{3}H-IP_3, after metabolic labeling. Our findings showed a significant increase (30%) in labeled IP_3 in cultures exposed to bright white light pulses of 90 seconds, when compared with control cultures kept in the dark (Fig. 3A). This effect was totally suppressed when the cells were treated with the specific PLC inhibitor U73122 (5 μM) before light stimulation.



Scheme 1. The photocascade operating in ipRGCs of the chicken begins when light activates a photopigment such as Opn4, which uses a vitamin A derivative.[15] Thus, light activation may cause the photoisomerization of 11-*cis* retinal to all-*trans* retinal, which may lead to the activation of a G protein (Gq), which in turn activates PLC. PLC hydrolyzes PI(4,5)P_2 to IP_3 and DAG. The elevation in levels of IP_3 by light is inhibited by U73122 (5 μM), a specific PLC inhibitor, and is visualized after LiCl pretreatment to avoid rapid metabolism. The DAG is rapidly transformed into PA (during the first 5–10 seconds of light stimulation) by activation of DAGK activity. PA is in turn converted into PI(4,5)P_2 through a multistep process that involves the concerted action of CDP-diacylglycerol synthase (CDS), PI synthase (PIS), and the two kinases PIK and PIPK. PIK and PIPK activities increase during the first seconds of stimulation. As a consequence of the light activation, there is also a significant increase in intracellular Ca^{2+} levels, which ultimately contributes cell depolarization, together with the potential activation of TRP-like channels.

It is highly probable that PLC stimulation occurs within milliseconds of exposure to light, just as it does in the known G protein-coupled signaling cascades.[29,31] However, this time scale is not compatible with our experimental design. Furthermore, the light–dark differences observed in levels of radiolabeled IP_3 in ipRGCs can be both underestimated as a consequence of the longer stimulation times examined and diluted by the IP content in nonphotosensitive RGCs in the cultures.

It is known that the stimulation of PLC activity leads to the diminution of $PI(4,5)P_2$ levels in favor of the generation of DAG and IP_3. DAG is converted into PA and PA into $PI(4,5)P_2$, through a multistep process involving the action of DAGK, CDP-DAG synthase (CDS), phosphatidylinositol synthase (PIS), and the two kinases PIK and PIPK. A complication in monitoring changes in PI alone is the ability of the cells to resynthesize PI rapidly and therefore also PIP and $PI(4,5)P_2$.[31,55,56]

We evaluated DAGK, PIK, and PIPK activities in chicken RGC cultures after brief light pulses ranging from 5 to 90 seconds. Our results show a significant activation of DAGK and PIK compared with controls kept in the dark. The quick increase in PA and PIP levels, taking only 5 to 15 seconds to peak, was transient, and the activation disappeared after 10 to 30 seconds of stimulation. In addition, light stimulated PIPK activity after 5 seconds, and PIP_2 levels showed a sustained increase after 30 seconds of stimulation. Overall, our findings strongly suggest that PA and PIP production is tightly regulated during the first seconds of light stimulation, most likely to reestablish the steady state concentration of PIP in the membrane, with a very rapid, transient, and sequential activation of lipid kinases. In addition, we have observed that the DAGK present in RGC cultures as assessed in a micelle-based assay system could be a cytosolic (type I) isoform (data not shown). Type I DAGKs (α, β, and γ) have calcium-binding EF hand motifs and are more active in the presence of this ion.[57–59] A DAGK activity strongly inhibited by Triton X-100 was also reported to be present in bovine rod outer segments (ROS).[60] A DAGK-γ was identified in rat and bovine ROS and a light-dependent association was reported.[61]

Other studies suggest that PLC triggers the opening of the light-gated channels by a membrane-associated mechanism,[34] with the direct interaction between the channels and $PI(4,5)P_2$ maintaining them in a closed state during darkness. In *Drosophila*, light stimulates PLC to hydrolyze $PI(4,5)P_2$, decreasing its concentration and releasing the channels into an open state.[32,36] Nevertheless, in ipRGCs, it is unclear whether IP_3, $PI(4,5)P_2$, or DAG plays a central role in cell depolarization. We can also speculate that replenishment of $PI(4,5)P_2$ levels by the sequential activation of PI and PIPKs regulates the concentration of $PI(4,5)P_2$ to modulate the channel state. These results are in agreement with previous findings in *Drosophila*, where disruption of $PI(4,5)P_2$ regeneration interrupts the phototransduction process.[35,62,63] Thus, mutations that affect $PI(4,5)P_2$ regeneration in vertebrates may disrupt phototransduction in the ipRGCs. Mechanisms that promote the regeneration of PIP_2 are therefore critical for maintaining signaling capacity.

Overall, our observations further support the idea that the phototransduction cascade operating in primary cultures of chicken RGCs and summarized in Scheme 1 involves a PIPs cascade.

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