

# Synthesis of Retinal Ganglion Cell Phospholipids Is Under Control of an Endogenous Circadian Clock: Daily Variations in Phospholipid-Synthesizing Enzyme Activities

E. Garbarino-Pico,<sup>1</sup> A.R. Carpentieri,<sup>1</sup> P.I. Castagnet,<sup>2</sup> S.J. Pasquaré,<sup>2</sup> N.M. Giusto,<sup>2</sup> B.L. Caputto,<sup>1</sup> and M.E. Guido<sup>1\*</sup>

<sup>1</sup>CIQUIBIC (CONICET)-Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

<sup>2</sup>INIBIBB (CONICET), Universidad Nacional del Sur, Bahía Blanca, Argentina

Retinal ganglion cells (RGCs) are major components of the vertebrate circadian system. They send information to the brain, synchronizing the entire organism to the light-dark cycles. We recently reported that chicken RGCs display daily variations in the biosynthesis of glycerophospholipids in constant darkness (DD). It was unclear whether this rhythmicity was driven by this population itself or by other retinal cells. Here we show that RGCs present circadian oscillations in the labeling of [<sup>32</sup>P]phospholipids both in vivo in constant light (LL) and in cultures of immunopurified embryonic cells. In vivo, there was greater [<sup>32</sup>P]orthophosphate incorporation into total phospholipids during the subjective day. Phosphatidylinositol (PI) was the most <sup>32</sup>P-labeled lipid at all times examined, displaying maximal levels during the subjective day and dusk. In addition, a significant daily variation was found in the activity of distinct enzymes of the pathway of phospholipid biosynthesis and degradation, such as lysophospholipid acyltransferases (AT II), phosphatidate phosphohydrolase (PAP), and diacylglycerol lipase (DGL) in cell preparations obtained in DD, exhibiting differential but coordinated temporal profiles. Furthermore, cultures of immunopurified RGCs synchronized by medium exchange displayed a circadian fluctuation in the phospholipid labeling. The results demonstrate that chicken RGCs contain circadian oscillators capable of generating metabolic oscillations in the biosynthesis of phospholipids autonomously. © 2004 Wiley-Liss, Inc.

**Key words:** phospholipid; circadian rhythm; retinal ganglion cells; lysophospholipid acyltransferases; phosphatidate phosphohydrolase; diacylglycerol lipase; retina

The vertebrate circadian system that controls most physiological and behavioral rhythms is the result of a highly complex interaction of specialized photoreceptors and oscillators (Hastings et al., 1991; Lowrey and Takashi, 2000).

The retina is responsible for photoreception of the ambient light-dark cycles that synchronizes the circadian system (Lowrey and Takahashi, 2000; Guido et al., 2002). Retinal neurons are linked in a layered anatomical arrangement comprising three nuclear layers, the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (RGC), and RGCs ultimately convey visual information to the brain (Tessier-Lavigne, 1991). Novel photopigments have been localized to the inner retina, which potentially may participate in the clock mechanism and its entrainment (Miyamoto and Sancar, 1998; Provencio et al., 2000; Berson et al., 2002). The visual system senses contrasts in illumination backgrounds; however, it is known that many adaptive responses are regulated by circadian clocks that induce changes as a time-of-day function, anticipating and predicting daily changes in the photic environment (Cahill and Besharse, 1995). The retina is capable of generating daily rhythms in a variety of physiological and metabolic functions (for review see Guido et al., 2002). Consequently, the retina contains an endogenous clock mechanism that works in the absence of other components of the circadian system

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\*Correspondence to: Dr. Mario E. Guido, CIQUIBIC-Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina.

E-mail: mguido@dqf.fcq.unc.edu.ar

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(Besharse and Iuvone, 1983; Pierce et al., 1993; Cahill and Besharse, 1995; Tosini and Menaker, 1996). The rhythm of melatonin synthesis and release constitutes one of the retinal rhythms most extensively studied in different animal species (Hamm and Menaker, 1980; Faillace et al., 1994; Iuvone et al., 2000). Clock genes are present in the retina and may control the rhythms of melatonin production (Green, 1998; Yoshimura et al., 2001; Hayasaka et al., 2002; Chong et al., 2003).

The metabolism of retinal phospholipids has been deeply examined in various animal species (Bazán and Bazán, 1976; Giusto and Bazán, 1979; Schmidt, 1983; Anderson et al., 1985; Marcheselli and Giusto, 1986; Birkle and Bazán, 1989; Caputto and Guido, 2000, 2002) and has especially been focused on rod outer segments (Rodriguez de Turco et al., 1997; for review see Giusto et al., 1998, 2000). However, not many studies had considered the metabolism of retinal phospholipids from a chronobiological viewpoint. A recent report has shown diurnal changes in the phospholipid synthesis of the rat retina, with enzyme activities decreased in animals subjected to a linolenic acid-deficient diet under a light-dark (LD) cycle (Ikemoto et al., 2000). However, these studies were carried out with the whole retina and under an LD cycle that did not necessarily represent circadian regulation. The biosynthesis of retinal phospholipids is differentially regulated from one type of cell to another (Guido and Caputto 1990; Guido et al., 1996; Bussolino et al., 1998): Particularly in RGCs, it is activated by light (Guido and Caputto 1990; Guido et al., 1996) through a mechanism mediated by *c-fos* expression (Guido et al., 1996; Bussolino et al., 1998; de Arriba-Zerpa et al., 1999; Caputto and Guido, 2000, 2002). More recently, we have observed that the biosynthesis of RGC phospholipids from animals maintained in constant darkness (DD) presents a significant daily variation (Guido et al., 2001). The highest levels of phospholipid labeling were observed at subjective dawn and during the day, whereas the lowest levels of labeling were seen at midnight after 48 hr in DD (Guido et al., 2001). To our knowledge this was the first report describing a daily rhythm in RGCs. Here we further investigated the circadian regulation of the phospholipid metabolism in RGCs, extending the investigation to 1) *in vivo* studies from animals maintained in constant light (LL) for 48 hr; 2) activity assay of two fundamental glycerophospholipid-synthesizing enzymes, the lysophospholipid acyltransferases II (AT II) and the phosphatidate phosphohydrolase (PAP), and of the diacylglycerol lipase (DGL) involved in fatty acid removal; and 3) *ex vivo* studies of phospholipid labeling in purified embryonic RGC cultures to establish the autonomy of these cells in generating daily metabolic oscillations.

## MATERIALS AND METHODS

### Materials

All reagents were of analytical grade. [ $^{32}\text{P}$ ]Na<sub>2</sub>orthophosphate was purchased from Amersham (Arlington Heights, IL) or NEN Life Science Products (Boston, MA). [ $^3\text{H}$ ]glycerol and

[ $^{14}\text{C}$ ]oleoyl-CoA were from NEN Life Science Products. Cobb Hardig chicks were purchased from Avícola Indacor (Córdoba, Argentina). HPTLC silica gel 60-precoated sheets were from Merck (Darmstadt, Germany). The protein assay kit was from Bio-Rad (Hercules, CA). Phospholipid standards were from Sigma (St. Louis, MO).

### Animal Handling

Cobb Hardig chicks were reared from hatching until day 7 on an LD cycle of 12 hr each, with food and water available *ad libitum* and a room temperature of 25°C. Then, animals were released to DD or LL (100 lux, cool white fluorescent light) for 48 hr. For the *in vivo* labeling, on day 10, animals in LL were given an intraocular injection of 10  $\mu\text{l}$  [ $^{32}\text{P}$ ]Na<sub>2</sub>orthophosphate (50  $\mu\text{Ci}$ ) at different zeitgeber times (ZTs) during the projected night or day and killed 1 hr later. For the enzyme activity assays, on day 10, chicks maintained in DD were killed at different ZTs during the subjective day or night. Animal handling in LL was carried out under cool white fluorescent light (100 lux), whereas, in DD, all animal handling was performed in dim red light (<5 lux). Animals were killed by decapitation, and both eyes were dissected out and opened at the iris, and the lens and vitreous humor were removed. The eye cups were rinsed twice in 4 ml of cold 0.25 M sucrose, immediately frozen in liquid N<sub>2</sub>, and lyophilized overnight at -40°C. Times of treatments are designated with respect to the previous entraining LD cycle (or ZT): ZT 0 corresponds to the phase of the previous dark-light transition (subjective dawn), whereas ZT 12 corresponds to the time of the light-dark transition (subjective dusk). All animal handling was performed in agreement with the standards stated in the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care and approved by the local animal care committee (Exp. 15-99-39796).

### Preparation of RGCs

The RGCs preparations were obtained as previously described (Guido and Caputto, 1990; Guido et al., 1999). RGC preparations were resuspended in 0.3 ml water, maintained on ice, and exhaustively sonicated for 30 sec. Aliquots from these preparations were used to quantify the protein content according to the Bio-Rad protein assay, based on the method of Bradford (1976), with bovine serum albumin as a standard.

### Determination of Radioactivity in Phospholipids, Phosphoproteins, and Acid-Soluble Precursors

The labeling of phospholipids in retinal cells was determined according to Guido and Caputto (1990) by the TCA-PTA method. Phospholipids were extracted with chloroform/methanol (2:1 vol/vol) until a single phase was obtained. Pellets were dissolved by resuspension in 10% sodium dodecyl sulfate (SDS) and heating at 60°C for 1 hr. Radioactivity from the acid-soluble fractions (precursors), dried lipidic extracts (phospholipids), and pellets (phosphoproteins) was determined in a liquid scintillation counter. Individual phospholipids were separated by a monodimensional two-solvent system procedure described by Weiss et al. (1982) with chloroform/methanol/acetic acid/water 40:10:10:1 (by volume) in the first development and 120:46:19:3 (by volume) in the second development. Standards and individual lipid species were visualized with io-

dine vapors. Radioactivity incorporated into individual lipids was assessed by autoradiography.

### In Vitro Determination of AT II in RGC Preparations

The activity of ganglion cell AT II was determined by measuring the incorporation of [ $^{14}$ C]oleate from [ $^{14}$ C]oleoyl-CoA (56 mCi/mmol) into different lysophospholipid acceptors as described by Castagnet and Giusto (1997). The reaction was started by the addition of the radioactive substrate or the RGC preparation. Lipids were extracted by the method of Folch et al. (1957), dried under  $N_2$ , resuspended in chloroform/methanol 2:1 (v/v), and spotted on silica gel 60-precoated plates. The chromatograms were developed in a solvent system containing chloroform/methanol/acetic acid/ $H_2O$  50:37.5:3.5:2 (by volume) and visualized with iodine vapors. The spots corresponding to PA, PC, PE, PI, and PS were scraped off, and radioactivity was determined by liquid scintillation.

### Determination of the PAP-1 Activity in Isolated RGC Preparations

PAP-1 activity was determined by monitoring the rate of release of 1,2-diacyl-[2- $^3$ H]glycerol (DAG) from [2- $^3$ H]phosphatidic acid as previously described by Pasquaré and Giusto (1986, 1993). PAP-1 activity was determined in an assay containing 50 mM Tris-maleate buffer, pH 6.5, 1 mM dithiothreitol (DTT), 1 mM EDTA and 1 mM EGTA, 0.2 mM  $Mg^{2+}$ , and 100  $\mu$ g of protein from the RGC preparations in a volume of 0.2 ml. The reaction was started by adding 0.6 mM [2- $^3$ H]phosphatidate plus 0.4 mM phosphatidylcholine. In addition, other incubations were carried out after preincubating the enzyme for 10 min with 4.2 mM NEM. The difference between the two types of activities was labeled as PAP-1. PAP activity was expressed as the sum of labeled monoacylglycerol plus diacylglycerol  $\times$  (hr  $\times$  mg protein) $^{-1}$ .

### Determination of DGL Activity in Isolated RGC Preparations

DGL activity was determined by monitoring the formation rate of monoacylglycerol (MAG), with PAP-generated diacylglycerol as substrate. Standard assays contained approximately 80 nM DAG; pH conditions, protein concentration, time, and final volume of incubation were the same as those described for the PAP assay (Pasquaré and Giusto, 1993).

### Purification and Culture of RGCs From Chicken Embryos

RGCs were purified from neural retinas at embryonic day 8 (E8) and dissected in ice cold  $Ca^{2+}/Mg^{2+}$ -free Tyrode's buffer containing 25 mM glucose according to Brocco and Panzetta (1999). Briefly, after dissociation, the cell suspension from 15–20 retinas was immunopurified by incubation at 37°C with anti-chicken Thy-1 polyclonal antibody raised in our laboratory. The remaining bound RGCs were harvested in Dulbecco's modified Eagle's medium (DMEM) and seeded on coverslips treated with polylysine and 5  $\mu$ g/ml laminin. The coverslips were transferred to Petri dishes containing 2.5 ml B27 (Gibco, Grand Island, NY)-supplemented DMEM and incubated at 37°C under constant 5%  $CO_2$ -air flow in a humid atmosphere. On the following day, cultures were synchronized

by exchange with fresh B27-DMEM medium (time 0). At different times from 0 to 32 hr, cultures were pulsed with 10  $\mu$ l [ $^{32}$ P]Na $_2$ orthophosphate (10  $\mu$ Ci) for 30 min and then rinsed twice with cold 10 mM phosphate-buffered saline and finally resuspended in 1 ml distilled water and frozen until protein quantification and lipid extraction. The immunocharacterization of these cells was carried out with specific markers, such as Thy-1, 200-kDa neurofilament, and GAP-43 (data not shown), and it was evident that the cultures were highly pure for RGCs, as shown by Brocco and Panzetta (1999). With these markers plus gangliotetraosylganglioside and RA4, more than 92% of the cells were mature RGCs.

### Statistical Analysis

Statistical analyses involved one-way analysis of variance (ANOVA), with Newman-Keuls post hoc tests when appropriate.

## RESULTS

### Circadian Variation in the In Vivo Labeling of Phospholipids in RGCs From Chicks Maintained in LL

We examined the in vivo labeling of phospholipids, phosphoproteins, and acid-soluble precursors in RGCs at different times during a 24-hr period in animals entrained to a 12:12 hr LD cycle and released to LL for 48 hr (Fig. 1). Chicks were given an intraocular injection of 10  $\mu$ l [ $^{32}$ P]Na $_2$ orthophosphate and were decapitated 1 hr later under the same illumination conditions at different phases. We found a significant daily variation in the labeling of total [ $^{32}$ P]phospholipids, with the lowest levels of radioactivity incorporation around midnight and the highest levels during subjective day and dusk (Fig. 1A). The statistical analysis revealed a major effect of time on the phospholipid labeling in LL as observed after normalization of results according to levels of radiolabeled precursors ( $P < .000004$  by ANOVA) or protein content ( $P = .045$  by ANOVA). Post hoc comparisons revealed that, for the phospholipid labeling normalized by the content of  $^{32}$ P precursors, levels of labeling at ZTs 20 and 22 during the night were significantly lower than those at ZTs 1, 5, 10, 12, and 14. After normalization by protein, levels of  $^{32}$ P radioactivity incorporated into RGC phospholipids at ZT 22 in the night were significantly different from those at all other times examined. Results normalized by the protein content mainly reflect the daily changes in the pool of precursors (compare Fig. 1A and C; the profile of phospholipid/mg protein follows the curve of labeled precursors). The percentile difference between maximum and minimum values of labeling over time ranges between 77% and 140% after protein and precursor normalizations, respectively.

As shown in Figure 1B, there were no major differences in the labeling of total RGC phosphoproteins over a 24-hr period in LL ( $F = 1.326$ ,  $P = .253$ , normalization by protein; and  $F = 0.963$ ,  $P = .475$ , normalization by precursors). In addition, Figure 1C shows the labeling of the acid-soluble precursors in RGCs at the different phases

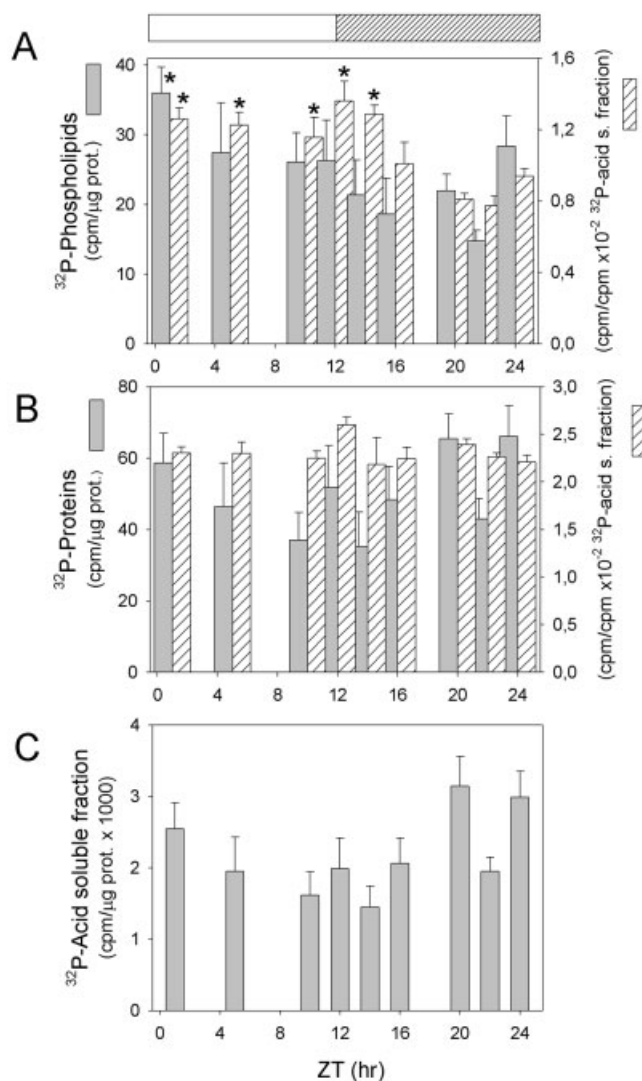


Fig. 1. Daily variation in the in vivo labeling of RGC [ $^{32}\text{P}$ ]phospholipids (A), [ $^{32}\text{P}$ ]phosphoproteins (B), and [ $^{32}\text{P}$ ]acid-soluble precursors (C) from chickens kept in LL for 48 hr. Animals were given an intraocular injection of [ $^{32}\text{P}$ ]orthophosphate at different ZTs and were killed after 1 hr of labeling under the same illumination condition. Data are mean  $\pm$  SEM ( $n = 5\text{--}7/\text{group}$ ) from one independent experiment representative of others with similar observations. In A, results are the ratio of the incorporation of  $^{32}\text{P}$  into phospholipids and the acid-soluble fraction (hatched columns) or the protein content (solid columns). Values with asterisks are significantly greater than basal levels of labeling at ZT 22 ( $*P < .05$ ). In B, no significant differences in the labeling of total [ $^{32}\text{P}$ ]phosphoproteins of RGCs in LL were found across time when expressed with respect to either the protein content (solid columns) or the acid-soluble fraction (hatched columns). In C, the [ $^{32}\text{P}$ ]acid-soluble fraction containing the precursors presents a significant daily variation without significant differences among groups by pairwise comparisons. The bar on top denotes the subjective day (open) and night (hatched) in LL.

in LL. Although the statistical analysis revealed a significant effect of time on the labeling of precursors ( $F = 2.318$ ,  $P = .03$ ), pos hoc comparisons did not show significant differences among groups and phases.

### $^{32}\text{P}$ Labeling of Individual Phospholipids in RGCs

The incorporation of radioactivity into individual RGC phospholipid classes was assessed in LL at several ZTs after 1 hr of precursor administration (Fig. 2). Closely related patterns of labeled phospholipids were observed at the various ZTs studied when the percentile distribution of each was considered (Fig. 2A). PI accounted for the highest percentage of  $^{32}\text{P}$  incorporation (28–38%), followed by PC (15–22%), PIs (15–17%), SPM/LPC (12–17%), PG/PE (5–14%), LPI (5–8%), and traces of PS and PA (<1%). Figure 2B shows the temporal variation in the specific activity for incorporation of  $^{32}\text{P}$  into individual phospholipids. All individual lipid classes assessed display a significant daily variation by ANOVA (PI:  $F = 12.060$ ,  $P < .000001$ ; PC:  $F = 11.373$ ,  $P < .000001$ ; PIs:  $F = 22.057$ ,  $P < .000001$ ; SPM/LPC:  $F = 13.746$ ,  $P < .000001$ ; PG/PE:  $F = 33.942$ ,  $P < .000001$ ). Interestingly, although each phospholipid class presented a differential pattern of variation over time, all of them exhibited the lowest levels of  $^{32}\text{P}$  incorporation around midnight (ZT 20). Whereas PI levels were significantly increased during the subjective day and dusk, PC presented the greatest levels at ZT 12, PIs at ZTs 14 and 24, SPM/LPC during the subjective day and dusk, and PG/PE during the day and at ZT16 in the night.

### Daily Variation in the Activity of Different RGC Phospholipid-Synthesizing Enzymes

We evaluated the possibility that the circadian changes observed in the in vivo labeling of total RGC phospholipids under constant illumination conditions were due to variations in the activity of particular enzymes of the pathways of phospholipid biosynthesis or degradation. For this, we assessed the in vitro activities of AT II, PAP, and DGL in preparations of RGCs from animals kept in DD for 48 hr and killed at different times during a 24-hr period. With this illumination condition, the incorporation of [ $^3\text{H}$ ]glycerol and  $^{32}\text{P}$  into phospholipids displayed a robust circadian oscillation (Guido et al., 2001).

**AT II activity.** The synthesis of glycerophospholipids starts with the subsequent esterification of a glycerol-3-phosphate molecule at the *sn*-1 and at the *sn*-2 positions with the corresponding fatty acids to form 1-acyl-*sn*-glycerol-3-phosphate (LPA) and 1,2-diacylglycerol-3-phosphate (PA), respectively (Vance and Vance, 1996). This last reaction is catalyzed by the AT II.

We found a remarkable daily variation in the activity of AT II from RGCs of animals maintained in DD, for the different lysophospholipids assessed (Fig. 3). Consistently, in most cases examined, activity levels were high during the subjective day and low at early subjective night or midnight. The statistical analysis revealed a significant main effect of time for AT II activity irrespective of the

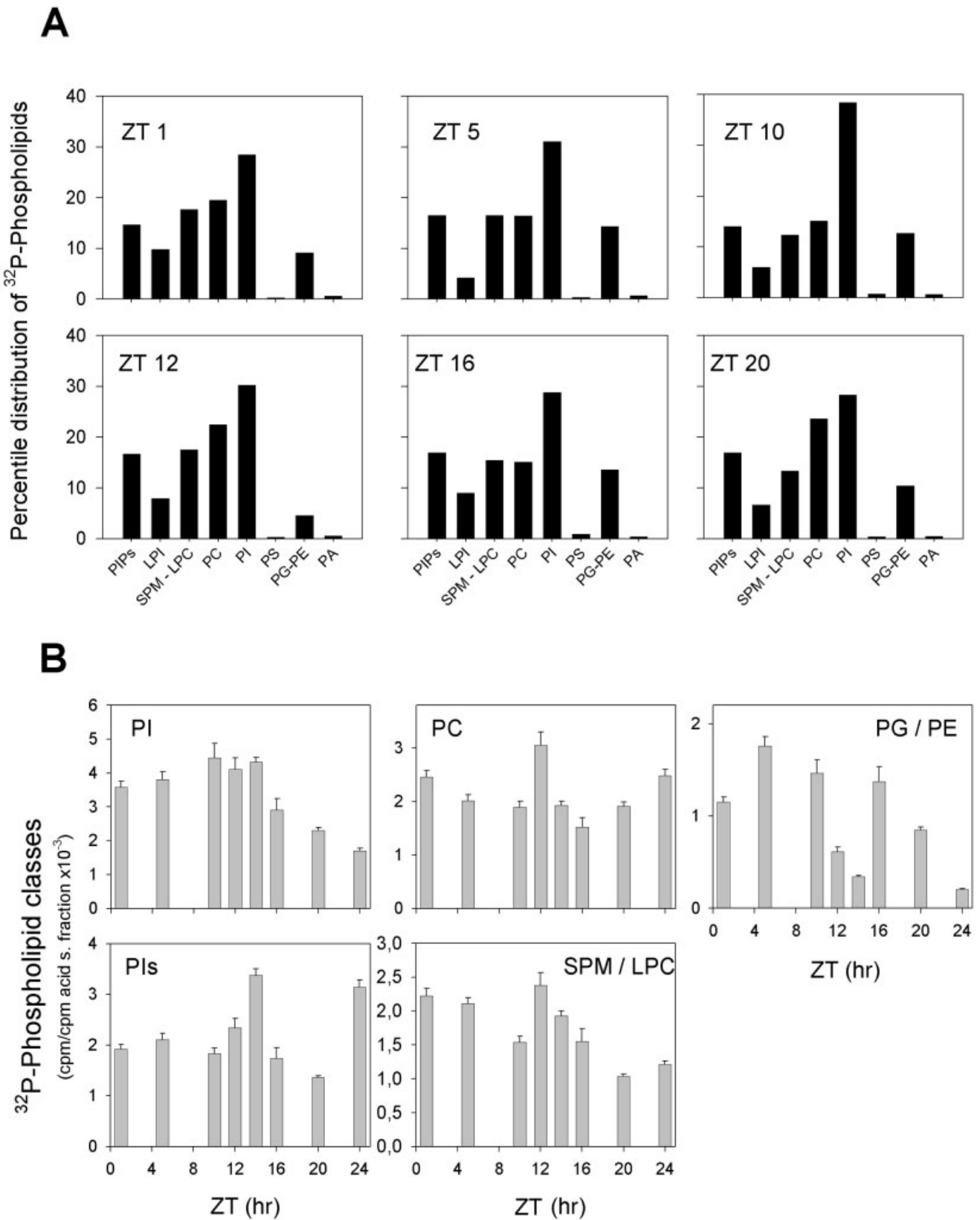


Figure 2.

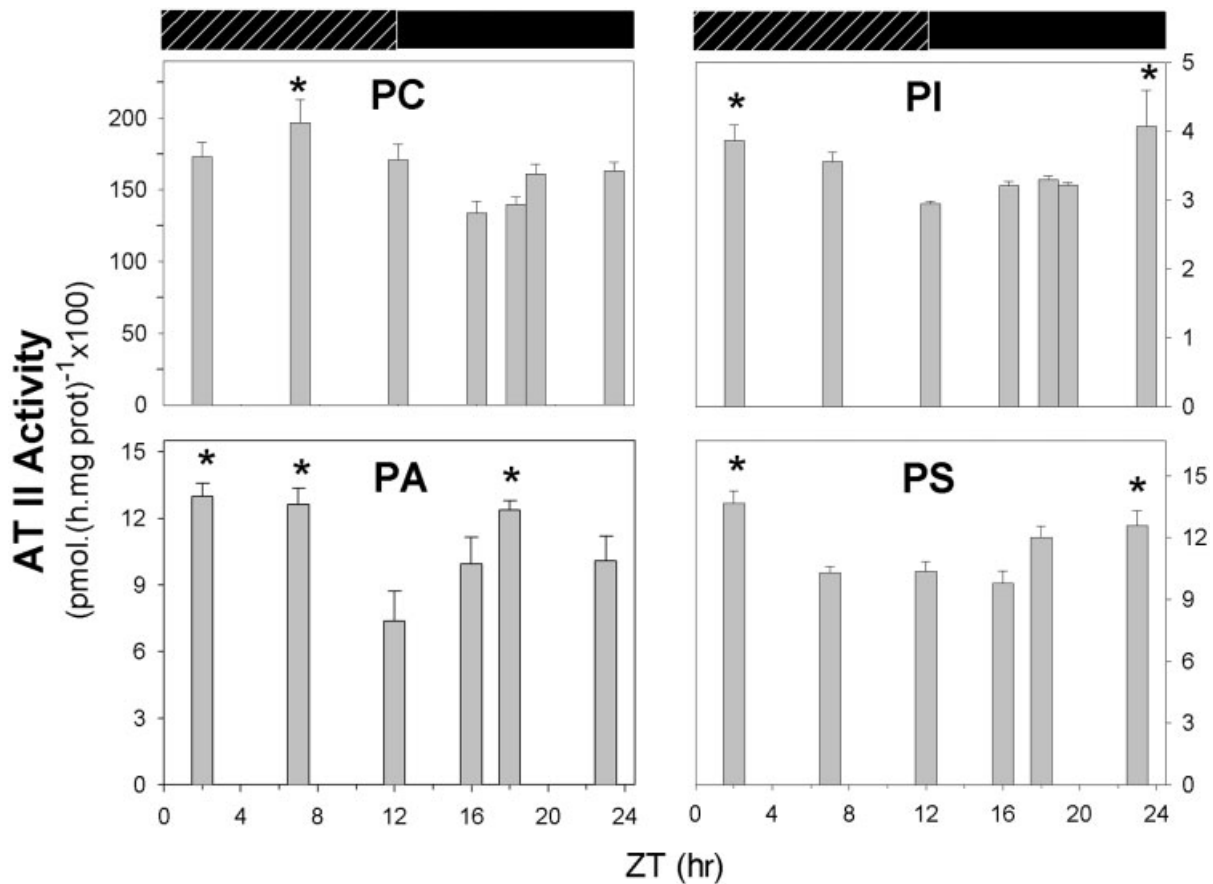


Fig. 3. Daily variation in the AT II activity in chick RGC preparations. AT II activity was determined in RGCs isolated from chicks killed at different times (ZT) in DD, measuring the incorporation of [<sup>14</sup>C]oleate into lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophosphatidylinositol (LPI), or lysophosphatidylserine (LPS). Results are the mean ± SEM (n = 3–9/group). At least six chick RGCs were

pooled for each determination at each time examined. Values with asterisks are significantly greater than basal levels at ZTs 16 for LPC, 12 for LPI, 12 for LPA, and 16 for LPS (\**P* < .05, Newman-Keuls test). The bars at the top represent the subjective day (hatched) and night (solid) in DD.

acceptor utilized (*P* < .008 by ANOVA). The percentile difference between the maximum and the minimum values of activity was close to 50%, ranging from 38% to 76% for the different AT IIs assessed. The highest values of activity were observed during a temporal window in the late night (ZT 22) and the early day (ZTs 1–6), whereas the lowest values of enzyme activity were found during subjective dusk and early night (ZTs 12–16). Post hoc comparisons demonstrated that activity levels for LPA AT

II at ZT 12 differ from those at ZTs 1, 6, and 18. For LPC AT II, activity levels at ZT 16 differ from those at ZTs 1, 6, 19, and 23. For LPI AT II, activity levels at ZT 12 differ from those at ZTs 1 and 22, and, for LPS AT II, activity levels at ZT 1 differ from those at ZTs 6, 12, and 16 and levels at ZT 23 differ from those at ZT 16.

**PAP activity.** After the AT II, the next step in the pathway of conversion of PA into more complex phospholipids, such as PC and PE, is its dephosphorylation catalyzed by PAPs that yield DAG (Brindley and Waggoner, 1998). Although two PAP isozymes have been described, only PAP-1 activity that is involved primarily in lipid synthesis in the endoplasmic reticulum has been found in chicken RGCs (de Arriba-Zerpa et al., 1999; Guido et al., 1999). In DD, PAP-1 activity of RGC preparations exhibited an important daily variation, with the highest levels of DAG production at midday (ZT 6) and low levels at night (ZTs 16–22; Fig. 4). The statistical analysis showed a major effect of time (*P* < .00004 by

Fig. 2. **A:** Percentile distribution of individual [<sup>32</sup>P]phospholipids in RGCs from retinas of chicks kept for 48 hr in LL at different ZTs after 1 hr of labeling. Lipids were extracted and subjected to high-performance TLC as described in the text. Results correspond to one experiment representative of two others, with similar percentile distribution. **B:** Incorporation of [<sup>32</sup>P]orthophosphate into individual phospholipid classes over a 24-hr period from animals maintained in LL after normalization by the content of radioactive precursors. Results are means ± SEM (n = 5–7/group).

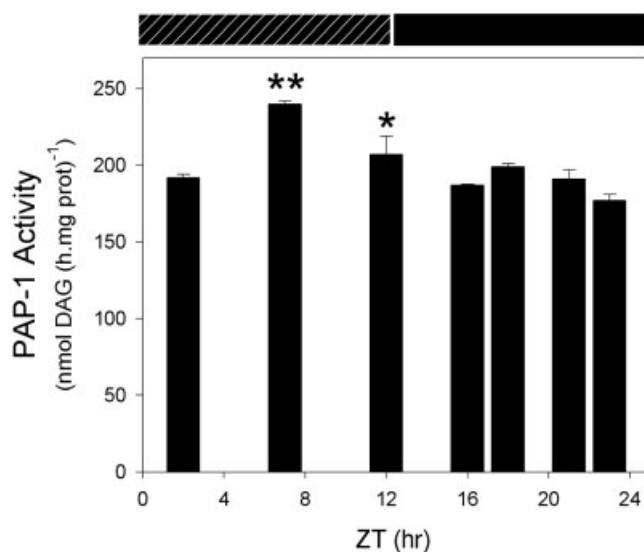


Fig. 4. Daily variation in the PAP-1 activity in isolated chick RGCs. PAP-1 activity was determined in RGCs isolated from animals maintained in DD for 48 hr and killed at different times (ZT). Results are the mean  $\pm$  SEM ( $n = 3-5$ /group). Pools of six chick RGCs were processed for each determination at the times indicated. Values with asterisks are significantly greater than basal levels of enzyme activity at ZTs 16 and 22 (\* $P < .05$ , \*\* $P < .01$ , Newman-Keuls post hoc comparisons). The bar at the top represents the subjective day (hatched) and night (solid) in DD.

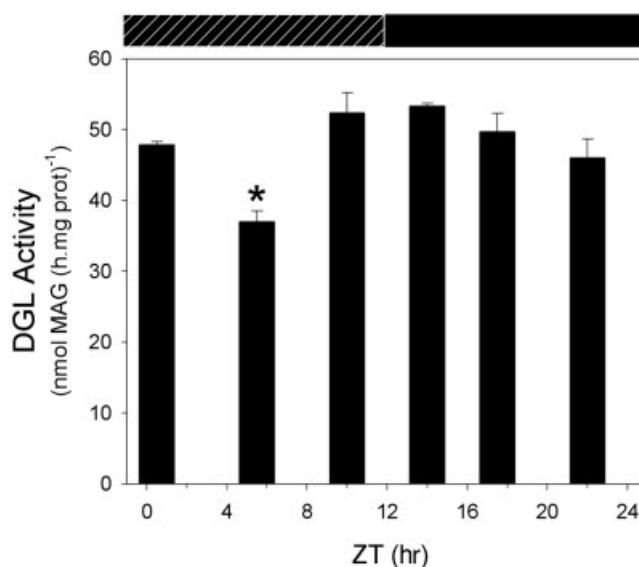


Fig. 5. Daily variation in the DGL activity in isolated chick RGCs from animals maintained in DD for 48 hr and killed at different times (ZT). Results are the mean  $\pm$  SEM ( $n = 3-6$ /group). Pools of six chick RGCs were processed for each determination at the different times examined. Values with asterisks at midday (ZT 6) significantly differ from those at all other times (\* $P < .025$ , Newman-Keuls comparisons). The bar at the top represents the subjective day (hatched) and night (solid) in DD.

ANOVA). Post hoc comparisons revealed that levels of activity at ZT 6 were significantly greater than those at all other times studied, whereas levels at ZT 12 differed from those at ZTs 1, 16, and 22.

**DGL activity.** Once PA is dephosphorylated by a PAP-catalyzed reaction that yields DAG, this intermediate metabolite can be used for the synthesis of more complex phospholipids, such as PC, PE, and TAG, or can be deacylated by means of the DGL activity (Pasquaré and Giusto, 1993). DGL activity in RGC preparations displayed a daily variation ( $P < .006$  by ANOVA), with higher levels of MAG production at subjective dusk and midnight and the lowest levels of activity at midday (ZT 6) in DD (Fig. 5). Pairwise comparisons revealed that the activity levels of DGL in RGC preparations collected at ZT 6 were significantly different from those observed at all other times examined.

#### Daily Variation of Phospholipid Labeling in Cultures of Purified Embryonic RGCs

To evaluate the autonomous capacity of RGCs in generating the daily changes in *in vivo* labeling of phospholipids, we examined the incorporation of [<sup>32</sup>P]phosphate into phospholipids in cultures of purified embryonic RGCs. After 1 day in culture, cells were synchronized by medium exchange (time 0) and pulsed with [<sup>32</sup>P]phosphate for 30 min at different times. As shown in Figure 6, the labeling of [<sup>32</sup>P]phospholipids in these cultures exhibits

a significant fluctuation ( $P < .00002$  by ANOVA), with the highest levels 5 hr after synchronization. The labeling of phospholipids remained elevated for several hours and decreased 20–25 hr later, increasing again 30 hr after the medium replacement. These findings showed that the daily oscillation observed in the labeling of phospholipids in these cultures has a period of  $\sim 24$  hr. The percentile difference between maximum and minimum values is close to 100%. Pairwise comparisons showed that levels at 5, 7, 15, and 30 hr did not differ from each other, but levels at 5 and 7 hr were significantly higher than those at 0, 1, 20, and 25 hr. Levels of labeling at 25 hr were also significantly different from those at 15 and 30 hr after medium exchange.

#### DISCUSSION

In the circadian timing system of vertebrates, it is well known that the retina is essential for the entrainment of the endogenous pacemaker located in the hypothalamic suprachiasmatic nucleus to the environmental light-dark cycles (for review see Guido et al., 2002). The retina also contains a precise clock mechanism that controls autonomous rhythms for a number of physiological and biochemical aspects even in the absence of environmental cues and of the rest of the organism (for review see Cahill and Besharse, 1995; Guido et al., 2002). This autonomous rhythmic capacity has been visualized especially in photoreceptor cells and under experimental conditions that selectively promote the survival of these cells (Pierce et al.,

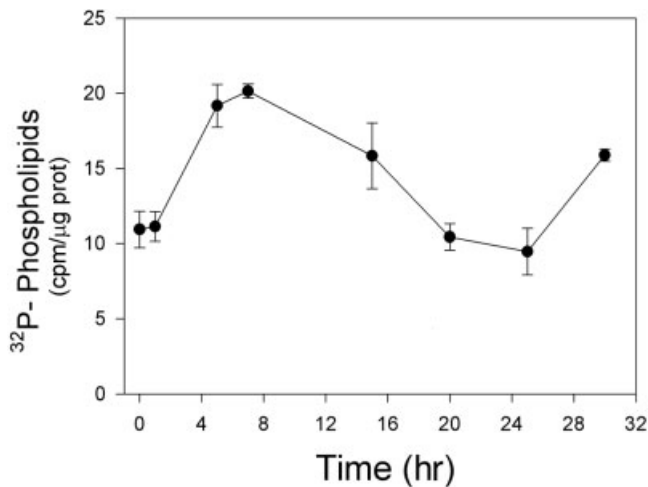


Fig. 6. Temporal course of the  $^{32}\text{P}$  labeling of phospholipids in primary cultures of chick RGCs immunopurified from retinas at embryonic day 8. After 1 day of culture, cells were synchronized by medium exchange with fresh B27-supplemented DMEM (time 0) and pulsed for 30 min with [ $^{32}\text{P}$ ]phosphate at different times from 0 to 30 hr. Results are the mean  $\pm$  SEM ( $n = 3-6$ ) from two independent experiments; values correspond to labeled phospholipids per protein. See the text for further details.

1993; Cahill and Besharse, 1995; Ko et al., 2001). However, new evidence has shown that the inner retina contains photopigments and endogenous oscillatory mechanisms (for review see Guido et al., 2002). We have recently reported that RGCs also exhibit daily variations in the biosynthesis and axonal transport of phospholipids in DD (Guido et al., 2001). It is in a constant illumination situation when a genuine biological rhythm becomes evident as an endogenous capacity, independently of any environmental change.

Phospholipids are major structural components of all biological membranes and precursors for second messengers involved in the signal transduction mechanisms (Vance and Vance, 1996; Araki and Wurtman, 1998; Caputto and Guido, 2002). In the present report, we demonstrate that the biosynthesis of [ $^{32}\text{P}$ ]phospholipids in chicken RGCs displayed a daily variation in animals maintained in LL for at least 48 hr. However, these findings do not appear to be a general variation in all metabolisms, insofar as the labeling of RGC phosphoproteins under LL did not exhibit significant temporal variations. It is noteworthy that the highest incorporation of  $^{32}\text{P}$  into phospholipids occurred during the subjective day and early night, whereas the lowest labeling took place at midnight. PI was the individual phospholipid species that accounted for the highest  $^{32}\text{P}$  labeling, displaying a daily variation with maximum levels during the subjective day and dusk. All individual lipid species assessed exhibit daily oscillations with differential patterns of variation over time; however, all of them presented the lowest levels of  $^{32}\text{P}$  incorporation around midnight (ZT 20). In addition, the percentile distribution of individual classes showed com-

parable changes in the different phospholipids over the period examined. Our findings revealed that, even under LL, an intrinsic clock mechanism is operating in these retinal cells. These observations add further evidence to our previous findings demonstrating a circadian rhythm in RGC glycerophospholipids in DD (Guido et al., 2001). Thus, it can be inferred that the biosynthesis of RGC phospholipids under constant illumination conditions is regulated by a circadian clock with high labeling levels during the day and low precursor incorporation into phospholipids at midnight. As observed in DD, the incorporation of both [ $^{32}\text{P}$ ]- and [ $^3\text{H}$ ]glycerol into phospholipids undergoes a robust circadian fluctuation (Guido et al., 2001). However, in LL, the daily variation of [ $^3\text{H}$ ]glycerol incorporation into phospholipids was substantially attenuated (Guido et al., 2001), in contrast with the findings reported in this paper concerning the incorporation of [ $^{32}\text{P}$ ]phosphate as precursor. This differential effect of the illumination situation may respond to a number of causes, such as oxidative stress after excessive photic exposure (Reiter, 2002) or the masking effect of light that reduces the spontaneous oscillations seen in DD, i.e., damping the expression of circadian oscillators (Aschoff, 1960). In this case, masking might involve direct stimulation by light of metabolic functions related to the incorporation and/or availability of [ $^3\text{H}$ ]glycerol (de novo biosynthesis) rather than that of  $^{32}\text{P}$  precursors during the night, a time at which animals would normally not be exposed to bright photic input.

Two key enzymes involved in the biosynthesis of phospholipids, AT II and PAP, have been shown to be highly regulated in mammalian rod outer segments (for review see Giusto et al., 2000) and in chick RGCs (de Arriba-Zerpa et al., 1999; Guido et al., 1999). Light modulates the activity of these enzymes in RGCs by a mechanism involving *c-fos* expression (de Arriba-Zerpa et al., 1999). Here, we found that the AT II and PAP activities exhibit significant daily variations in vitro when assessed in samples that come from RGC preparations collected at different times in DD. Interestingly, these activities are high during the subjective day and low at midnight, as were the metabolic changes observed in the in vivo labeling of phospholipids in these cells. We hypothesize based on these results that a coordinated and complex activity of different enzymes acts in RGCs to generate a circadian rhythm of lipid synthesis. Two PAP isoforms, PAP-1 and lipid phosphate phosphohydrolase (LPP; formerly called *PAP-2*) have been identified (Walton and Possmayer, 1986; Brindley and Waggoner, 1998) and differentiated on the basis of the NEM sensitivity and  $\text{Mg}^{2+}$  dependence. The DAG generated by this reaction is distinctly utilized for PC, PE, and triacylglycerol synthesis, whereas PA is used for PI synthesis through the CDP-diacylglycerol pathway (Kennedy, 1961). Different studies indicate a potential role for LPP in signal transduction mechanisms (Brindley and Waggoner, 1998; Giusto et al., 2000). PA generated in the plasma membrane either by DAG kinase (DGK; Ide and Weinhold, 1982) or by phos-



pholipase (PL) D (Sciorra and Morris, 1999) activities may be hydrolyzed by LPP. In addition, DAG generated by LPP but not by PAP-1 can be phosphorylated to generate PA. In this respect, Kai et al. (1994) have observed that the human retina presents a phosphatidylserine-dependent DGK isoform that appears to function in PI regeneration from DAG arising through PLC activation. A central role played by DGK epsilon in the PI-PLC signaling pathway has been recently reported (Rodriguez de Turco et al., 2001). We found only PAP-1 activity in our cell preparations (de Arriba-Zerpa et al., 1999), so the DAG formed in chick RGCs would not be employed as a substrate by DGK. The generation of lysophospholipids is the result not only of the prior esterification of glycerol-3-phosphate but also of PLA<sub>2</sub> activity as part of the well-known deacylation-reacylation cycle. AT II may reflect the activity of this cycle, and we cannot discard at this point a differential temporal regulation of PLA<sub>2</sub> in RGCs. In addition, DGL presents the highest levels of activity during the subjective dusk and midnight and the lowest levels at that phase well into the day (ZT 6) at which time the PAP and AT IIs are elevated. Altogether, the data suggest that enzymes involved in lipid biosynthetic pathways would be more active during the subjective day, consistent with the [<sup>32</sup>P]phospholipid labeling discussed above. It is unknown at present whether the changes described herein are generated by 1) changes in levels of enzyme mRNA and/or protein, 2) a precise temporal regulation of enzyme activities, 3) accessibility of particular substrates, or 4) a combination of all these.

Another major finding was the circadian variation observed in the labeling of phospholipids in cultures of purified embryonic RGCs. At this stage, RGCs are post-mitotic and mostly mature, whereas photoreceptors differentiate later (Mey and Thanos, 2000). Insofar as these cultures are free of other retinal cell types or systemic signals coming from central pacemakers, the metabolic oscillation found was autonomously generated in the RGCs themselves. We may infer based on these results that RGCs contain intrinsic clocks that endogenously regulate their metabolic activities on a circadian basis. The amplitude of rhythms for both the in vivo and the ex vivo labeling is similar, with high levels during both the first hours of the subjective day, in the in vivo experiments and the onset of the synchronization by medium exchange in RGC cultures. These metabolic oscillations may constitute an output of the circadian oscillators located in RGCs and may reflect a differential requirement of phospholipids recently synthesized for the biogenesis of membrane, axonal transport, or generation of distinct waves of lipid-derived second messengers varying across time. Taken together, these findings support the idea that RGCs constitute primary retinal oscillators capable of autonomously generating daily fluctuations in the synthesis of their phospholipids. By comparison of the oscillations found in the lipid biosynthesis in both photoreceptors and RGCs, we may infer that, although they are driven by a circadian clock mechanism, they showed some marked differences

in DD (Guido et al., 2001). For example, the rhythm of labeling of RGC [<sup>32</sup>P]phospholipids begins a few hours earlier than that described for lipids of photoreceptors and declines at a phase during the late day at which levels of labeling in photoreceptors remain elevated. Thus, these rhythms can be somewhat independent and are likely regulated by the differential neuronal/electrical activity of these cell populations. They may reflect responses to internal demands related to membrane biogenesis or renewal, and axonal transport in anticipation of those phases at which the entire organism is preparing to start daily activity by increasing its metabolism. Photoreceptor outer segments exhibit a daily rhythm of shedding and renewal of their membranes, which, in chick cones, peaks at the beginning of the night, whereas rod shedding peaks at dawn (Kunz, 1990). Although this phenomenon takes place only in a secluded part of the cell, and the metabolic reactions participating there involve mainly the partial renewal of lipid moieties (for review see Giusto et al., 1998, 2000), this may contribute to demands for differential activation of de novo biosynthesis of phospholipids in the cell soma at specific day times. Although circadian rhythmicity in gene expression has been previously reported in a variety of cell lines and peripheral tissues (Reppert and Weaver, 2002), our findings are the first to describe metabolic oscillations in the biosynthesis of retinal phospholipids (Guido et al., 2001; this study). The results presented here may be considered to be of special significance based on the identity and potential roles played by the RGCs (Miyamoto and Sancar, 1998; Provencio et al., 2000; Berson et al., 2002), upon which could converge oscillators and photoreceptors together to regulate the physiology of the circadian system (Lowrey and Takahashi, 2000). In addition, we must consider the complexity of the vertebrate retina, the differential electrical activity and light responsiveness of its cells, and the animal species differences within it, in terms of circadian oscillators: Not all the retinal cell types display circadian rhythms, at least in melatonin synthesis and clock gene expression. For example, in the frog retina, the circadian oscillators are located in photoreceptor cells (Cahill and Besharse, 1995; Green 1998; Hayasaka et al., 2002), whereas, in the mouse retina, the clock genes *Period1* and *Cryptochromes 1* and *2* were localized to inner nuclear and RGC layers but were absent in the photoreceptor layer (Miyamoto and Sancar, 1998; Witkovsky et al., 2003). Birds probably represent a more complex and highly intermediate, interesting state because circadian clocks are found in photoreceptor cells (Pierce et al., 1993; Ko et al., 2001) as well as in RGCs (this study). Remarkably, it was reported that there is expression of clock genes in chick photoreceptors and RGCs (Haque et al., 2002; Bailey et al., 2002). On average, considerable numbers of RGCs show depolarization in response to the photic input, whereas photoreceptors are depolarized in the dark. It is in this context that light could be the main signal acting on RGCs, whereas, in the absence of light, an unknown clock-controlled mechanism operates in these cells. In

addition, we have recently shown that fibroblasts in culture after synchronization by a serum shock exhibit robust daily rhythms in the biosynthesis of their phospholipids regulated by an endogenous clock mechanism involving the expression of the clock protein *Period1* (Marquez et al., 2004). Further studies will be required to elucidate whether similar mechanisms are taking place in RGCs to control the metabolic oscillations described.

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