

Circadian Regulation of cGMP-Gated Cationic Channels of Chick Retinal Cones: Erk MAP Kinase and Ca²⁺/Calmodulin-Dependent Protein Kinase II

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Summary

cGMP-gated channels are essential for phototransduction in the vertebrate retina. Here we show that the affinity of these channels for cGMP in chick cones is substantially higher during the subjective night than during the subjective day. This effect persists in constant environmental conditions after entrainment to 12:12 hr light–dark cycles *in vitro* or *in ovo*. Circadian modulation of ligand affinity is a posttranslational effect and is driven by rhythms in the activities of two protein kinases: Erk and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII). Erk is maximally active during the subjective night, whereas CaMKII is maximally active during the subjective day. Acute inhibition of these signaling pathways causes phase-dependent changes in the affinity of the channels for cGMP.

Introduction

Vertebrate phototransduction is mediated by a G-protein-coupled cascade that results in changes in the gating of cyclic GMP (cGMP)-gated cationic channels expressed at high density in photoreceptor outer segments (reviewed by Zagotta and Siegelbaum, 1996). Intracellular cGMP concentrations are relatively high in the dark, causing tonic activation of these channels and a steady transmembrane cation flux known as the dark current. Light causes a fall in intracellular cGMP, closure of these channels, and membrane hyperpolarization. cGMP-gated channels are therefore an essential component of the mechanism that couples photon absorption by vertebrate visual pigments to changes in photoreceptor membrane potential, neurotransmitter release, and subsequent neural processing.

The gating of photoreceptor cGMP-gated channels can be modulated by multiple processes, including direct phosphorylation of the channel molecules and binding of Ca²⁺/calmodulin or related molecules. The physiological significance of this modulation is unknown, but it has been proposed to contribute to adaptation (Rebrik and Korenbrot, 1998). Adaptation refers to a class of processes by which the sensitivity to light decreases in proportion to an increase in background illumination, which allows ensembles of photoreceptors to detect the contrast between an object and its background over a wide range of ambient illumination intensities (Shapley and Enroth-Cugell, 1984).

The steady-state phosphorylation of cGMP-gated channels appears to be regulated by a dynamic interplay between protein kinases and phosphatases, as perturbations of these dynamics alter the gating behavior of the channels. For example, the apparent affinity of cGMP-activated channels for their activating ligand increases with time after excision of inside-out patches from amphibian rod outer segments (Gordon et al., 1992; Gordon et al., 1995; Hackos and Korenbrot, 1997). This change in affinity can be blocked by application of protein phosphatase inhibitors to the patch membrane (Gordon et al., 1992), suggesting that cGMP-activated channels of rods are closely associated with membrane-bound protein phosphatases. Patch excision eliminates interactions between the channels and intracellular kinases and ATP leading to gradual protein dephosphorylation and changes in gating behavior. Direct application of protein kinases and protein phosphatases to the cytoplasmic face of patch membranes causes opposite changes in the affinity of channels for cGMP (Gordon et al., 1992; Molokanova et al., 1997, 2000a, 2000b).

The cGMP-gated channels of rods (Hsu and Molday, 1993; Gordon et al., 1995; Bauer, 1996; Kosolapov and Bobkov, 1996; Rebrik and Korenbrot, 1998) can also be modulated by binding of Ca²⁺/calmodulin such that channels shift to a lower affinity state for cGMP. In patches excised from cones, modulation by Ca²⁺/calmodulin is quite small (Hackos and Korenbrot, 1997) or is undetectable (Haynes and Stotz, 1997) and does not fully mimic the endogenous modulation that occurs after patch excision (Rebrik and Korenbrot, 1998). It has been suggested that a molecule that is related to but not identical to Ca²⁺/calmodulin may provide a mechanism for feedback modulation of cone phototransduction (Rebrik and Korenbrot, 1998).

Visual system function is also regulated by long-term processes, that is, changes in structure, physiology, and biochemistry, that take place over time scales of hours to days. This includes regulation by intrinsic retinal circadian oscillators (reviewed by Cahill and Besharse, 1995). Circadian oscillators are biological clocks with a period of close to 24 hr (circadian) that regulates a host of biochemical, physiological, and behavioral processes, including visual system function in a wide range of species. Circadian oscillators provide a mechanism for visual systems to anticipate the large daily changes in ambient illumination and thereby initiate more sustained adaptive changes that entail changes in gene expression.

The most extensively studied circadian output in vertebrate retinal photoreceptors is the synthesis and secretion of melatonin, which is controlled, at least in part, by oscillators located in the photoreceptors themselves (Cahill and Besharse, 1993). In addition, circadian oscillators control several morphological features, including retinomotor movements, disk shedding, and outer segment membrane renewal (reviewed by Cahill and Besharse, 1995). Photoreceptor circadian oscillators also regulate gene expression (e.g., Yoshida et al., 1993; Green and Besharse, 1996), including the expression of visual pigment genes (Korenbrot and Fernald, 1989;

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Pierce et al., 1993). Circadian rhythms in cell structure and biochemistry are associated with changes in physiological responses. For example, circadian oscillators control rod-cone dominance (Wang and Mangel, 1996; Manglapus et al., 1999), and the photoreceptor components of the electroretinogram exhibit a circadian rhythm in several species, including birds (Lu et al., 1991; Manglapus et al., 1998; McGoogan and Cassone, 1998).

The extent to which retinal circadian oscillators control changes in the gating properties of photoreceptor cGMP-gated channels has not been investigated but is an important question given the central role of these channels in phototransduction. In this study, we show that the affinity of cGMP-gated channels of embryonic chick cones for activating ligand exhibits a circadian rhythm that persists in constant environmental conditions. The apparent affinity of channels for cGMP is increased during the subjective night, and this effect is driven at least in part by circadian rhythms in the Erk form of mitogen-activated protein kinase (Erk) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). Perturbation of the activities of these enzymes causes phase-dependent changes in the gating properties of cGMP-gated channels.

Results

In initial experiments, we characterized cGMP-gated channels of chick cones and examined their affinity for activating ligand at different times of day. Chick retinal cells were dissociated at embryonic day 6 (E6) and were maintained in cell culture for 5 days. Considerably more than half of the cells growing under these conditions exhibit cone morphology; that is, the cells are elongated and bipolar, with one or more prominent oil droplets located at the end of the soma (Figure 1A). Cultured photoreceptors also express nonselective cationic channels that can be activated by bath application of cGMP to inside-out patches excised from the soma (Figure 1B). These channels show a slight outward rectification when activated by 50 μM cGMP and reverse at 0 mV in symmetrical 145 mM NaCl. The reversal potential shifted ~ 10 mV to the right when the cytoplasmic face of the patch membrane was exposed to salines containing 125 mM CsCl and 20 mM NaCl (Figure 1C), indicating that the channels are more permeant to Na^+ than to Cs^+ , as described for other cone channels (Haynes, 1995). The cGMP-gated channels of chick cones have complex gating kinetics. Because excised inside-out patches from chick retinal cells invariably contain multiple channels with rapid kinetics, we have not been able to calculate unitary conductance or mean channel open or closed times from direct observations of open-closed transitions. Instead, we have obtained estimates of kinetic behavior from steady-state fluctuation analysis of currents evoked by cGMP. Briefly, inside-out patch currents were recorded in the presence and absence of cGMP. The mean power spectra of the current fluctuations were calculated under both conditions, and the resulting spectra were subtracted digitally (Figure 1D). Subtracted power spectra were fitted with one or more Lorentzian curves of this form:

$$S(f) = S(0)/[1 + (2\pi f_c \tau)^2]$$

where $S(f)$ is power as a function of frequency, $S(0)$ is the extrapolated power at frequency = 0 Hz, f_c is the frequency at which power is $S(0)/2$, and τ is a time constant defined as $1/2\pi f_c$ and is related but not precisely equal to the mean open time (Anderson and Stevens, 1973). We consistently observed that the sum of two Lorentzian curves was necessary to provide adequate fits to the subtracted power spectra (Figure 1D), suggesting that these channels have somewhat complex kinetics or that more than one population of channels is present. In this regard, it bears noting that the time constants obtained in fluctuation analyses of chick retinal cone channels ($\tau_1 = 0.39 \pm 0.15$ ms and $\tau_2 = 15.2 \pm 4.3$ ms; mean \pm SEM) are comparable to open time constants observed directly in mature chick pineal cells, which express a homogeneous population of cone-type, cGMP-gated channels, but at much lower density (Dryer and Henderson, 1991). The high-frequency components of channel gating account for most of the noise in the power spectrum in retinal cones, and are consistent with observations of chick pineal cells. In other words, cGMP-gated channels in both cell types exhibit a great deal of flicker, as reported previously for native cGMP-gated channels of rod (Torre et al., 1992) and cone (Haynes and Yau, 1990) outer segments.

In order to determine whether the properties of cGMP-gated channels exhibit a daily rhythm, dissociated chick cones were maintained on 12:12 hr light-dark cycles (LD 12:12) for 5 days in vitro. On the 5th day of LD, patches were excised from cones 4–7 hr after lights on (ZT4–7) or 4–7 hr after lights off (ZT16–19), and cGMP concentration-response curves were generated immediately after patch excision, fitted with the Hill equation (Figure 2A). The mean K_D for cGMP varied with the time of day at which patches were excised and was significantly ($p < 0.001$) greater during the day than during the night in cells observed on the 5th day of LD 12:12 (Figure 2B). Identical results ($p < 0.001$) were obtained from patches excised on the 2nd day of constant darkness (DD) after 4 days of entrainment to LD 12:12, indicating that daily changes in channel gating properties free run in constant environmental conditions (Figure 2B), suggesting circadian control. Experiments with greater temporal resolution are described later in this article.

Daily changes in the gating properties of cGMP-gated channels are not direct effects of light. To ascertain this, chick photoreceptors were dissociated at E6 and maintained for 5 days in the presence of aperiodic LD transitions, continuous light (LL), or DD. The aperiodic LD lighting regimen exposed cells to the same total duration of light as cells maintained on LD 12:12 hr over the 5-day culture period (i.e., 60 hr of light), but the light pulses were applied randomly, with individual photoperiods ranging from 0.5–4.0 hr. This lighting paradigm was designed to prevent entrainment of circadian oscillators. Cells maintained in LL received 120 hr of continuous light, whereas cells in DD received no light after dissociation. None of these groups received entraining LD 12:12 cycles at any time prior to cell dissociation or electrophysiology. The mean K_D for cGMP obtained from cells maintained in a periodic LD was not significantly different regardless of whether recordings were made at times of lights on or lights off (Figure 2C). Moreover,

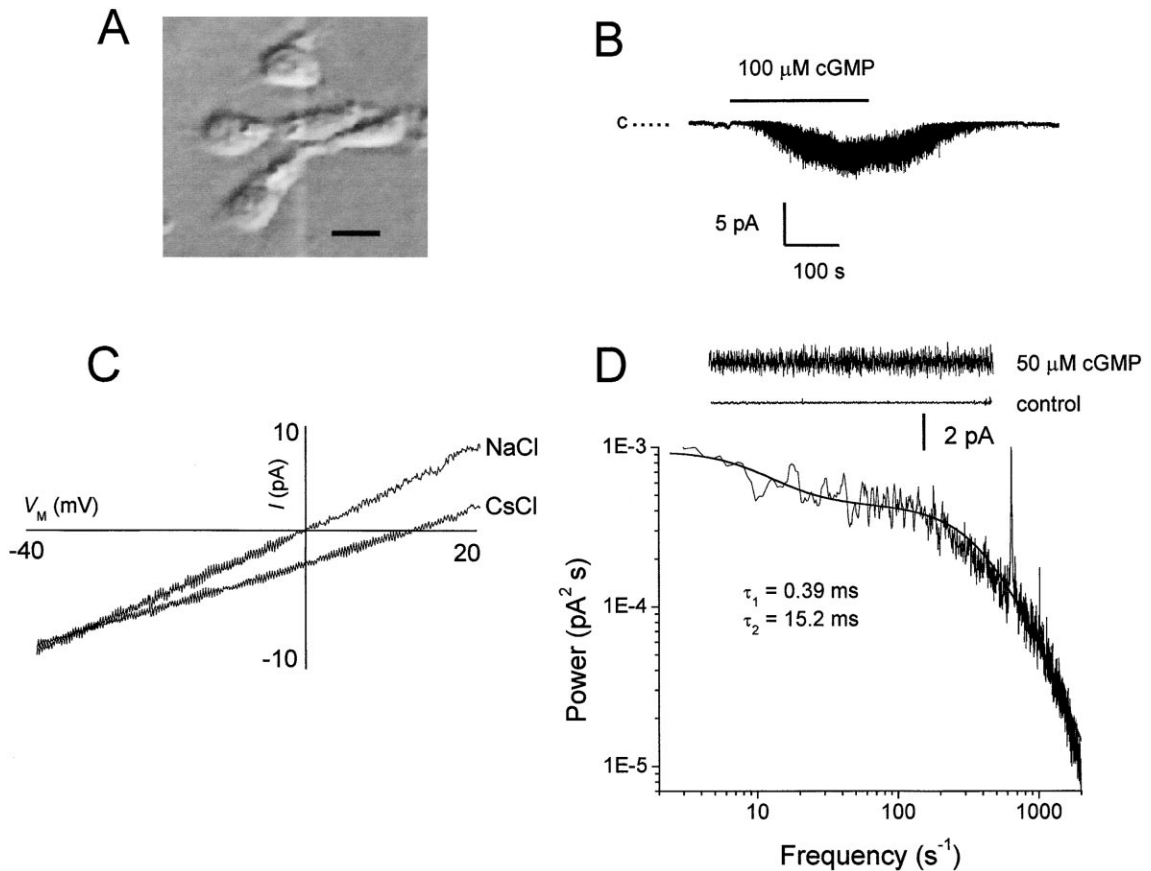


Figure 1. Properties of Cone Photoreceptors Dissociated from Chick Embryos

(A) Modulation contrast optics image of a cluster of cones dissociated from an E6 chick embryo and maintained in vitro for 5 days.
 (B) Typical response to cGMP in an inside-out patch excised from a cultured cone photoreceptor. This patch contained ~ 10 cGMP-gated cationic channels. A fully closed state is indicated to the left of the trace. The slow onset of the current response represents the dead time of the bath perfusion system.
 (C) Current voltage relationship for cGMP-gated channels in two different ionic conditions. Ramp voltage commands were applied in the presence and absence of $50 \mu\text{M}$ cGMP. Traces are leak subtracted (cGMP-control) in normal bath saline (containing 145 mM NaCl) and in bath saline containing 20 mM NaCl and 125 mM CsCl. Note the slight outward rectification in symmetrical NaCl. Reversal potential changes to more positive voltages in CsCl bath salines, indicating that Cs^+ is less permeant.
 (D) Power spectral analysis of current fluctuations evoked by $50 \mu\text{M}$ cGMP in excised patches. Top traces are 30 s DC-coupled traces of typical currents recorded in the presence and absence of cGMP. Subtracted power spectrum calculated from these data (cGMP spectrum-control spectrum) is shown later in this article with a superimposed fitted curve representing the sum of two Lorentzians with the indicated time constants (see text).

the mean K_D for cGMP was not different in cells maintained continuously on LL or DD (Figure 2C).

The circadian oscillator that controls the properties of cGMP-gated channels can be entrained in ovo. In these experiments, E6 eggs were allowed to develop in two incubators equipped with lights and timers such that entraining LD 12:12 cycles were 12 hr antiphase. On the 5th day, E11 retinæ were excised from embryos developing in both chambers, dissociated at the same time, and maintained in vitro in DD. Recordings were made at ZT4-7 and ZT16-19 on the 2nd day of DD from photoreceptors. As with cells entrained in vitro, mean K_D obtained immediately after patch excision was significantly ($p < 0.0001$) greater during the subjective day than the subjective night (Figure 2D). Thus, embryonic chick photoreceptors can be entrained in ovo, and cell dissociation does not cause major disruptions in entrainment.

In order to characterize circadian changes in channel-gating properties in more detail, recordings from inside-out patches were made every six hours. A significantly ($p < 0.001$) lower affinity (higher K_D) for cGMP was observed during the middle of the subjective day (ZT4-7) in photoreceptors free running on the 2nd day of DD (Figure 3A). In contrast, the Hill coefficient for channel activation (n) did not vary as a function of the time of day in photoreceptors maintained on LD 12:12 (data not shown) or on the 2nd day of DD (Figure 3B) and was similar to values reported previously for cloned chick cone-type, cGMP-gated channel α subunits (Bonigk et al., 1996). Moreover, there was no difference in the maximum currents or mean patch capacitance at different times of day, and in a series of separate experiments, we observed that mean patch capacitance (data not shown). Taken together, these results demonstrate that a retinal circadian oscillator regulates the apparent affin-

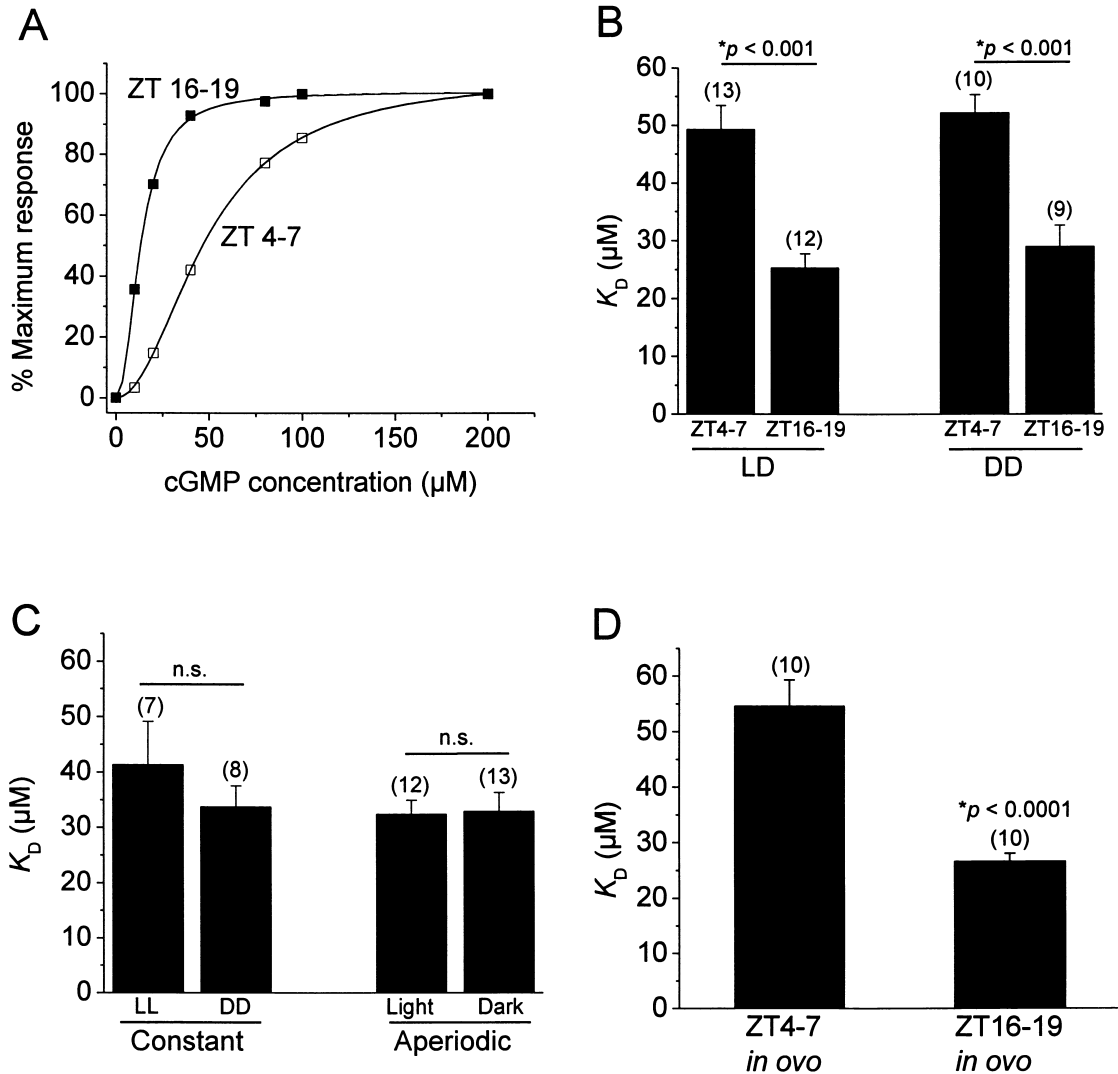


Figure 2. Circadian Rhythm in the Apparent Affinity of Cone cGMP-Gated Channels for Their Activating Ligand

(A) Typical cGMP concentration response curves obtained from patches excised during the subjective day (ZT4–7, hollow squares) and during the subjective night (ZT16–19, filled squares). The lines represent least-squares fit to the Hill equation (see text).

(B) Summary of mean K_D s obtained from many patches excised at ZT4–7 and ZT16–19. Data were obtained from cells on the 5th day of LD 12:12 (LD) and from cells free running on the 2nd day of DD after 4 days of *in vitro* entrainment to LD 12:12 (DD) suggesting circadian control. Error bars represent SEM, and numbers in parentheses are the number of patches tested.

(C) Changes in channel affinity are not direct effects of light. Data are mean K_D s obtained from cells cultured in continuous darkness (DD), in continuous light (LL), or from the light or dark phases in cells exposed to aperiodic LD protocols for 5 days *in vitro*. The aperiodic LD cycle was exposed cells to a total of 60 hr of light during the 5-day culture period but was designed to prevent entrainment of circadian clocks. These cells were never exposed to an entraining LD 12:12 cycle prior to electrophysiology.

(D) Robust changes in mean K_D occur in cells entrained to LD 12:12 cycles *in ovo*. Eggs were entrained to LD 12:12 for 5 days, and cells were then dissociated and cultured in DD for 2 days. Recordings were made at ZT4–7 and ZT16–19 on the 2nd day of DD *in vitro*.

ity of cGMP-gated channels for their activating ligand, but does not regulate binding stoichiometry or produce an acute effect on the density of functional plasma membrane channels.

Previous studies have shown that rod-type cGMP-gated channels exhibit increases in apparent ligand affinity with time after patch excision because of changes in the phosphorylation state of the channels (Gordon et al., 1992; Molokanova et al., 1997) and/or changes in the binding of other ligands (Hsu and Molday, 1993). We have also observed a significant ($p < 0.05$) increase in the apparent affinity of cGMP-gated channels with time

after patch excision, but only in recordings made during the subjective day (Figure 3C). In contrast, the K_D for channel activation remained stationary when patches were excised during the subjective night (Figure 3C). In these experiments, the first cGMP concentration-response curves were constructed immediately after patch excision (within 1–4 min) and again 15–20 min later from the same patch. These results indicate that posttranslational modification of plasma membrane cGMP-gated channels underlies the circadian changes in their affinity for cGMP.

Circadian regulation of ligand affinity by posttransla-

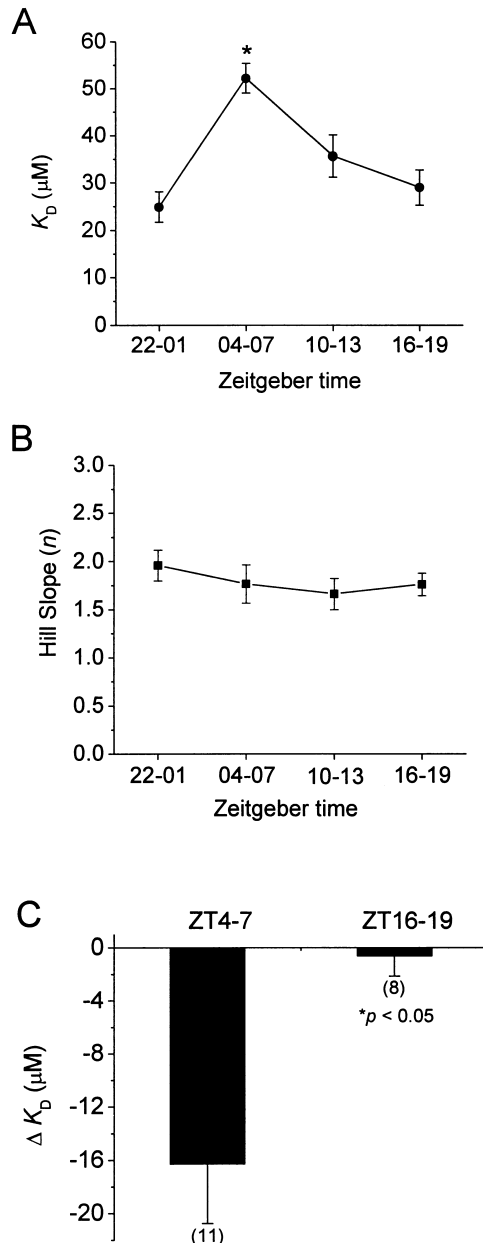


Figure 3. Characteristics of the Circadian Changes in Channel-Gating Properties

(A) Mean K_D obtained at four different times of day in cone photoreceptors on the 2nd day of DD. Note the significant increase in mean K_D during the middle of the subjective day.

(B) From the same patches, the mean Hill slope did not change as a function of the time of day.

(C) Nonstationarity of channel-gating properties depends on the time of day. Channels in patches excised during the subjective day (ZT4–7) exhibited a gradual increase in affinity for cGMP (a negative ΔK_D shown on the ordinate) that is apparent by 15–20 min after patch excision. In contrast, mean K_D did not change after patch excision during the subjective night (ZT16–19). Note that the channels studied during the night were already in their high-affinity state.

tional modification raises the possibility that this phenomenon is mediated by one or more clock-controlled protein kinases. Moreover, the activity of the MAP kinase Erk shows a peak of activity occurring during the subjective night in chick pineal photoreceptors (Sanada et al.,

2000). We have observed a similar pattern in chick retinal cells on the 2nd day of DD in ovo (Figure 4A) or in vitro (Figure 4B). In the in ovo experiments, E6 eggs were placed in incubators and exposed to LD 12:12 for 5 days followed by 2 days of DD. For in vitro experiments, retinæ were dissociated at E6 and were cultured under LD 12:12 for 4 days followed by 2 days of DD. The activity of diphosphorylated Erk was examined at various times on the 2nd day of DD by immunoblot analysis. We observed a greater ratio of diphosphorylated Erk (the active form of the enzyme) to total Erk during the middle of the subjective night than the subjective day in cells developing in ovo (Figure 4A) or in vitro (Figure 4B). It bears noting that cultures of retinal cells dissociated at E6 are highly enriched in oil droplet-containing photoreceptor cells as originally noted by Adler and Hatlee (1989). The majority of the remaining cells appear spherical and undifferentiated, and only a small number of cells exhibit bipolar or multipolar neuronal phenotypes. This suggests that at least a component of the rhythmic signal in cultured retinal cells is derived from photoreceptors, but the caveat remains that these measurements were made from cultures containing several cell types.

On the other hand, if rhythms in Erk activity play a role in regulating cGMP-gated channels, then perturbation of these rhythms would be expected to alter the gating behavior of these channels. This hypothesis also predicts larger effects on channel gating at times of day when Erk activity is normally high. In order to test this hypothesis, cultured retinal photoreceptors were entrained to LD 12:12 for 4 days in vitro and then switched to DD. On the 2nd day of DD, cells were treated at ZT3 or ZT15 with 50 μ M PD98059, a selective inhibitor of MEK1, the enzyme required for phosphorylation and activation of Erk in most signaling pathways (Alessi et al., 1995). Inside-out patch recordings were made after 1.5–2.0 hr of drug treatment. Treatment with PD98059 caused channels to exhibit a lower ligand affinity compared with controls ($p < 0.001$) when the drug was applied during the subjective night (ZT16–17), but not when PD98059 was applied during the subjective day (ZT4–5; Figure 4C). Direct biochemical evidence for the efficacy of PD98059 is presented later in this article. PD98059 had no effect on maximum response amplitude, Hill slope, or other properties of cGMP-gated channels (data not shown). Treatment with vehicle control (saline containing 0.5% dimethyl sulfoxide [DMSO]) had no effect on channel affinity at any time of day. It also bears noting that acute application of PD98059 had no effect on the behavior of cGMP-gated channels.

We have also observed a circadian rhythm in CaMKII phosphorylation in chick retinal cells developing in ovo and in vitro. As with experiments on Erk phosphorylation, it bears noting that these biochemical measurements were made from samples containing multiple cell types. We observed a greater ratio of phosphorylated (Thr-286) CaMKII α subunit (the active form of CaMKII) to total Erk (used as a loading control) during the subjective day than during the subjective night both in ovo (Figure 5A) and in vitro (Figure 5B). These results are consistent with a circadian rhythm in the activity of this protein kinase, approximately antiphase to the Erk MAP kinase rhythm. In order to determine whether a CaMKII activity rhythm plays a role in regulating cGMP-gated channels, cultured retinal photoreceptors were entrained to LD

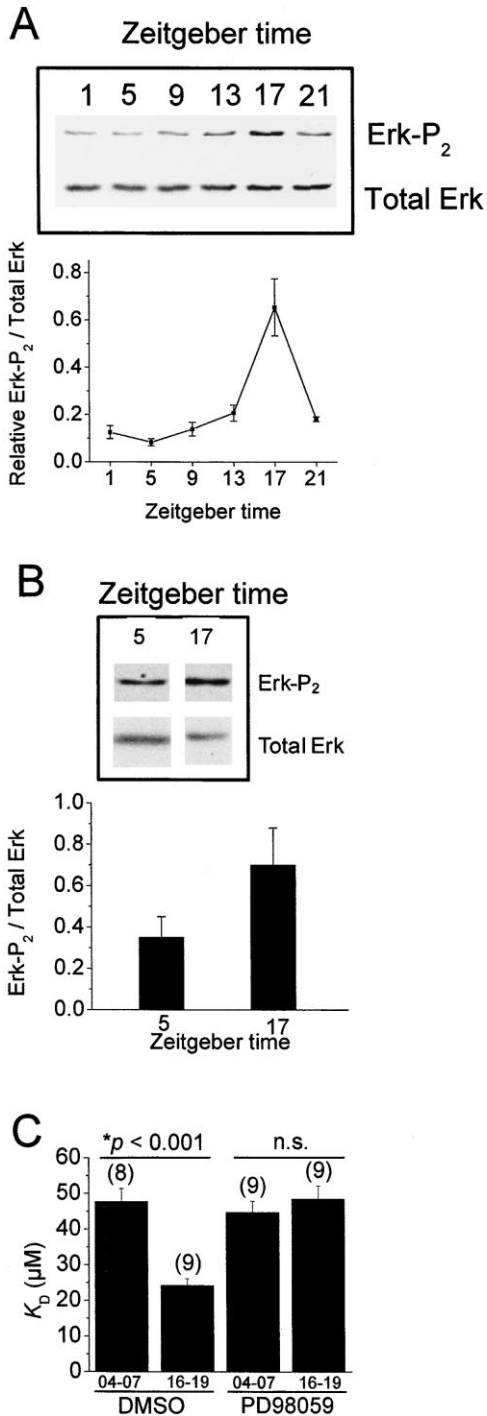


Figure 4. Erk Mediates Circadian Changes in Channel-Gating Properties

(A) Immunoblot analysis of Erk phosphorylation in whole retina at different times on the 2nd day of DD. The top panel shows results of a typical experiment in which a single blot was probed with an antibody against diphospho-Erk (Erk-P₂), stripped, and reprobbed with an antibody against total Erk. Note the increase in Erk-P₂ signal relative to total Erk during the subjective night (ZT13–21). The graph in the lower panel shows the results from three such experiments. (B) A similar pattern is observed in cultured photoreceptors on the 2nd day of DD after 4 days of in vitro entrainment to LD 12:12. (C) Inhibition of Erk signaling during the subjective night, but not during the subjective day, alters the affinity of cGMP-gated channels

12:12 for 4 days in vitro and were then switched to DD. On the 2nd day of DD, cells were treated at ZT3 and ZT15 with the structurally dissimilar CaMKII inhibitors KN-62 or KN-93, or with KN-92, an inactive analog of KN-93. Drugs were applied at a concentration of 10 µM, and inside-out patch recordings were made after 1.5–2.0 hr of drug treatment. Treatment with either KN-62 or KN-93 caused the channels to move toward a higher apparent ligand affinity ($p < 0.001$) when the drugs were applied during the subjective day (ZT4–5) but not during the subjective night (ZT16–17; Figure 5C). The inactive analog KN-92 had no effect on channel gating at either time of day. The two structurally distinct CaMKII inhibitors and their inactive analog had no effect on maximum response amplitude, Hill slope, or other properties of cGMP-gated channels. Taken together, these data suggest that CaMKII is part of the circadian output pathway that leads to modulation of cGMP-gated channels in cones.

Do Erk MAP kinase and CaMKII interact in chick cone photoreceptors? In order to test this hypothesis, retinal photoreceptors were entrained to LD 12:12 for 5 days in ovo, dissociated, and cultured in DD. On the 2nd day of DD, cells were treated at ZT3 and ZT15 with the CaMKII inhibitor KN-93 (10 µM), the inactive analog KN-92 (10 µM), the MEK1 inhibitor PD98059 (50 µM), or 0.5% DMSO (a vehicle control for PD98059) (Figure 6). Cells were harvested 1.5–2.0 hr after drug treatment and were used for immunoblot analysis of Erk and CaMKII phosphorylation. Treatment with the CaMKII inhibitor KN-93 eliminated the daytime increase in CaMKII phosphorylation and caused a marked reduction in CaMKII phosphorylation at both times of day. However, this inhibitor had no effect on the circadian rhythm in Erk phosphorylation (Figure 6A). As expected, the inactive analog KN-92 had no effect on either protein kinase at either time of day. In contrast, treatment with the MEK1 inhibitor PD98059 virtually eliminated Erk phosphorylation at both times of day and also blocked the daytime increase in CaMKII phosphorylation such that circadian rhythms in this enzyme could no longer be detected. Phosphorylated CaMKII was detectable but present at low levels at both times of day in PD98059-treated cells, that is, at levels comparable to those observed during the nighttime in vehicle-treated control cells (Figure 6B). These results suggest that the circadian rhythm of Erk activity drives a rhythm in CaMKII activity such that the maximum activities of these enzymes are in antiphase. Consistent with these biochemical data, inhibitors of these kinase pathways exert opposing effects on channel-gating behavior.

for cGMP. Cells were treated with the MEK1 inhibitor PD98059 (50 µM) or vehicle control (0.5% DMSO) 1.5–2.0 hr before inside-out patch recordings were made during the subjective day or the subjective night, as indicated. Cells were on the 2nd day of DD at the time of drug treatments and electrophysiology. Note the robust rhythm in mean K_D in DMSO-treated control cells. In contrast, the circadian rhythm in K_D was abolished in PD98059-treated cells, such that the mean K_D during the nighttime was increased to levels normally observed during the subjective day. PD98059 treatment had no effect during the subjective day.

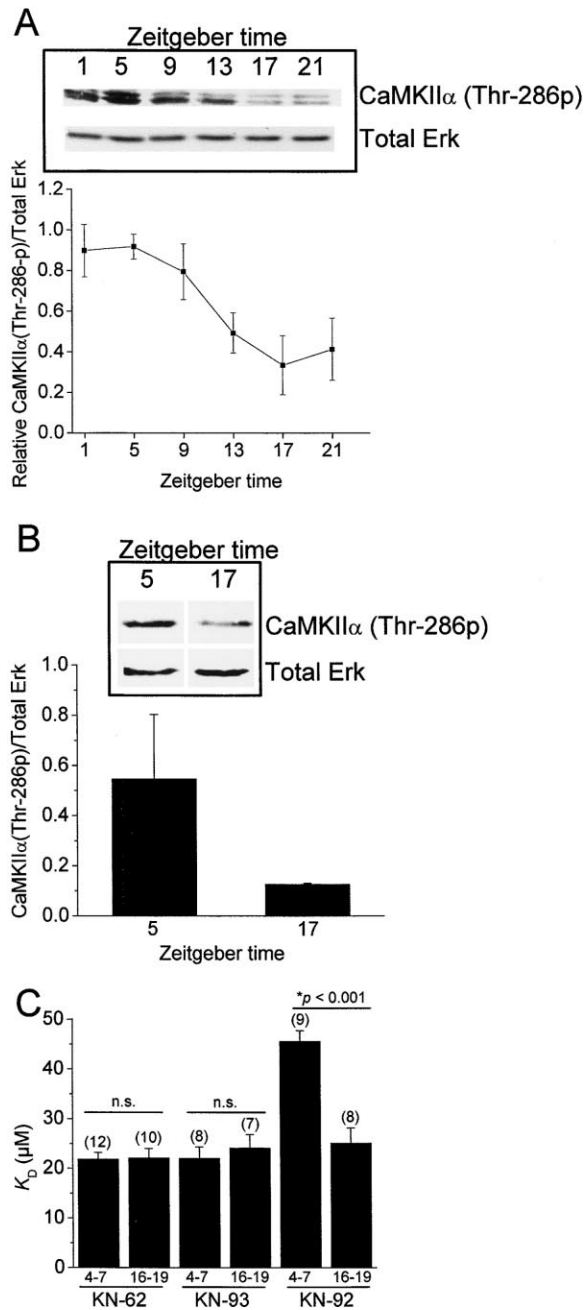


Figure 5. Role of CaMKII in Mediating Circadian Changes in Channel-Gating Properties

(A) Immunoblot analysis of CaMKII α subunit phosphorylation in whole retina at different times on the 2nd day of DD. The top panel shows results of a typical experiment in which a single blot was probed with an antibody against phosphorylated CaMKII- α (Thr-286-phosphate) and was stripped and reprobbed with an antibody against total Erk, used as a loading control. Note the increase in phosphorylated CaMKII signal relative to total Erk signal during the subjective day (ZT1–9). The graph in lower panel shows results from three such experiments.

(B) A similar pattern is observed in cultured photoreceptors on the 2nd day of DD.

(C) Inhibition of CaMKII signaling during the subjective day, but not during the subjective night, alters the affinity of cGMP-gated channels for cGMP. Cells were treated with KN-62 and KN-93 (10 μ M) or with KN-92 (10 μ M), an inactive analog of KN-93. Drugs were

Discussion

In this study, we have demonstrated that cGMP-gated cationic channels of chick cones exhibit a circadian rhythm in affinity for their activating ligand. The apparent affinity of the channels for cGMP is substantially higher during the subjective night than during the subjective day in photoreceptors maintained on LD 12:12 cycles, as well as in photoreceptors free running on the 2nd day of DD after entrainment to LD 12:12. These changes occur when photoreceptors are entrained either in ovo or in vitro, do not represent direct effects of light, and clearly entail posttranslational modifications of the channel molecules.

The phosphorylation of two different signaling enzymes, Erk MAP kinase and the α subunit of CaMKII, also shows a circadian rhythm in chick photoreceptors such that Erk phosphorylation is several-fold greater during the subjective night, whereas CaMKII α subunit phosphorylation is several-fold greater during the subjective day. Because these enzymes are active only when phosphorylated, these data suggest that the peak activities of Erk and CaMKII are approximately antiphase throughout the circadian cycle in retinal photoreceptors. Consistent with these observations, inhibition of Erk signaling during the subjective night (when Erk phosphorylation is maximal) causes the channels to move from their normal high-affinity state to a low-affinity state characteristic of the subjective day. Inhibition of Erk signaling during the day has no effect on channel gating. Conversely, inhibition of CaMKII signaling during the subjective day, when this enzyme is maximally active, causes the channels to move from a low-affinity state to the high-affinity state more typically seen during the nighttime. Inhibition of CaMKII signaling during the nighttime has no effect on channel gating behavior.

These data are consistent with a model in which both Erk and CaMKII are components of the output pathway leading from the core circadian oscillator to the cGMP-gated channels (Figure 7A). This diagram shows CaMKII downstream from Erk, which is based on the observation that acute inhibition of Erk signaling abolishes the daytime increase in CaMKII phosphorylation, thereby causing this enzyme to remain in its nighttime state throughout the day. In contrast, inhibition of CaMKII has no effect on the Erk phosphorylation rhythm. It bears noting that the number of steps that intervene between the core circadian oscillator, Erk, and CaMKII is not known. However, because the peak activities of these enzymes are in antiphase, it appears that Erk exerts some type of direct or indirect inhibition of CaMKII activation. These data do not indicate whether one or both of these enzymes act directly on the cGMP-gated channels or whether they instead act indirectly on other molecules that ultimately bind to the channels and lead to modula-

applied 1.5–2.0 hr before inside-out patch recording on the 2nd day of DD. Note the robust rhythm in mean K_D in KN-92 treated control cells but not in cells treated with KN-93 or KN62. The mean K_D during the day was decreased to levels normally observed during the night. Inhibitors of CaMKII had no effect during the subjective night.

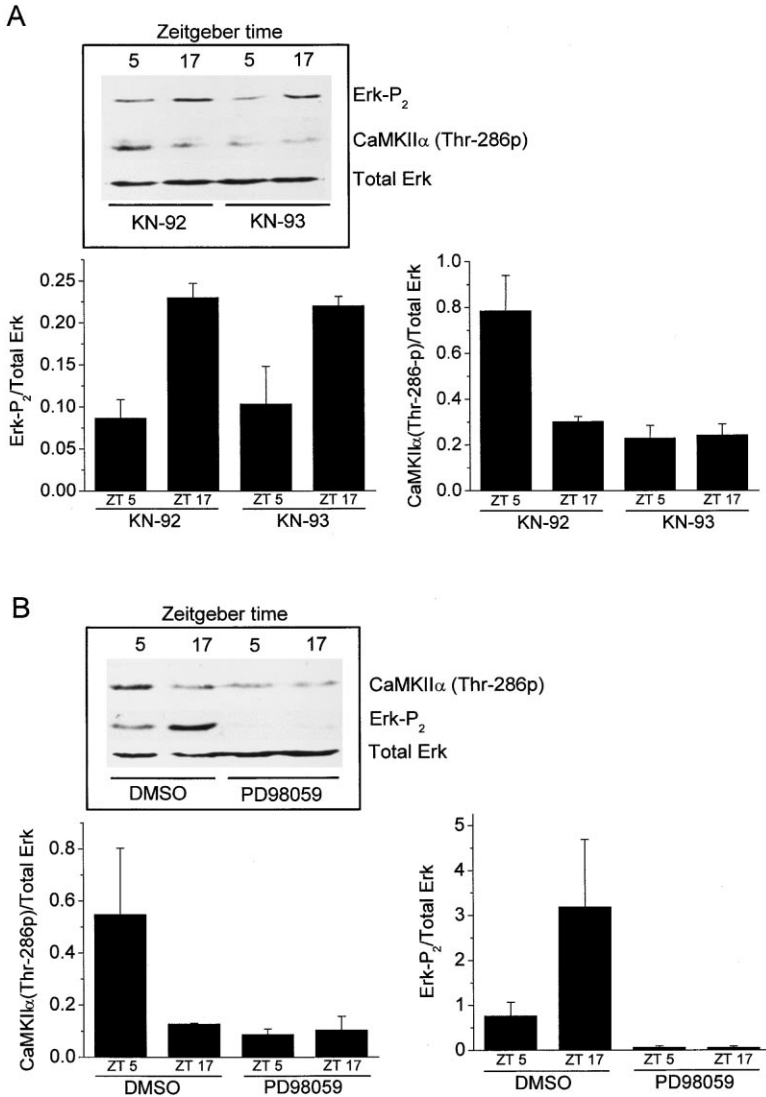


Figure 6. Circadian Rhythm in Photoreceptor Camkii Phosphorylation Is Driven by Erk MAP Kinase

(A) The nocturnal increase in Erk phosphorylation persists after inhibition of CaMKII. Embryos were entrained to LD 12:12 in ovo for 5 days. Retinal cells were then isolated and cultured for 2 days in DD. On the 2nd day of DD, cells were treated with the CaMKII inhibitor KN-93 or the inactive analog KN-92 (10 μ M) during the subjective day or the subjective night as indicated. After 1.5–2.0 hr of drug treatment expression of total Erk, Erk-P₂ and phosphorylated CaMKII were determined by immunoblot analysis. The CaMKII inhibitor KN-93 had no effect on the rhythm in Erk phosphorylation (blot on the top and bar graph to lower left) but abolished the nocturnal increase in CaMKII phosphorylation (blot on the top and bar graph to lower right).

(B) The daytime increase in CaMKII phosphorylation is eliminated by inhibition of Erk signaling. Cells were treated on the 2nd day of DD with the MEK1 inhibitor PD98059 (50 μ M) or vehicle control (0.5% DMSO) during the subjective day or the subjective night. Treatment with PD98059, but not DMSO, eliminated the daytime increase in CaMKII phosphorylation, although signal was still detectable at both times of day (blot on the top and bar graph to lower left). PD98059 caused nearly complete inhibition of Erk phosphorylation at both times of day (blot on the top and bar graph to lower right).

tion of gating. Nevertheless, these data provide more information than is currently available regarding clock control of neuronal or sensory cell membrane properties.

Can a circadian modulation of this magnitude, a roughly 2-fold change in apparent channel affinity, produce physiologically meaningful changes in cone phototransduction? The increase in current predicted from a given change in apparent ligand affinity is given by the following:

$$I_N/I_D = ([cGMP]^n + K_D^n)/([cGMP]^n + K_N^n)$$

where I_N is the current evoked by a given [cGMP] during the subjective night, I_D is the current evoked by the same [cGMP] during the subjective day, K_N and K_D are the apparent affinity constants observed during the night and day, respectively, and n is the Hill coefficient, equal to 2.0 at all times of day (Hackos and Korenbrot, 1997; Rebrik and Korenbrot, 1998). A theoretical plot of I_N/I_D versus [cGMP] is shown in Figure 7B, assuming that $K_N = 30 \mu$ M and $K_D = 50 \mu$ M. These calculations predict a substantially greater dark current during the subjective

night than during the subjective day, especially at the lower range of cGMP concentrations ($\leq 7 \mu$ M in the dark) that are physiological in photoreceptors (Cobbs and Pugh 1985; Cobbs et al., 1985). It should be noted that our measurements have been made in excised membrane patches, as opposed to intact or semi-intact preparations, which raises the possibility that we are actually underestimating the magnitude of circadian changes in channel affinity (Rebrik and Korenbrot, 1998).

These simple calculations assume that no other adaptive processes occur. Clearly, this last assumption is unlikely to be true, as adaptation in rods is associated with Ca^{2+} -dependent regulation of guanylate cyclase (Miller and Korenbrot, 1994; Miller et al., 1994), cGMP phosphodiesterase (Kawamura and Murakami, 1991), and the catalytic efficiency of visual pigments (Lagnado and Baylor, 1994), all of which could contribute to feedback regulation of resting cGMP concentrations. Consequently, it is impossible to predict the precise effects of clock regulation of channel gating on cone photoreponses without a knowledge of a number of other parameters that could themselves be under circadian con-

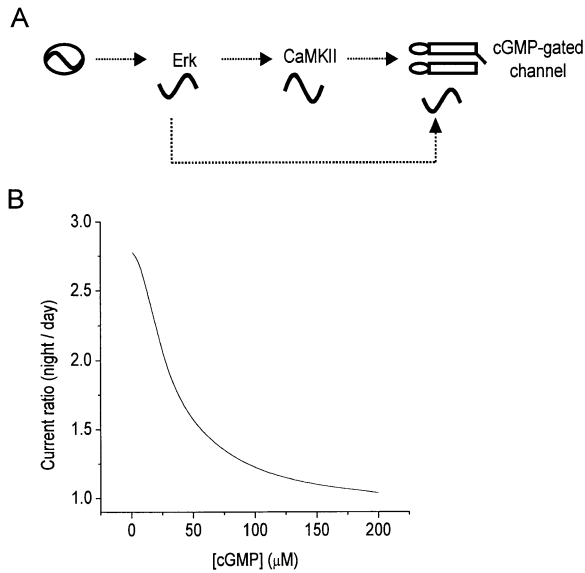


Figure 7. A Model for Circadian Regulation of cGMP-Gated Channels and Potential Physiological Consequences of This Modulation

(A) A rhythm in Erk MAP kinase activation is driven by a circadian oscillator in the photoreceptors. The Erk rhythm drives an antiphase rhythm in CaMKII activation. In this model, both kinases are depicted as regulating the cGMP-gated channels, although this effect is likely to be indirect. The number of steps that intervene between the various model components is not known.

(B) Theoretical plot of potential consequences of changes in the affinity of cGMP-gated channels for their activating ligand. Ordinate shows the relative increase in current that would flow during the subjective night compared with day as a function of cGMP concentrations. Note that the effect of circadian changes in channel affinity is much greater at physiologically free cGMP concentrations (~5 μ M). This plot ignores any other adaptive phenomena that would lead to changes in intracellular cGMP concentration as a function of time of day or changes in background illumination.

trol (Lamb and Pugh, 1992). Nevertheless, a circadian rhythm in channel affinity certainly predicts that phototransduction components will exhibit markedly different dynamic interactions as a function of the time of day. Consistent with this prediction, several features of the photoreceptor components of the avian electroretinogram are known to be under circadian control (Lu et al., 1991; Manglapus et al., 1998; McGoogan and Cassone, 1998). These observations also suggest that studies on the molecular dynamics of phototransduction and adaptation need to control for the time of day as well as the recent illumination history of the experimental preparation.

Circadian oscillators expressed in neurons and sensory cells are known to drive physiologically and behaviorally relevant outputs, including rhythms in spontaneous spike discharge (Corrent et al., 1978; Shibata et al., 1982; Block et al., 1995) and rhythms in sensory cell responses (Cahill and Besharse, 1995; Krishnan et al., 1999). However, relatively few studies have investigated circadian clock regulation of ion channel function. In molluscan basal retinal neurons, circadian changes in spontaneous firing and input resistance appear to be caused by clock regulation of a delayed rectifier K^+ current that inactivates just before the start of the sub-

jective day (Michel et al., 1993). In chick pineal photoreceptors, a clock-regulated cationic channel known as I_{LOT} is active during the subjective night but not during the subjective day (D'Souza and Dryer, 1996). Mammalian suprachiasmatic nucleus neurons show an increase in spontaneous firing during the subjective day that is associated with an increase in resting input resistance (Jiang et al., 1997), although the ionic channels that mediate this effect have not been identified. None of the steps between the circadian clock and the changes in channel gating have been identified in these systems. In the present study, it is clear that clock regulation of cone cGMP-gated channels entails posttranslational modification of the channel molecules, proteins that interact with channel molecules, or both.

We have not yet determined the nature of the posttranslational mechanisms responsible for circadian regulation of cGMP-gated channels. Previous studies of rod-type cGMP-gated channels indicate that direct phosphorylation of the channel molecules (Gordon et al., 1992; Molokanova et al., 1997, 2000a) can evoke changes in ligand affinity similar to those observed here. Examination of the primary sequence of the α subunits of chicken cone cGMP-gated channels (Bonigk et al., 1996) does not reveal any consensus CaMKII phosphorylation sites, but modulation could entail modification of β subunits. Because sequence information on the avian forms of these subunits is not available, we cannot exclude that CaMKII, and possibly Erk, may cause direct modification of the functional channel molecules.

Roenneberg and Merrow (1999) have recently presented models of circadian oscillator systems in which pathways that lead to entrainment of the "core oscillator" (i.e., the input pathways) can themselves be regulated by the oscillators (i.e., they are also components of physiologically relevant output pathways). One feature of this class of models is that they contain additional feedback loops that can markedly enhance the stability of the overall oscillator system. The cGMP-gated channels of rods and cones are essential components of visual phototransduction cascades, and it is possible that they also play a role in the light entrainment of photoreceptor circadian oscillators. Because the gating of cGMP-gated channels is under circadian control in cones, this could represent another example of a single gene product that is both an input to and an output from the clock. Therefore, it is possible that clock regulation of cGMP-gated channels of retinal photoreceptors represents an adaptation to enhance the stability of retinal circadian oscillators.

Experimental Procedures

Cell Isolation and Culture

Chick retinae were dissociated at E6 essentially as described by Adler and Hatlee (1989). This protocol was chosen because it results in a population of cells enriched in photoreceptors (Adler and Hatlee, 1989; Repka and Adler, 1992; Belecky-Adams et al., 1996). Cone photoreceptors, which comprise close to 75% of the cells in these cultures, can be readily identified by morphological criteria, as noted later in this article. Most of the remaining cells are spherical cells that appear to be undifferentiated precursor cells (Repka and Adler, 1992). A small number of cells (<5%) extend one or more neurites and appear to be neurons. For cell dissociation, E6 retinae were dissected and incubated in a solution consisting of 123 mM NaCl,

5.36 mM KCl, 9.51 mM Na₂HPO₄, 1.48 mM NaH₂PO₄, 0.1 g/ml glucose, and 0.5 mg/ml trypsin at 37°C for 25 min and then dissociated by trituration using a fire-polished Pasteur pipette. Retinal cells were grown for 5 days on poly-D-lysine-coated glass coverslips (molecular weight 276,000) in a medium consisting of Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 40 ng/ml recombinant rat ciliary neurotrophic factor. Cell culture incubators (39°C and 5% CO₂) were equipped with lights and timers, which allowed for entrainment of retinal circadian oscillators in vitro, as described previously for studies of chick pineal cells (D'Souza and Dryer, 1996). Sister cultures were maintained in two separate incubator chambers in which LD cycles were 12 hr antiphase so that recordings from cells at different clock phases could be interleaved. Throughout, all times of day are expressed relative to the original entraining LD 12:12 cycle (Zeitgeber time [ZT]). In most experiments, electrophysiology or biochemistry was carried out on the 5th day of exposure to LD 12:12 or on the 2nd day of DD, after 4 days of entrainment to LD 12:12. In order to test for direct effects of light or dark, recordings were made on the 5th day of in vitro exposure to aperiodic LD transitions in which the lights were on for a total of 12 hr each day with individual photoperiods varying from 0.5 to 4 hr or from cells maintained in continuous LL or DD for 5 days after dissociation. In these experiments, cells were never exposed to an entraining LD 12:12 cycle. For in ovo entrainment of circadian oscillators, retinae were excised from E11 embryos obtained from intact eggs exposed for 5 previous days to LD 12:12. Cells were then dissociated, cultured in the dark, and used for electrophysiology on the 2nd day of culture in DD. In other experiments, intact eggs were entrained to LD 12:12 for 5 days and switched to DD, and retinae were excised for immunoblot analyses of Erk phosphorylation or autophosphorylated (Thr-286) CaMKII α subunit on the 2nd day of DD. Dissociation of retinal cells did not cause a detectable change in the phase of circadian rhythms in protein phosphorylation or channel gating behavior.

Electrophysiology

Recordings were made from cells with elongated cell bodies, a truncated outer segment, and one or more prominent oil droplets in the soma. These cells express cone photopigments (Pierce et al., 1993) and can be stained with peanut lectin (Gleason et al., 1992). A substantial number of cone-type cells were obtained in all culture conditions, but this was the predominant cell type after cells dissociated at E6 were kept in culture for 5–7 days (Adler and Hatlee, 1989; Repka and Adler, 1992). Methods for inside-out patch recordings of cGMP-gated channels have been described elsewhere (Dryer and Henderson, 1991, 1993). Recordings were carried out at room temperature (22–23°C). Only one patch was excised from any given cell. Patches were excised from all portions of the soma, including soma regions adjacent to the presumptive synaptic end, the nuclear region, and the regions above the oil droplets. The location from which patches were excised had no obvious effect on maximum response amplitude or affinity for activating ligand at any time of day. Channels were activated by gravity-fed bath application of varying concentrations of cGMP dissolved in bath saline. Approximately half of the successfully excised patches had functional channels in cells dissociated at E6. In some experiments, cultures were pretreated with drugs at ZT3 or ZT15 for 1–2 hr prior to recording. PD98059, KN-62, KN-93, and KN-92 were obtained from Calbiochem (La Jolla, CA). Data were stored on magnetic tape in FM mode prior to off-line digitization at 20 kHz (Axoscope; Axon Instruments, Foster City, CA) and analysis (Fetchan; Axon Instruments). Concentration-response curves were fitted with this Hill equation:

$$I_s = I_{\text{Max}} [S^n / (K_D^n + S^n)]$$

where S is the concentration of cGMP, K_D is the dissociation constant, and n is the Hill coefficient. Fluctuation analysis was carried out as described previously (Cameron and Dryer, 2000). Briefly, currents in each patch were observed in the presence and absence of 50 µM cGMP, and 30 s of data in each condition were digitized at 4 kHz. DC components of the evoked currents were removed. Signals were cosine-tapered, and power spectra were calculated from 131,072 digital samples using routines implemented in Pclamp

software (Clampfit v. 8.0; Axon Instruments). The resulting spectra were subtracted (cGMP spectrum–control spectrum), smoothed by adjacent point averaging, and then fitted with a single Lorentzian curve of this form:

$$S(f) = S(0) / [1 + (2\pi f_c \tau)^2]$$

or as the sum of two Lorentzians. IV curves were generated as described previously (Dryer and Henderson, 1991). All statistical analyses were carried out using Statistica software (Statsoft, Tulsa, OK) and consisted of Student's unpaired t test or one-way analysis of variance followed by Tukey's post hoc test for unbalanced n (when comparisons were made between multiple independent groups). Throughout, p < 0.05 was regarded as significant.

Immunoblot Analysis

Dissociated retinal cells were washed in ice cold phosphate-buffered saline (PBS) and lysed in 2× Laemmli sample buffer. Intact retinae were homogenized in a buffer consisting of PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM sodium molybdate, 50 mM NaF, 2 mM NaPO₄, and 1 mM sodium orthovanadate (pH 7.4). Samples were boiled for 5 min, separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and transferred to nitrocellulose membranes. Membranes were blocked overnight in PBS containing 0.3% Tween-20 and 6.5% non-fat dried milk before incubation with a monoclonal antibody specific for phosphorylated (Thr-286) CaMKII α subunit (RBI/Sigma, St. Louis, MO), a monoclonal antibody specific for diphospho-Erk (Sigma), or a polyclonal antibody insensitive to the phosphorylation state of Erk (Santa Cruz Biochemicals) all at 1:1000 dilution. Blots were analyzed using anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and an ECL detection system (Amersham, Buckinghamshire, UK). The ratio of diphospho-Erk to total Erk, or phosphorylated CaMKII to total Erk, in each sample was determined by densitometry. All experiments were repeated three to six times. In some experiments, analyses of total Erk, diphospho-Erk, and/or phospho-CaMKII were carried out on a single blot, which was stripped and reprobed with multiple antibodies. Chickens appear to express a single form of Erk (on the basis of molecular weight) so that antibodies against diphospho-Erk and total Erk label a single band on Western blots (Sanada et al., 2000). The monoclonal antibodies directed against phosphorylated (Thr-286) CaMKII α subunit often labeled two bands of slightly different molecular weights in our immunoblot analyses of total retina, although only a single band was typically observed in samples isolated from cultured retinal cells. These bands appear to correspond to the two different CaMKII α subunit splice variants described previously in chicken (Li et al., 1998).

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