Temporal coupling of cyclic AMP and Ca2+/CaM-stimulated adenylyl cyclase to the circadian clock in chick retinal photoreceptor cells

Shyam S. Chaurasia, Emory University
Rashidul Haque, Emory University
Nikita Pozdeyev, Emory University
Chad R. Jackson, Emory University
P Michael Iuvone, Emory University

Journal Title: Journal of Neurochemistry
Volume: Volume 99, Number 4
Publisher: Wiley: 12 months | 2006-11, Pages 1142-1150
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1111/j.1471-4159.2006.04154.x
Permanent URL: http://pid.emory.edu/ark:/25593/fjd1x

Final published version:

Copyright information:

Accessed September 13, 2020 12:26 PM EDT
Temporal coupling of cyclic AMP and Ca\textsuperscript{2+}/CaM-stimulated adenylyl cyclase to the circadian clock in chick retinal photoreceptor cells

Shyam S. Chaurasia\textsuperscript{1}, Rashidul Haque\textsuperscript{1}, Nikita Pozdeyev\textsuperscript{1}, Chad R. Jackson\textsuperscript{1}, and P. Michael Iuvone\textsuperscript{1,2,*}

\textsuperscript{1}Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322
\textsuperscript{2}Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA 30322

Abstract

Cyclic AMP signaling pathways play crucial roles in photoreceptor cells and other retinal cell types. Previous studies demonstrated a circadian rhythm of cyclic AMP level in chick photoreceptor cell cultures that drives the rhythm of activity of the melatonin synthesizing enzyme arylalkylamine N-acetyltransferase (Ivanova and Iuvone, 2003a) and the rhythm of affinity of the cyclic nucleotide-gated channel for cyclic GMP (Ko et al., 2004). Here we report that the photoreceptor circadian clock generates a rhythm in Ca\textsuperscript{2+}/calmodulin-stimulated adenylyl cyclase activity, which accounts for the temporal changes in the cyclic AMP levels in the photoreceptors. The circadian rhythm of cyclic AMP in photoreceptor cell cultures is abolished by treatment with the L-type Ca\textsuperscript{2+} channel antagonist nitrendipine, while the Ca\textsuperscript{2+} channel agonist, Bay K 8644, increased cyclic AMP levels with continued circadian rhythmicity in constant darkness. These results indicate that the circadian rhythm of cyclic AMP is dependent, in part, on Ca\textsuperscript{2+} influx. Photoreceptor cell cultures exhibit a circadian rhythm in Ca\textsuperscript{2+}/calmodulin-stimulated adenylyl cyclase enzyme activity with high levels at night and low levels during the day, correlating with the temporal changes of cyclic AMP in these cells. Both of the Ca\textsuperscript{2+}/calmodulin-stimulated adenylyl cyclase genes, type 1 and type 8 (Adcy1 and Adcy8), displayed significant daily rhythms of mRNA expression under a light-dark cycle, but only the Adcy1 transcript rhythm persisted in constant darkness. Similar rhythms of Adcy1 mRNA level and Ca\textsuperscript{2+}/calmodulin-stimulated adenylyl cyclase activity were observed in retinas of 2 week old chickens. These results indicate that a circadian clock controls the expression of Adcy1 mRNA and Ca\textsuperscript{2+}/calmodulin-stimulated adenylyl cyclase activity; and calcium influx into these cells gates the circadian rhythm of cyclic AMP, a key component in the regulation of photoreceptor function.

Keywords
circadian clock; cyclic AMP; adenylyl cyclase; photoreceptors; calmodulin; retina

Introduction

Circadian clocks are complex biological machines in which a set of programmed processes generate ~24 h rhythms in physiology and behavior (Reppert and Weaver 2002; Hastings et al. 2003; Iuvone et al. 2005). Vertebrate retinal neurons, including photoreceptor cells, are characterized by the presence of self-sustaining autonomous circadian clocks (Cahill and
Circadian clocks have been implicated in several retinal functions including visual sensitivity; expression of immediate early genes and visual pigment genes; affinity of the cone cyclic nucleotide-gated channel (CNGC) for cyclic GMP; and the synthesis of the neuromodulators dopamine and melatonin (for reviews see Tosini and Fukuhara 2002; Iuvone et al. 2005). The chick retina is an excellent model system for studying circadian organization because of its robust rhythmic expression of clock genes and clock controlled genes, as well the availability of a photoreceptor cell culture preparation that retains photosensitivity and circadian clock function (reviewed in Iuvone et al., 2005). The level of melatonin in chicken retina is high at night and low during the day (Hamm and Menaker, 1980). This rhythm of melatonin is derived from circadian rhythms of tryptophan hydroxylase and arylalkylamine N-acetyltransferase (AANAT), the first and penultimate enzymes in the biosynthetic pathway of melatonin from tryptophan (Bernard et al. 1997; Chong et al., 1998; Iuvone et al., 1999). In chicken retina, AANAT is expressed primarily in photoreceptor cells and is intricately controlled by light and by the circadian clock. Cyclic AMP contributes to the transcriptional and posttranslational control of AANAT activity in retina (reviewed in Iuvone et al., 2005). Cyclic AMP has also been implicated in the circadian control of the cone photoreceptor CNGC (Ko et al., 2004), and may serve as a coordinating clock signal for many circadian rhythms in photoreceptor cells (reviewed in Iuvone et al., 2005). Recently, chick photoreceptor cell cultures were shown to express circadian rhythms of cyclic AMP (Ivanova and Iuvone, 2003a). Levels of the second messenger are high at night and low during the day in cells exposed to a light-dark (LD) cycle and, with slightly reduced amplitude, in constant (24 h/day) darkness (DD). These temporal changes in cyclic AMP couple the circadian oscillator to the rhythms of AANAT activity (Ivanova and Iuvone, 2003a) and CNGC affinity for cyclic GMP (Ko et al., 2004).

K+-induced depolarization of chick retinal photoreceptors stimulates cyclic AMP formation and AANAT activity (Avendano et al., 1990; Iuvone et al., 1991; Gan et al., 1995). These effects of depolarization require Ca2+ influx through L-type voltage-gated channels and are blocked by inhibitors of calmodulin (CaM) and adenylyl cyclase (Gan et al., 1995; Alonso-Gomez and Iuvone, 1995), suggesting the involvement of Ca2+/CaM-stimulated adenylyl cyclases. The type 1 (ADCY1) and type 8 (ADCY8) adenylyl cyclases are the principal isoforms of the enzyme stimulated by Ca2+ and CaM (reviewed by Wang and Storm, 2003). Transcripts encoding the neural-specific ADCY1 are prominently expressed in retinal photoreceptor cells and ganglion cells of mammalian retina (Xia et al., 1993; Fukuhara et al., 2004), and Adcy1 mRNA expression is circadian in rat retina (Fukuhara et al., 2004). To our knowledge, expression of Adcy8 mRNA in photoreceptors has not been examined. The present study was undertaken to examine the temporal expression patterns of Adcy1 and Adcy8 in chick photoreceptor cells and to determine if a circadian rhythm of Ca2+/CaM-stimulated adenylyl cyclase activity contributes to the circadian rhythm of cyclic AMP in these cells.

Materials and methods

Animals and tissue collection

Male White Leghorn chickens (Gallus gallus domesticus) were acquired (Hyline International, Convington, GA) on the day of hatching. Animals were maintained for a minimum of two weeks in heated brooders on a 12h light : 12h dark (LD) cycle with lights on from zeitgeber time (ZT) 0 to ZT12. Following this, the animals were subjected to constant (24h/day) darkness (DD). All experiments under conditions of darkness were performed under dim red light (No. 92 filter; Eastman Kodak, Rochester, NY). Chickens were killed by decapitation and retinas were rapidly dissected and frozen on dry ice. The experimental protocols were approved by the Emory University Institutional Animal Use and Care Committee and conform to the
Cell preparation, culture conditions and drug treatments

Fertilized eggs were purchased from Hyline International (Covington, GA) and incubated for six days in a 14h light-10h dark cycle. Monolayer cultures of retinal cells are prepared from neural retinas of 6-day-old chicken embryos (E6) as described by Adler et al. (1984) with modifications (Ivanova and Iuvone, 2003b; Chaurasia et al., 2006). Neural retinas were dissociated in 0.25% trypsin and cells were seeded at a density of ~3.6 × 10^6 cells on polyornithine-coated 35 mm Primaria six-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in 3 ml medium 199 supplemented with 20 mM HEPES, linoleic acid-BSA (110 μg/ml), 2mM glutamine, penicillin-streptomycin (100 U/ml) and 10% fetal bovine serum. Cells were maintained at 39.5±0.4°C under a humidified atmosphere of 5% CO_2 in air. Illumination was provided by an 8W cool white fluorescent lamp (General Electric, Ohio, USA); the irradiance at the level of the culture dishes was 30–60 μW/cm^2. Days in vitro (DIV) are numbered successively from the day of dissection (DIV 0). On the DIV 1, S-(p-nitrobenzyl)-6-thioinosine (NBTI) was added in a final concentration of 5 μM. Medium was replaced on DIV 4 and 7 with medium 199, 1% fetal bovine serum, 1% equine serum, 5 nM insulin-like growth factor-1, 5 μM 9-cis-retinoic acid, and HEPES, glutamine, linoleic acid-BSA, NBTI and penicillin-streptomycin at the concentrations described above. Cultured cells were exposed to a daily lighting regime of 14 h light (L) and 10 h dark (D) from day 1 of incubation, with light onset at Zeitgeber time (ZT) 0. On DIV 9, cells were transferred to DD and maintained in darkness for the remainder of the experiment. The cells were sampled on DIV 8–10 during the hours indicated in the figures. Approximately 80% of the cells at this stage express a photoreceptor phenotype (Chaurasia et al., 2006).

Cyclic AMP Assay

Cultured cells were collected on DIV 8 in LD and DIV 9 in DD and homogenized in 6% trichloroacetic acid (TCA). To determine the recovery of cyclic AMP during extraction, ~2,000 dpm of [3H]cyclic AMP marker (Perkin-Elmer Life and Analytical Sciences, Inc. Shelton, CT, USA) was added to each sample. Homogenates were centrifuged at 2,500 × g at 4°C for 15 min, and the supernatant fluid removed and extracted three times with 5 ml of ethyl ether saturated with water. The extracted aqueous phase was evaporated and the residue dissolved in sodium acetate buffer, pH 6.2, which was used directly in the immunoassay. Cyclic AMP levels were measured by radioimmunoassay according to manufacturer’s instructions (Perkin-Elmer Life and Analytical Sciences, Inc.). The protein in the pellet was solubilized in 0.1 N sodium hydroxide and quantified (Lowry et al. 1951) using bovine serum albumin as standard. The cyclic AMP levels were normalized to the protein concentration and expressed as pmol of cyclic AMP per mg of protein.

Preparation of membranes and measurement of adenylyl cyclase activity

Cultured cells were collected at ZT 5 and ZT 17 on DIV 8 in LD and DIV 9 in DD. Cell from six dishes were pooled in a microfuge tubes for each sample and 5–6 samples were taken in each group. Cells were washed twice with Ca^{2+},Mg^{2+}-free Hanks balanced salt solution, harvested and homogenized in 150 μl of buffer TME, which contains 200 mM Tris-maleate, pH 7.5, 1 mM MgSO_4, 1.2 mM EGTA, 0.5 mM dithiothreitol, and Protease Inhibitor Cocktail III (5μl/ml; EMD Biosciences, La Jolla, CA, USA). Neural retinas were dissected from 2 week old chickens at ZT 4 and ZT 16 in DD and homogenized in the solution described above. Partially purified membranes were prepared by centrifugation at 20,000 × g for 20 min. Supernatant fraction was discarded, and membranes were resuspended in 300 μl of buffer TME and washed twice. The pellet was resuspended in 100 μl of buffer TME and stored at −80°C.
Measurement of adenylyl cyclase activity involves assessing the conversion of [α-32P]ATP to [32P]cyclic AMP using the method of Salomon et al. (1974) with modifications. The incubation mixture contained 80 mM Tris-HCl, pH 7.5, 10 μM GTP, 1 mM DTT, 5 mM MgSO4, 0.5 mM cyclic AMP, 5 mM phosphocreatine, 0.1 mg/ml creatine phosphokinase, 4 units/ml adenosine deaminase, 0.2 mM EGTA, 50 μM ATP, [α32P]ATP (0.5 μCi/tube) in the presence or absence of 195 μM CaCl2, 120 nM calmodulin and 10 μM forskolin. Free [Ca2+] with 195 μM CaCl2 added, was calculated to be 2 μM using WEBMAXCLITE v1.15 (http://www.stanford.edu/~cpatton/webmaxc/webmaxclite115.htm). The reaction was initiated by the addition of 20–30 μg membrane protein in a final reaction volume of 250 μl and incubated at 37°C for 10 min. The reaction was stopped by addition of 750 μl of 10% TCA containing [3H]cyclic AMP (~2000 dpm). After centrifugation, cyclic AMP was extracted by employing sequential chromatography on columns of Dowex AG50-W4 cation exchange resin (200–400 mesh) and neutral alumina. 32P and 3H in the resulting elute were quantified by liquid scintillation counting.

Laser Capture Microdissection (LCM)

The retinal photoreceptor layer was isolated by LCM as described previously (Haque et al. 2002; Chaurasia et al. 2005). Briefly, the eye cups with retinas were embedded in Tissue-Tek OCT Compound, frozen on dry ice and stored at −80°C. Frozen tissues were cut at 8–10 μm using a cryostat (CN 1850, Leica Microsystems, Deerfield, IL, USA) and mounted on uncharged glass slides (VWR International, West Chester, PA, USA). Sections were fixed in nuclease-free 75% ethanol, rehydrated in nuclease-free distilled water for 30 s, stained for 20 s (HistoGene, Arcturus Engineering, Mountain View, CA, USA) and rinsed in nuclease-free water for 30 s. They were then dehydrated by sequential immersion into 75, 95 and 100% ethanol for 30 s each and cleared in xylene for 5 min. After air-drying for 30 min, the slides were kept in a vacuum dessicator for 45 min-2 h. Laser capture was performed by lifting the photoreceptor cell layer (outer nuclear layer plus inner segments) of retina onto HS-CapSure non-contact LCM film (Arcturus Engineering). The PixCell IIe LCM system (Arcturus Engineering) was set to the following parameters: 7.5 μm laser spot size, 100 mW power, 1–2 ms duration. One photoreceptor layer, including central and peripheral retina, was obtained from each animal. Microdissected cells were immediately processed using the PicoPure RNA isolation kit (Arcturus Engineering). To eliminate possible genomic DNA contamination, RNA samples were treated with RNase-free DNase I (Qiagen, Inc., Valencia, CA) as described by the manufacturer. The total extracted RNA was used for first strand cDNA synthesis and real-time PCR was performed as described below.

RNA isolation and first strand cDNA synthesis

Retinal tissue and photoreceptor-enriched cultured neural retinal cells were extracted in buffer RLT™ (Qiagen Inc.) and processed for RNA isolation by a silica-based filter-binding RNaseasy™ mini kit (Qiagen Inc.). Samples were treated with RNase-free DNase I following the manufacturer’s instructions (Qiagen Inc.). First strand cDNA synthesis was performed as described earlier (Chaurasia et al. 2005). Briefly, total RNA (2 μg) was reverse transcribed in a 20 μl reaction using oligo-dT primer (Invitrogen, Carlsbad, CA), RNase inhibitor, T4 gene 32 protein (Ambion, Austin, TX, USA), and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reaction proceeded for 1 hr at 50°C, followed by 15 min at 72°C to inactivate the enzyme.

Quantitative real-time PCR

Real-time PCR amplification of cDNA was performed with SYBR Green master mix (Bio-Rad, Hercules, CA, USA) in a Bio-Rad iCycler (Bio-Rad) as described in previous reports (Chong et al. 2003; Chaurasia et al. 2005). Briefly, the reaction mixture included 2 μl of cDNA,
1X SYBR Green mix and 300nM gene specific forward and reverse primers. PCR reaction includes initial denaturation at 95°C for 3 min followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Each sample was assayed in triplicate and normalized to the expression of a housekeeping gene, hypoxanthine phosphoribosyl transferase (Hprt). cDNA fragments of Adcy1, Adcy8, and Hprt transcripts were generated by PCR, gel purified, quantified by spectrophotometer, and used as standards in the real-time PCR assays. The primers used for generation of standard cDNAs and expression analysis of Adcy1, Adcy8, and Hprt (Table 1) were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA) and validated for a single PCR product by melting curve analysis and agarose gel electrophoresis.

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM). Comparison between two groups was accomplished with Student's t-test. Comparisons of multiple groups were made by analysis of variance (ANOVA) followed by Student-Newman-Keul's multiple comparison test. All data were subjected to normality and equal variance tests; if either test failed, data were analyzed by Mann Whitney Rank Sum test (2 groups) or Kruskal-Wallis ANOVA on Ranks with Dunn's multiple comparison test.

**Results**

**Calcium influx is required for cyclic AMP circadian rhythm generation in photoreceptor cell cultures**

Cells incubated under LD displayed a statistically significant rhythm in cyclic AMP level (Fig. 1), peaking at night (ZT 20 vs. ZT 10, p<0.001). This rhythm in cyclic AMP level persisted on the first day of constant darkness (DD; ZT 20 vs. ZT 10, p<0.001). Treatment of cells with nitrendipine (3 μM) completely abolished the cyclic AMP rhythms in LD and DD, reducing cyclic AMP levels at night to daytime values. In contrast, Bay K 8644 (3 μM) significantly enhanced the cyclic AMP levels in these cells at all times of the day tested with a sustained rhythm in LD and DD (p<0.05, ZT 20 vs. ZT 10 on both days) (Fig. 1). These findings indicate that the circadian rhythm of cyclic AMP is dependent, at least in part, on Ca^{2+} influx.

**Ca^{2+}/CaM-stimulated adenylyl cyclase activity in photoreceptor cell cultures**

The most parsimonious interpretation of the results described above is that Ca^{2+} influx results in stimulation of one or more Ca^{2+}/CaM-stimulated adenylyl cyclases and that the circadian rhythm of cyclic AMP arises from a rhythm in the abundance of the cyclic AMP synthesizing enzymes. To test this hypothesis, adenylyl cyclase activity was measured in crude membrane preparations of homogenized cells collected during the daytime (ZT 5) and nighttime (ZT 17) in LD on DIV 8 and in DD on DIV 9. There was no significant difference in basal adenylyl cyclase activity between daytime and nighttime samples in either LD or DD (Fig. 2). Adenylyl cyclase activity was significantly stimulated by addition of Ca^{2+} and CaM. Moreover, stimulation of cyclase activity by Ca^{2+}/CaM in nighttime samples (9 fold) was significantly greater than that in daytime samples (5 fold) in LD (p<0.001; Fig. 2). The difference in Ca^{2+}/CaM-stimulated adenylyl cyclase activity persisted in DD, with stimulation in nighttime samples approximately twice as large as that in daytime samples. The stimulation of adenylyl cyclase by Ca^{2+}/CaM was potentiated by forskolin, and the night/day difference in stimulation was maintained in the presence of the adenylyl cyclase activator in both LD (p<0.001) and DD (p<0.05; Fig. 2).
Daily rhythms of Adcy1 and Adcy8 transcript levels in photoreceptor cell cultures

To evaluate whether the circadian rhythm of cyclic AMP levels, which is dependent on Ca\textsuperscript{2+} influx into the cultured photoreceptor cells, is derived from the rhythmic transcription of adenylyl cyclase genes, we investigated the mRNA levels of two Ca\textsuperscript{2+}/CaM-stimulated adenylyl cyclases- Adcy1 and Adcy8 (Fig 3). The cultures cells expressed transcripts for both Adcy1 and Adcy8, with higher levels of Adcy1 mRNA. Photoreceptor cell cultures incubated under an LD cycle displayed prominent daily fluctuations of Adcy1 transcript, peaking at ZT16 on DIV 8 (p<0.001). The rhythmic expression of the Adcy1 transcript persisted for two days in constant darkness (DD) on DIV 9 (p<0.001) and DIV 10 (p=0.022), indicative of circadian control (Fig. 3a). In contrast, the expression of Adcy8 mRNA was rhythmic in LD with high levels during the early morning hours of the day and low levels during the night on DIV 8 in cultured photoreceptor cells (Fig. 3b, p<0.001). On day 1 of DD, there was a trend towards continued rhythmicity of Adcy8 transcript levels, but it was not statistically significant (p=0.112). On the second day of DD, Adcy8 mRNA levels increased from ZT0 to ZT8 (p=0.018), and remained at that level for the remainder of the day.

Adcy1 and Adcy8 are expressed in chicken photoreceptor cells in vivo

The photoreceptors cell cultures used in this study are from embryonic neural retina and are not completely pure, with approximately 80% of the cells expressing the photoreceptor phenotype (Chaurasia et al., 2006). To determine if photoreceptors express Adcy1 and Adcy8 in vivo in posthatch chickens, the photoreceptor layer was isolated by laser capture microdissection of frozen sections of retina obtained from 2 week old chickens killed during the daytime (ZT 4) and night (ZT 16) in LD (Fig. 4). Both transcripts were readily detectable in the isolated photoreceptor layers, with significantly higher expression at ZT16 compared to ZT4 for Adcy1 (p≤0.005) and at ZT4 compared to ZT16 for Adcy8 (p≤0.005). The relative levels of Adcy1 transcript were higher than those of Adcy8 mRNA at both times of day.

Adcy1 transcript and adenylyl cyclase activity in neural retina is under the control of an endogenous circadian clock in vivo

Adcy1 mRNA expression was examined in neural retina of chickens entrained to LD for 2 weeks and then released into DD for two days. A significant daily rhythm of Adcy1 mRNA expression was observed in LD (Fig. 5; p=0.008), with highest levels of transcript at night (ZT 16) and lowest levels during early hours of the daytime (ZT0–4). In DD, we found a significantly daily variation in the expression of Adcy1 transcript in neural retina for at least 2 days (p≤0.001 for both days in DD). Adcy8 transcript levels displayed significant variation only in LD (p<0.001), with highest levels during the daytime; this pattern was not maintained on either day in DD (data not shown).

Membranes prepared from neural retina in DD also displayed a circadian fluctuation in Ca\textsuperscript{2+}/CaM-stimulated adenylyl cyclase activity (Fig. 6), similar to that observed in photoreceptor cell culture, with higher activity at night (p<0.001). As a control, membranes were treated with nitrendipine (3 μM), which had no effect on the circadian rhythm of adenylyl cyclase activity. This result indicates that the effect of the calcium channel blocker on the circadian rhythm of cyclic AMP is not due to a direct effect on adenylyl cyclase.

Discussion

Our data provide the first experimental evidence for a requirement of Ca\textsuperscript{2+} influx in the generation of the circadian rhythm of cyclic AMP level in photoreceptor cells. They also demonstrate for the first time the existence of daily rhythms of Ca\textsuperscript{2+}/CaM-stimulated adenylyl cyclase activity in retinal cells, and have identified regulation of Adcy1, and possibly Adcy8, transcript levels as factors in the regulation of these rhythms.
The levels of cyclic AMP in photoreceptor cells, as well as the activity of AANAT, are subject to dual control by a circadian clock and light. The levels of the cyclic nucleotide and AANAT activity in photoreceptor cell cultures oscillate in constant darkness with peaks during the subjective night (Ivanova and Iuvone, 2003a,b). Light exposure at night lowers cyclic AMP and AANAT to daytime levels. In the present study, we demonstrated that the circadian rhythm of cyclic AMP in photoreceptor cell cultures is abolished by nitrendipine, a dihydropyridine L-type Ca\(^{2+}\) channel blocker, with low levels observed both day and night. In contrast, the L-type Ca\(^{2+}\) channel agonist, Bay K 8644, elevated cyclic AMP levels during both subjective day and subjective night. The plasma membrane of vertebrate retinal photoreceptor cells are relatively depolarized in darkness, and hyperpolarized in a graded fashion in response to light of increasing intensity (Hagins et al., 1970). Photoreceptors have dihydropyridine-sensitive Ca\(^{2+}\) channels (Taylor and Morgans, 1998; Nachman-Clewner et al., 1999; Krizaj, 2005), which open upon depolarization (Uchida and Iuvone, 1999) and mediate the Ca\(^{2+}\) influx required for the synthesis of cyclic AMP (Iuvone et al., 1991). Moreover, the induction of AANAT activity requires sustained Ca\(^{2+}\) influx in photoreceptors and the enzyme activity decreases upon closure or inhibition of channel activity (Iuvone and Besharse, 1986; Avendano et al., 1990). Collectively, these observations indicate that the suppression of cyclic AMP level and AANAT activity by light is mediated by hyperpolarization of the plasma membrane and closure of the Ca\(^{2+}\) channels. Thus, differential Ca\(^{2+}\) influx can explain the light-dark difference in cyclic AMP level, but alone does not account for the sustained oscillation of the cyclic nucleotide in constant darkness, when photoreceptors would be depolarized and voltage-gated Ca\(^{2+}\) channels activated both day and night.

Two possible mechanisms for the sustained oscillation of cyclic AMP are circadian control of intracellular Ca\(^{2+}\) and circadian gating of the availability of Ca\(^{2+}\)/CaM-stimulated adenylyl cyclases. The latter mechanism was explored in this study. We show here that Ca\(^{2+}\)/CaM-stimulated adenylyl cyclase activity is higher in membranes prepared from photoreceptor cell cultures during the subjective night than in membranes prepared during subjective day, in both LD and constant darkness. Thus, in the presence of a fixed Ca\(^{2+}\) concentration in vitro, cyclic AMP production is higher at night than during the day, indicative of a rhythm of cyclase availability. This observation does not preclude a possible contribution of circadian control of intracellular Ca\(^{2+}\), which has yet to be explored.

Of the 10 adenylyl cyclases known in vertebrates, ADCY1 and ADCY8 are directly stimulated by Ca\(^{2+}\)/CaM in vitro and in vivo (Choi et al., 1992; Cali et al., 1994). ADCY1 is synergistically stimulated by intracellular Ca\(^{2+}\) and G protein-coupled receptor activation in vivo (Wayman et al., 1994), whereas ADCY8 is not. We found that Adcy1 and Adcy8 transcripts are present in the chicken photoreceptor cell cultures and in neural retina. Using laser capture microdissection (LCM), we also showed that Adcy1 and Adcy8 are expressed in photoreceptors of two week old chickens in vivo. Previous studies in mammalian retina have shown the expression of Adcy1 mRNA in photoreceptors (Xia et al., 1993; Fukuhara et al., 2004). Photoreceptor cells cultured under LD showed rhythmic patterns of Adcy1 and Adcy8 expression. Similar results were observed in whole neural retina and in LCM-isolated photoreceptors of two week old chickens in vivo. Rhythms of Adcy1 mRNA, which peaked during the subjective night, persisted in constant darkness in vitro and in vivo, indicative of circadian control. In contrast, Adcy8 mRNA levels were higher during the daytime and arrhythmic in constant darkness. The amount of Adcy1 transcript was ~10 fold higher in cultured photoreceptor cells and ~5 fold higher in LCM dissected photoreceptors compared to Adcy8 transcripts. These results clearly suggest that the circadian rhythm of Ca\(^{2+}\)/CaM-stimulated adenylyl cyclase activity and cyclic AMP level is generated, at least in part, by a rhythm in the abundance of ADCY1 protein. Unfortunately, we were unable to find an antibody that specifically recognizes ADCY1 in chicken retina to directly test this hypothesis. A recent study has also shown the circadian expression of Adcy1 mRNA in the rat retina, and provided
evidence for a role of ADCY1 in the circadian gating of melatonin synthesis (Fukuhara et al. 2004).

In addition to regulation of melatonin biosynthesis and CNGC affinity for cyclic GMP, other circadian processes in photoreceptor cells may be gated or driven by the circadian rhythm of cyclic AMP. For example, induction of c-Fos mRNA and protein in photoreceptors is regulated in a circadian fashion, with peak levels during the subjective night (Yoshida et al., 1993; Humphries and Carter, 2004). Similar to cyclic AMP, light exposure at night suppresses c-Fos transcript levels in photoreceptors (Yoshida et al., 1993). In many cell types, c-Fos expression is induced by a cyclic AMP-dependent mechanism (Greenberg et al., 1985; Webster and Kedes, 1990; Buckmaster et al., 1991). Induction of nocturin, an RNA deadenylase (Baggs and Green, 2003), in Xenopus photoreceptors is stimulated by phospho-CREB (Liu and Green, 2002), which undergoes a circadian rhythm with peak levels at night. It must be noted, however, that CREB can be phosphorylated and c-fos transcription can be activated by multiple protein kinases and a definitive link between the rhythm of cyclic AMP and those of phospho-CREB and c-Fos have yet to be established.

In summary, we have demonstrated the circadian expression of Ca\(^{2+}\)/calmodulin-stimulated adenylyl cyclase activity in chicken retinal photoreceptors. The clock-controlled expression of Adcy1 appears to contribute to the light-sensitive, Ca\(^{2+}\)-dependent rhythm of cyclic AMP, which provides a circadian clock output capable of regulating multiple aspects of cellular physiology. Coupled with the diurnal expression of Adcy8, this regulatory system may be responsible for the many light-driven and circadian rhythms associated with photoreceptor cell biology.

**Acknowledgements**

The authors thank Hong Zhou and Amy Visser for excellent technical assistance. This work was supported by grant R01EY004864 and P30EY006360 from the National Institute of Health. Preliminary reports of some of these data were presented at the 2004 meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, FL, and the Xth Congress of the European Pineal and Biological Rhythm Society, Frankfurt, Germany, 2005.

**Abbreviations**

AANAT, arylalkylamine N-acetyltransferase  
Adcy1, adenylyl cyclase type 1  
Adcy8, adenylyl cyclase type 8  
BSA, bovine serum albumin  
CaM, calmodulin  
CNGC, cyclic nucleotide-gated channel  
DD, constant darkness  
DIV, days in vitro  
DTT, Dithiothreitol  
EGTA, ethylene glycol tetraacetic acid  
GCL, ganglion cell layer  
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
Hpht, hypoxanthine guanine phosphoribosyltransferase  
INL, inner nuclear layer  
LCM, laser capture microdissection  
LD, light-dark  
ONL, outer nuclear layer  
PCR, polymerase chain reaction  
PhR, photoreceptors  
TCA, trichloroacetic acid
TME, tris-maleate EDTA
ZT, zeitgeber time

REFERENCES


Figure 1. **Ca^{2+}** influx is required for cyclic AMP circadian rhythm generation

Cells were prepared from E6 retinas and incubated for 8 days under a 14 h light and 10 h dark (LD) cycle and 1 day in constant darkness (DD) as described in Materials and methods. On DIV7, culture medium was replaced with fresh media containing the L-type **Ca^{2+}** channel blocker, nitrendipine (3 μM), the **Ca^{2+}** channel agonist, Bay K 8644 (3 μM), or vehicle. Cyclic AMP was determined in the cells harvested on DIV 8 in LD and DIV 9 in DD. Cells treated with vehicle exhibited a circadian rhythm of cyclic AMP level. Levels of the cyclic nucleotide were significantly lower during the day (ZT10) than at night (ZT 20) in LD (P<0.001; n=10 –12) and in DD (P<0.001; n=12). Treatment of cells with nitrendipine totally suppressed the nocturnal increase in cyclic AMP concentration and abolished the circadian rhythm of cyclic AMP in LD and DD (n=5–6). In contrast, the circadian rhythm of cyclic AMP level persisted in the presence of Bay K 8644 (p<0.05; n=11–12), with higher levels of cyclic AMP compared to vehicle controls. The horizontal white and black bars above the x-axis represent times of light and darkness, respectively. Data are presented as mean ± SEM.
Figure 2. Adenylyl cyclase activity in photoreceptor cell cultures
Cells were prepared from E6 retinas and incubated as described in Materials and methods.
Cells were harvested at ZT 5 and ZT 17 on DIV 8 in LD and DIV 9 in DD. Adenylyl cyclase activity was determined in crude membrane preparations by measuring the formation of radiolabelled cyclic AMP from $[^{32}P]ATP$, as described in Materials and methods. Cells from 6 dishes were pooled together to prepare membranes for each sample, which was assayed for activity under three conditions: basal, Ca$^{2+}$ (2μM) + CaM (120 nM), and Ca$^{2+}$ + CaM + forskolin (10μM). Each time point is the mean ± SEM from 5–6 membrane preparations. No significant differences of basal activity were observed between ZT 5 and ZT 17 in either LD or DD. A circadian fluctuation in Ca$^{2+}$/CaM-stimulated adenylyl cyclase activity was observed in LD (**p≤0.001) and DD (*p<0.05), with activity higher at night (ZT 17) than during the daytime (ZT 5). A similar circadian fluctuation was observed in activity measured in the presence of Ca$^{2+}$, CaM, and forskolin (LD, **, p≤0.001; DD, *, p<0.05).
Figure 3. Adcy1 and Adcy8 mRNA rhythm in photoreceptor cell cultures

Cells were prepared from E6 retinas and incubated as described in Materials and methods. Cells were harvested at the times indicated in the figure in LD on DIV 8 and DD on DIV9 and 10. Relative mRNA levels for Adcy1 and Adcy8 were quantified by real-time RT-PCR. Transcript levels were normalized with Hprt mRNA. (a) Adcy1 mRNA expression was rhythmic in LD (p<0.001, n=5–6 dishes / time point) and in constant darkness (p<0.001 for DD1, n=6; p=0.022 for DD2, n=6) with highest levels during the late afternoon hours. (b) In LD, Adcy8 transcript level displayed a daily rhythm with high values during the early hours of the day (p<0.001, n=6) relative to those at night. On day 1 of DD, there was a trend towards continued rhythmicity of Adcy8 transcript levels, but it was not statistically significant.
On the second day of DD, Adcy8 mRNA levels increased from ZT0 to ZT8 (p=0.018), and remained at that level for the remainder of the day. The horizontal white and black bars above the x-axis represent times of light and darkness, respectively.
Figure 4. Expression of Adcy1 mRNA in retinal photoreceptors dissected by laser capture microdissection (LCM)

One-day old chicks were housed in a 12-h light: 12-h dark (LD) cycle with lights on at ZT 0 for 2 weeks. Eyes were collected in LD at the times indicated in the figure and prepared for LCM as described in Materials and methods. (a) Photomicrograph of representative retinal section prior to dissection. (b) Photomicrograph of a retinal after removal of the photoreceptor layer by LCM. (c) Relative mRNA levels in the photoreceptor layers were quantified by real-time RT-PCR. Adcy1 and Adcy8 transcript levels were normalized with Hprt mRNA. Adcy1 mRNA expression was significantly higher at ZT 16 than at ZT4 (\(**p \leq 0.005\), n=7) whereas
Adcy8 transcript showed higher levels at ZT4 (**p ≤ 0.005, n=7). RPE, retinal pigment epithelium; PhR, photoreceptors; INL, inner nuclear layer; GCL, ganglion cell layer.
Figure 5. *Adcy1* transcript in neural retina is regulated by a circadian clock in vivo

One-day old chicks were housed in a 12-h light: 12-h dark (LD) cycle with lights on at ZT 0 for 2 weeks and later kept under constant darkness (DD) for 2 days. Neural retina was collected in LD, DD1 and DD2 at the times indicated in the figure. Relative mRNA levels were quantified by real-time RT-PCR. Each data point represents *Adcy1* transcript from the six individual chickens. *Adcy1* transcript levels are normalized with *Hprt* mRNA. *Adcy1* mRNA expression was rhythmic in LD (p=0.008, n=6) and in constant darkness (p<0.001 for DD1, n=6; p=0.001 for DD2, n=6) with lowest levels in the early morning. The horizontal white and black bars above the x-axis represent times of light and darkness, respectively.
Figure 6. Circadian Regulation of Ca\textsuperscript{2+}/CaM-stimulated adenylyl cyclase activity in neural retina
One-day old chicks were housed in a 12-h light: 12-h dark (LD) cycle with lights on at ZT 0 for 2 weeks and then transferred to DD. Neural retina was collected on DD1 at the times indicated in the figure. Adenylyl cyclase activity was determined in crude membrane preparations as described in Materials and methods. Retinal membranes were prepared and assayed for activity under four conditions: basal, basal + nitrendipine (3 μM), Ca\textsuperscript{2+} (2μM) + CaM (120 nM), and Ca\textsuperscript{2+} + CaM + nitrendipine. Each time point is the mean ± SEM from 5 membrane preparations. No significant differences of basal or basal + nitrendipine activity were observed between ZT 4 and ZT 16 in DD. A circadian fluctuation in Ca\textsuperscript{2+}/CaM-stimulated adenylyl cyclase activity was observed in DD (**, p<0.001), with activity higher at night (ZT 16) than during the daytime (ZT 4). Nitrendipine added to the retinal membranes in vitro did not affect the circadian fluctuation observed in activity measured in the presence of Ca\textsuperscript{2+} and CaM (**, p<0.001 at ZT16 compared to ZT4).


### Table 1

**PCR primers**

Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) using the GenBank accession numbers listed. Primers were designed to generate PCR products that span exon-intron boundaries. Each primer pair yielded a single product of the correct size as determined by agarose gel electrophoresis and melt curve analyses. Amplicons from the "Standard" primers were gel purified and served as standard cDNAs for the "Real-time" PCR assays.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
<th>GenBank Accession Number</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adcy1</td>
<td>F: AGGTGACTGTTACTATTGTATCAGGC&lt;br&gt;R: GCCATATCCTGGTTCTACTTCATAGTC</td>
<td>XM_418882</td>
<td>328</td>
</tr>
<tr>
<td>Adcy8</td>
<td>F: GGAGAAACAGACTTCCTGGGTACAA&lt;br&gt;R: AAGCAAAACATCACTCCAACCGCATC</td>
<td>XM_418437</td>
<td>292</td>
</tr>
<tr>
<td>Hprt</td>
<td>F: CCCTCCATGGCGACTCACA&lt;br&gt;R: AGTGAGGGCGTATCCCAAACAA</td>
<td>AJ132697</td>
<td>590</td>
</tr>
<tr>
<td><strong>Real-time:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adcy1</td>
<td>F: AGGTGACTGTTACTATTGTATCAGGC&lt;br&gt;R: CTAGGACACCACAAAGCACCCTTC</td>
<td>XM_418882</td>
<td>179</td>
</tr>
<tr>
<td>Adcy8</td>
<td>F: GGAGAAACAGACTTCCTGGGTACAA&lt;br&gt;R: CATTTGCTTCCTAAACTCTTGATTT</td>
<td>XM_418437</td>
<td>175</td>
</tr>
<tr>
<td>Hprt</td>
<td>F: CATGGAGAAGAGAGAGAGAGAG&lt;br&gt;R: CTCTTCAACCTAATGAGTCCACAG</td>
<td>AJ132697</td>
<td>186</td>
</tr>
</tbody>
</table>