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Invest Ophthalmol Vis Sci. Author manuscript; available in PMC 2008 May 12.

Published in final edited form as:
Invest Ophthalmol Vis Sci. 2007 February ; 48(2): 901–906.

Tyrosine Phosphorylation of cGMP-Gated Ion Channels Is under Circadian Control in Chick Retina Photoreceptors

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Abstract

Purpose—To investigate the role of tyrosine phosphorylation in circadian regulation of cGMP-gated cation channels (CNGCs) of chicken cone photoreceptors.

Methods—Chick retinas were studied on the second day of constant darkness (DD) after several days of entrainment to 12:12 hr light–dark (LD) cycles in vitro. Inside-out patch recordings were made during the subjective day and subjective night to quantify circadian changes in the sensitivity of CNGCs to activation by cGMP after treatment with various tyrosine kinase and tyrosine phosphatase inhibitors. Immunoprecipitation and immunoblot analysis were also used to examine tyrosine phosphorylation of CNGCs and closely associated proteins after separation by conventional and two-dimensional SDS-PAGE.

Results—Treatment with tyrosine kinase inhibitors caused a significant decrease in $K_{1/2}$ for cGMP activation of CNGCs in patches excised from cones during the subjective day, but had no effect on $K_{1/2}$ during the subjective night. Conversely, treatment with a tyrosine phosphatase inhibitor caused a significant increase in the $K_{1/2}$ of CNGCs in patches excised during the subjective night but had no effect on channel $K_{1/2}$ during the subjective day. Broad spectrum serine-threonine phosphatase inhibitors had no effect. An 85-kDa tyrosine polypeptide that coimmunoprecipitated with CNGC α-subunits was detectable at higher levels during the subjective day than during the subjective night. CNGC α-subunits were not tyrosine phosphorylated as a function of the time of day.

Conclusions—Circadian control of cone CNGCs appears to entail elevated daytime tyrosine phosphorylation of an ~85-kDa auxiliary protein or another subunit of the CNGCs.

Visual systems must detect contrasts in the face of large daily changes in ambient illumination. Circadian oscillators in the retina provide a mechanism that allows the visual system to anticipate these daily changes in photon flux by modulating the structure and physiology of the retina.[1,2] Retinal circadian oscillators control retinomotor movements,[3–7] outer segment disc shedding[8] and membrane renewal,[1,9] morphologic changes at synaptic ribbons,[10] melatonin synthesis and release,[11–13] gene expression,[14–18] activities of protein kinases and small GTPases,[19,20] and gating properties of ionic channels.[19] At least some of these processes are controlled by circadian oscillators within the photoreceptors themselves.[11,16,19]

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Disclosure: **K.-S. Chae**, None; **G.Y.-P. Ko**, None; **S.E. Dryer**, None

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We have reported that circadian oscillators within photoreceptors modulate cGMP-gated ion channels (CNGCs) in chick cones.[19–21] The apparent affinity of CNGCs for cGMP is significantly higher during the subjective night than during the subjective day, such that considerable changes in channel gating would be expected to occur at physiological concentrations of cGMP.[19,20] Circadian regulation of CNGCs is a posttranslational process, and it qualitatively resembles effects that others have observed after phosphorylation on specific CNGC serine-threonine or tyrosine residues or binding of calcium and calmodulin.[22–24] The circadian modulation of CNGCs is driven in part by a nocturnal rhythm in intracellular cAMP, which in turn drives the activation of PKA, Ras, MEK, and Erk.[19–21] We have found that the pathway from cAMP to Erk occurs rapidly, in less than 10 minutes, whereas the modulation of CNGCs does not occur until ~2 hours after activation of adenylate cyclase.[19–21] The details of the late stages of the posttranslational events leading to modulation of CNGC gating are unknown. However, it is likely that these steps entail posttranslational modification of the channels themselves or their associated proteins.

We report that one of the final steps in the circadian regulation of CNGCs is the tyrosine phosphorylation of a protein that is tightly associated with the CNGC α subunit. Tyrosine phosphorylation of this CNGC-associated protein is higher during the subjective day (when the apparent affinity for cGMP is lowest), whereas phosphorylation is significantly lower during the subjective night.

Materials and Methods

Cell Isolation and Culture

Chick retinas were dissociated at embryonic day (E)6 and cultured as described previously.[19–21] Cultures prepared in this way in the presence of ciliary neurotrophic factor (R&D Systems, Minneapolis, MN) yield a highly enriched population of cone photoreceptors.[25,26] Cell culture incubators (39°C and 5% CO_2) were equipped with timers and lights, which allowed for the entrainment of retinal circadian oscillators to 12-hour light–dark (LD) cycles in vitro or in ovo, as described previously.[19–21] All measurements were made on the second day of constant darkness (DD) after 4 to 5 days of entrainment to LD cycles.

Electrophysiology

Recordings were made from cells with elongated cell bodies, an outer segment, and one or more prominent oil droplets on the distal side of the soma, as described in detail elsewhere.[19–21] Inside-out patch recordings of CNGCs were made on the second day of DD, after 4 days of prior entrainment to LD cycles. Briefly, recordings were made in symmetrical 145 mM NaCl, 10 mM Na-HEPES, 10 mM glucose, and 1 mM EGTA (pH 7.4), at −65 mV at room temperature (22–23°C), and under normal visible light illumination. Cyclic GMP concentration–response curves were fitted with the Hill equation: $I_s = I_{max}\,[S_n/(K_D{}^n + S_n)]$ where S is the concentration of cGMP, K_D is the dissociation constant, and n is the Hill coefficient. Statistical analyses consisted of one-way ANOVA followed by the Tukey post hoc test for unbalanced n. Throughout, $P < 0.05$ was regarded as significant.

Preparation of Retinal Membrane Proteins and Immunoprecipitation

On the second day of DD after 5 days of entrainment in ovo, four retinas for each group were isolated and homogenized in a 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM sodium molybdate, 50 mM NaF, 2 mM $NaPO_4$, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma-Al-drich, St. Louis, MO) and centrifuged (14,000*g*, 20 minutes, 4°C). Pelleted membrane fragments were resuspended, solubilized in buffer A consisting of RIPA buffer (20 mM Tris-HCl, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS;

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pH 7.4]) with 1% Triton X-100, 10 mM sodium molybdate, 50 mM NaF, 2 mM $NaPO_4$, 1 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail for 4 hours at 4°C with gentle shaking and centrifuged (14,000*g*, 20 minutes, 4°C), and then the supernatant was processed for immunoprecipitation. Solubilized retina membrane proteins were first incubated with 5 μg of normal rabbit IgG and 20 μL of protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) to eliminate nonspecific binding. The precleared membrane proteins and 5μg of antibody against the chicken cone photoreceptor CNGC α-subunit were mixed in a microtube and inverted gently for 1 hour, and then 20 μL of protein A/G plus-agarose was added and incubated for another hour. Agarose beads were spun down (10,000*g*, 5 minutes, 4°C) and washed with buffer A three times. Proteins were eluted from precipitated agarose beads in 2× Laemmli buffer and boiled (95°C, 5 minutes) for SDS-PAGE. For two-dimensional (2D)-PAGE, the loaded proteins were a pool of several immunoprecipitates.

Immunoblot Analysis

This procedure has been described in detail elsewhere.[19–21] Briefly, whole-cell extracts of retinas or immunoprecipitated retinal membrane proteins were lysed in 2× Laemmli buffer. Samples were boiled for 5 minutes, separated by SDS-PAGE on 10% gels, transferred to nitrocellulose membranes, blocked overnight in a Tris-buffered saline solution containing 0.1% Tween-20 and 3% nonfat dried milk, and then incubated with an antibody against phosphotyrosine (Cell Signaling Technology, Beverly, MA) or chicken cone photoreceptor α-subunit. Blots were visualized using anti-rabbit secondary antibodies conjugated to horseradish peroxidase and an enhanced chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) capable of detecting proteins at the femtogram level, which is approximately 1000 times more sensitive than standard chemiluminescent substrates. Protein bands on the blots were quantified using Scion Image (available by ftp at zippy.nimh. nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). All experiments were repeated three times, and the data reported as the mean ± SEM. Two dimensional-SDS-PAGE analysis of immunoprecipitates from retinal membrane proteins was performed at the Kendrick Laboratory Inc. (Madison, WI) according to the method of O'Farrell.[27] Briefly, protein samples from a pool of five immunoprecipitates for each group were resolved by isoelectric focusing on 20-cm immobilized pH gradient strips (pH 4–8) followed by SDS-PAGE gels (10%) for two-dimensional separation. The pH gradient for the first dimension was determined by a surface pH electrode. After electrophoresis, the proteins on the gels were transferred to PVDF membranes by standard protein transferring methods and immunoblot analysis proceeded.

Results

Tyrosine Kinases and Phosphatases and the Circadian Modulation of Cone CNGCs

Chick cone photoreceptors were entrained to 12-hour LD cycles for 4 to 5 days in vitro and then switched to constant darkness (DD). On the second day of DD, inside-out patches were excised from cone photoreceptors during the subjective day (circadian time [CT] 4–7) or the subjective night (CT 16–19). Cyclic GMP concentration–response curves were determined immediately after patch excision. As described previously,[19–21] the average apparent $K_{1/2}$ for cGMP estimated from fitted Hill curves was significantly greater in patches excised during the subjective day than during the subjective night (Fig. 1A, control), and the apparent affinity of cGMP-gated ion channels (CNGCs) for their normal activating ligand was under circadian control. As noted previously, the average maximum current density elicited by 200 μM cGMP and the Hill slope for channel activation do not change throughout the day.[19,20]

The cGMP sensitivity of rod photoreceptor CNGCs is modulated by tyrosine phosphorylation and dephosphorylation.[23,28–31] Treatment with the tyrosine kinase inhibitors genistein,

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lavendustin A, or erbstatin increased the apparent affinity of CNGCs for cGMP as indicated by a decrease in the $K_{1/2}$ of concentration–response curves, whereas inhibition of endogenous tyrosine phosphatases by pretreatment with pervanadate or orthovanadate caused a decrease in cGMP sensitivity.[28,29] While lavendustin A and erbstatin do not affect the CNGC maximum current amplitude evoked by saturating cGMP, genistein inhibits the maximal current by 50% to 85%.[28,29] Therefore, we used genistein and lavendustin A to test whether phosphorylation by tyrosine kinases contributes to circadian modulation of CNGCs in cone photoreceptors. In these experiments, drugs were applied to intact cells 2 hours before inside-out patch recordings were made from cones on the second day of DD. Treatment with genistein (10 μM) or lavendustin A (10 μM) caused a significant decrease in the $K_{1/2}$ of CNGCs in patches excised during the subjective day (CT 4–7) but had no effect on channel $K_{1/2}$ during the subjective night (Fig. 1A). Conversely, we observed that treatment with a tyrosine phosphatase inhibitor, sodium orthovanadate (200 μM), for 2 hours caused a significant increase in the $K_{1/2}$ of CNGCs in patches excised during the subjective night (CT 16–19) but had no effect on channel $K_{1/2}$ during the subjective day (Fig. 1B). We obtained similar results with pervanadate treatment, which also inhibits tyrosine phosphatases (data not shown). These data raise the possibility that tyrosine phosphorylation and dephosphorylation of CNGCs is under circadian control in cone photoreceptors. It is worth noting that Gordon et al.[24] showed that serine-threonine phosphatases (PP1 and PP2A) modulate the apparent cGMP sensitivity of CNGCs in rod photoreceptors. Therefore, we tested whether inhibition of PP1 and PP2A cause a circadian phase-dependent modulation of CNGCs. Treatment with okadaic acid (1 μM) or calyculin A (50 nM) for 2 hours did not have any effect on the $K_{1/2}$ of cone CNGCs in patches excised during either the subjective night (CT 16–19) or the subjective day (CT 4–7; Fig. 1C). Thus, it does not appear that PP1 and PP2A are involved in CNGC sensitivity to cGMP in cone photoreceptors.

Circadian Control of Tyrosine Phosphorylation of a CNGC-Associated Protein

The following experiments directly addressed our hypothesis that tyrosine phosphorylation and dephosphorylation of CNGCs is under circadian control, as suggested by pharmacological experiments. In these experiments, retinal membrane proteins were solubilized and immunoprecipitated with an antibody against the chicken cone photoreceptor CNGC α-subunit. Profiles of tyrosine-phosphorylated proteins in immunoprecipitates were then analyzed by immunoblotting with an anti-phosphotyrosine antibody. We detected an 85-kDa band with the anti-phosphotyrosine antibody in retinas collected during the subjective day (CT 5). However, this band was present at a significantly lower intensity in retinas collected during the subjective night (CT 17, $n = 3$ for each blot, Figs. 2B, 2C). Of note, in this experiment, signal from the CNGC α-subunit was not significantly different as a function of the time of day, and we could not detect phosphorylation of tyrosine residues on that subunit (Fig. 2B). It is also important to note that we observed a similar pattern with several different combinations of detergents, including 1% NP-40, 1% Triton X-100, RIPA, or RIPA+Triton X-100, as well as with incubation times ranging from 1 hour to overnight. These results indicate that the circadian clock-regulated phosphoprotein observed in these experiments is tightly associated with the CNGC α-subunit, as the association persists under quite harsh conditions. Silver staining of membrane and immunoprecipitated protein samples indicate that total protein between CT 5 and CT 17 samples were loaded evenly (Fig. 2A).

In an additional set of experiments, we used 2D-PAGE to further characterize the tyrosine phosphoproteins that coimmunoprecipitated with the CNGC α-subunit. This analysis resolved the phosphotyrosine band into two separate proteins (Fig. 3A, symbols) with slightly different molecular weights that were completely distinct in mass and isoelectric point from the CNGC α-subunit. Both spots were present at greater intensity at CT 5 than at CT 17 (Fig. 3A). The lower molecular weight tyrosine phosphoprotein (*) may be glycosylated, since it has a strong

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ellipsoid appearance typically associated with glycosylated proteins. It is possible that the two signals represent two different posttranslational modifications of the same protein, or they may be splice variants. At this time, we have not been able to obtain enough of these proteins to identify them by conventional proteomic methods (Figs. 2A, 3B). As noted earlier, samples for 2D-PAGE were a pool of at least five immunoprecipitate profiles, and immunoblots became visible only through the use of an enhanced chemiluminescent substrate. Nonetheless, the available biochemical results are consistent with the possibility that these bands could be CNGC β-subunits. However, these proteins have not been characterized in birds, and antibodies are not currently available for the CNGC β-subunit.

Discussion

The CNGCs of chick cone photoreceptors are under circadian control, so that cGMP activates CNGCs with a lower $K_{1/2}$ during the subjective night.[19–21] Computational[19,32] and experimental[33,34] evidence suggests that modulation of CNGCs should markedly affect the dynamics of phototransduction, at least in part by altering the dynamic range over which the photoreceptors can operate.[32] Circadian regulation of CNGCs is the final step of an output pathway comprised in part by cAMP, PKA, Ras, and Erk.[19–21] Moreover, the pathway from cAMP to Erk is rapid, because increased Erk phosphorylation can be detected within minutes of adenylate cyclase activation. By contrast, the physiological modulation of CNGCs does not occur until ~2 hours later,[20,21] suggesting the existence of either a large number of additional steps or significant diffusion barriers. The circadian changes in CNGC gating appear to entail a posttranslational change on the channel complex, because they gradually subside over a period of tens of minutes after patch excision.[19] In the present study, we present data suggesting that one of the final steps in the circadian regulation of CNGCs is the tyrosine phosphorylation of proteins tightly associated with the CNGC α-subunit. Tyrosine phosphorylation of these CNGC associated proteins changes during the circadian cycle in constant darkness, and is higher during the subjective day, a time when the apparent affinity of CNGC channels for cGMP is at its lowest. In addition, we observed that the pore-forming α-subunits of CNGCs do not appear to be regulated by tyrosine phosphorylation.

Molokanova et al.[28,29,35] previously showed that the gating properties of CNGCs in rod photoreceptors are modulated by tyrosine phosphorylation. In our study, treatment with the same tyrosine kinase and phosphatase inhibitors respectively increased and decreased the apparent affinity of cone photoreceptor CNGCs for cGMP measured using electrophysiological methods. The CNGCs of rods and cones contain structurally similar but distinct α- and β-subunits.[36–38] Phosphorylation of tyrosine 498 on the rod CNGCα1 subunit or tyrosine1097 on the CNGC β1 subunit causes a decrease in the apparent affinity for cGMP, in part by reducing Ca^{2+}/calmodulin binding to CNGCs.[31] However, the tyrosine phosphorylation site on the rod β1 subunit appears to play a more important role in Ca^{2+}-calmodulin modulation than does that on the α1 subunit, since heteromeric CNGCα1-CNGCβ1 channels expressed in oocytes are inhibited by Ca^{2+}/calmodulin binding, whereas homomeric CNGCα1 channels are unaffected.[31] Calcium-calmodulin modulation of cone CNGCs is also β-subunit dependent.[39]

The pattern of tyrosine phosphorylation of the proteins tightly associated with the chick cone CNGCα subunit as well as the changes in gating properties, suggests a model where tyrosine phosphorylation of the β-subunit produces gating effects similar to those observed in rods. In addition, we found that tyrosine phosphorylation of these proteins is under circadian control. Thus, we observed that inhibition of tyrosine kinases during the subjective day increased the apparent affinity of CNGCs for cGMP, whereas inhibition of tyrosine phosphatases during the subjective night produced the opposite effect. Also, the molecular weight of the clock-controlled phosphoprotein and its persistent association with the α-subunits even after strong

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detergent treatments strongly suggest that these bands represent CNGC β-subunits.[40,41] Unfortunately, we have been unable to obtain enough of these proteins to allow sequencing by standard proteomic methods. Moreover, avian CNGC β-subunits have not been characterized yet, and appropriate antibodies and other reagents are not available.

These data do not address whether this CNGCα-associated protein is coupled with CNGCα and then becomes phosphorylated, whether tyrosine phosphorylation of this CNGCα-associated protein causes the coupling, or whether it is a combination of both. However, we favor the first simply because the phosphorylation of the CNGCα-associated protein persists in harsh detergent treatment. Normally, we could analyze the precipitates after SDS-PAGE with a total protein stain to determine whether the ~85-kDa CNGC-associated proteins remain associated at CT 5 and CT 17 regardless of the phosphorylation state. Unfortunately, the low abundance of the CNGCα-associated protein makes it impossible to visualize this protein even by silver staining procedures, and so we were unable to readily visualize any bands at the appropriate molecular weight. Limitations in protein amount also prohibited us from analyzing the effects of tyrosine kinase and phosphatase inhibitors on tyrosine phosphorylation of these CNGC-associated proteins in cone cultures. Also, the in vivo administration of any inhibitors would require opening the egg shells or using shell-less cultures, then injecting directly into the eyes, which requires the use of light. Exposing the retinas to light during the DD period could cause light-induced modulation of CNGC phosphorylation[42] and circadian phase-shifting.[43–45] In this case, we would not be able to determine whether any changes seen on CNGC phosphorylation is a light effect, circadian phase-shifting (as circadian input changes), or circadian regulation (as circadian outputs). However, we demonstrated that the circadian entrainment of cone photoreceptors in vitro and in ovo both showed the same patterns in CNGC affinity and MAP kinase pErk activity rhythms using patch-clamp recordings and Western blot analysis.[19] In subsequent studies on the signaling pathway of the circadian regulation of CNGCs and the effects of dopa-mine on CNGC rhythms, we demonstrated that circadian entrainment in cultured photoreceptors (in vitro) and in chick embryos (in ovo) showed the same patterns.[20,21] Therefore, we are confident that our in vitro and in vivo results obtained in this study correlate.

Previous studies have implicated a role for tyrosine kinases in circadian regulatory mechanisms. In the eye of the marine mollusk *Bulla gouldiana*, tyrosine kinase inhibitors cause a phase-dependent shift of the core oscillator.[46] In the mammalian suprachiasmatic nucleus, the nonreceptor Src-family tyrosine kinase Fyn appears to be involved in the mechanics of the circadian core oscillator, as $Fyn^{-/-}$ mutant mice have a significantly longer circadian period than that of normal mice.[47] Moreover, Src-family tyrosine kinases have been shown to be activated in the retina on photic stimulation.[48] At the moment, it is not clear which circadian output pathway(s) tyrosine kinases and phosphatases are a part of, but there are a host of potential mechanisms whereby these kinases could be on pathways downstream of Erk.

In summary, we have shown that tyrosine phosphorylation of an ~85-kDa protein closely associated with the CNGC channel α-subunit of cone photoreceptors is essential for phase-dependent circadian modulation of their gating properties. This finding is novel, since phosphorylation of these proteins may significantly alter the dynamics of phototransduction. Therefore, the circadian regulation of CNGCs in photoreceptors provides a cellular mechanism that allows the visual system to anticipate daily ambient changes.

Acknowledgements

Supported by National Eye Institute Grant EY 11973 (SED) and NIH postdoctoral fellowship EY 13920 (GY-PK).

The authors thank Jacqueline Tanaka (Temple University, Philadelphia, PA) for generously providing the chick CNGC α subunit antibody.

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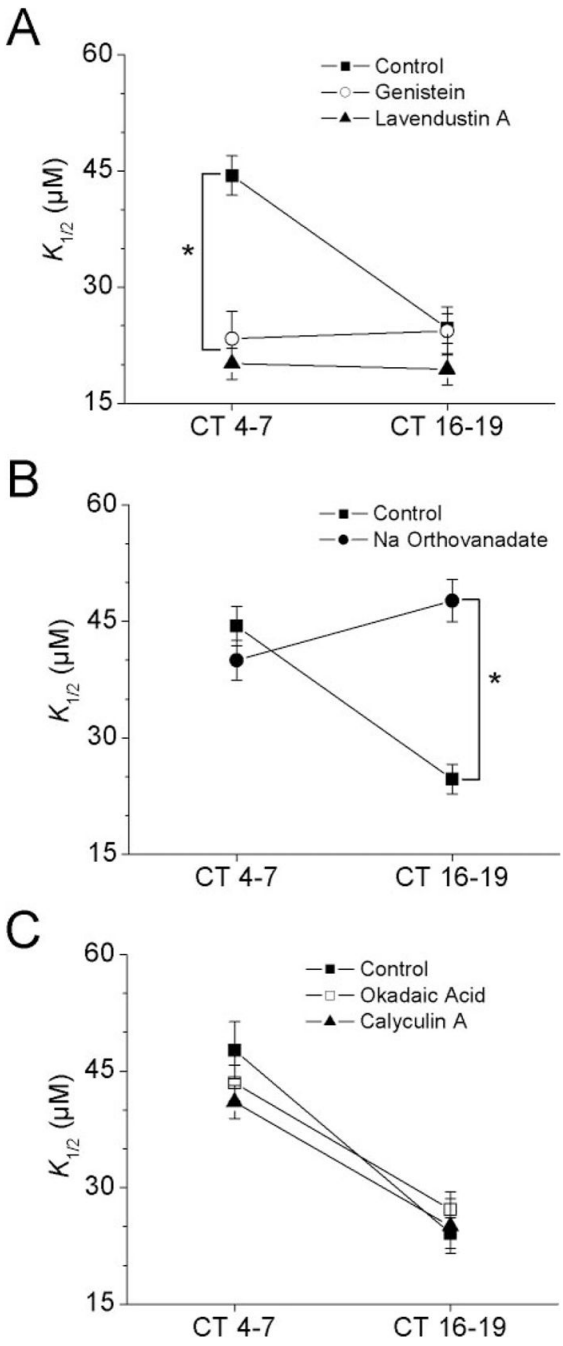

Figure 1.

Roles of tyrosine kinases and phosphatases in mediating circadian changes in channel-gating properties. (**A**) Inhibition of tyrosine kinase during the subjective day (CT 4–7), but not the subjective night (CT 16–19), altered the apparent affinity ($K_{1/2}$) of CNGCs. Cells were treated with a tyrosine kinase inhibitor genistein (10 μM) or lavendustin A (10 μM) for 2 hours before the inside-out patches were excised on the second day of DD. The mean $K_{1/2}$ during the subjective day was significantly reduced to the levels normally observed during the subjective night in tyrosine kinase inhibitor–treated groups. Genistein and lavendustin A had no effect during the subjective night. (**B**) Inhibition of tyrosine phosphatase during the subjective night, but not the subjective day, changed the $K_{1/2}$ of CNGCs. Treatment with a tyrosine phosphatase

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inhibitor, sodium orthovanadate (200 μM), caused an increase in the mean $K_{1/2}$ during the subjective night, but had no effect during the subjective day. (**C**) Serine-threonine phosphatase inhibitors had no effect on the circadian regulation of CNGCs. Treatment with a serine-threonine phosphatase (PP1 and PP2A) inhibitor okadaic acid (1 μM) or calyculin A (50 nM) for 2 hours before inside-out patch recordings had no effect on the mean $K_{1/2}$ from cells recorded during the subjective day or the subjective night.

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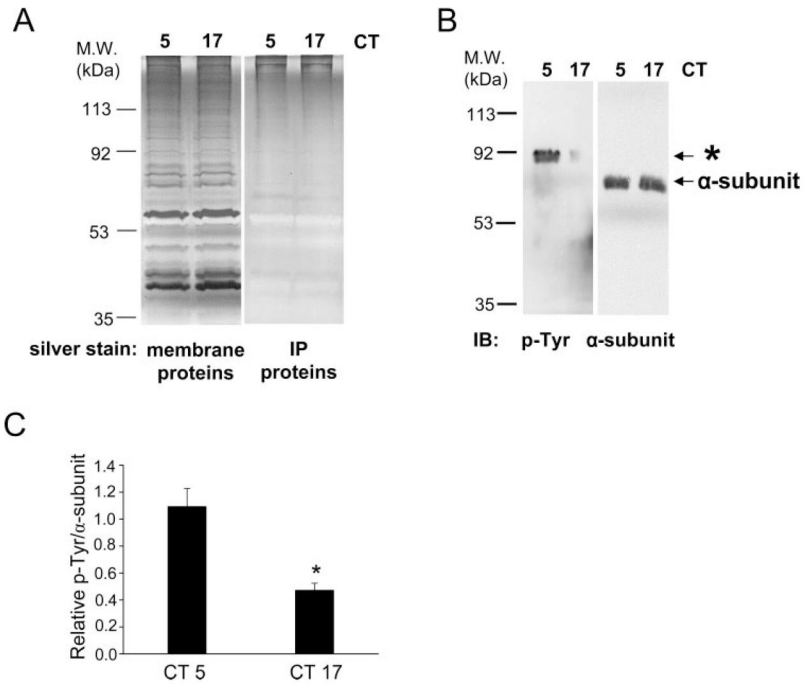

FIGURE 2.

Tyrosine phosphorylation of a protein strongly associated with the CNGC *α*-subunit is under circadian control. (**A**) Solubilized retinal membrane proteins and immunoprecipitates (with an antibody against the chicken cone photoreceptor CNGC *α*-subunit) were loaded on 10% gels and silver stained. Total protein for the CT 5 and CT 17 samples were nearly equal. (**B**) Retina samples were first immunoprecipitated with an antibody against the chicken cone photoreceptor CNGC *α*-subunit, and immunoprecipitates were then analyzed by immunoblot with an anti-phosphotyrosine antibody. Tyrosine phosphorylation of a protein associated with the CNGC *α*-subunit near 85 kDa was more prevalent in samples harvested during the subjective day (CT 5), but very weak during the subjective night (CT 17). Protein levels of the CNGC *α*-subunit do not change as a function of circadian time. (**C**) The level of tyrosine phosphorylation on the CNGC *α*-subunit associated protein was significantly higher in samples harvested during the subjective day (CT 5) than during the subjective night (CT 17). $n = 3$ in each group. $^*P < 0.05$.

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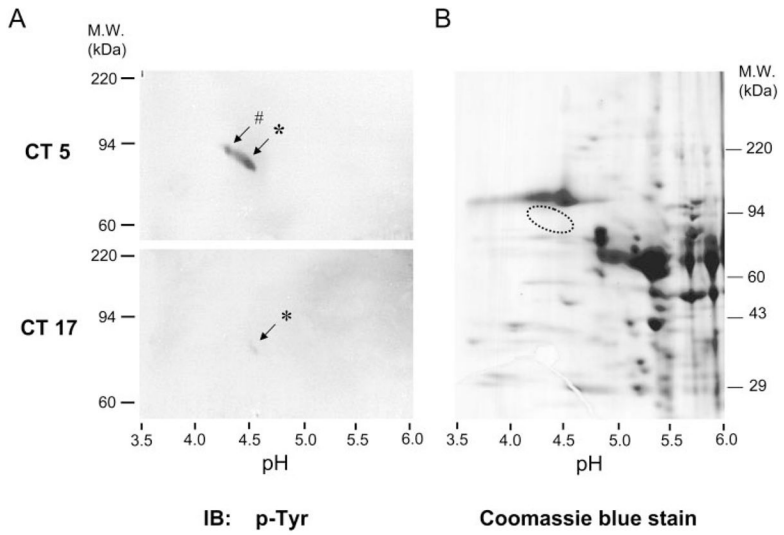

Figure 3.

2D-PAGE analysis of the tyrosine phosphorylation of the CNGC α-subunit associated proteins. Retina samples were first immunoprecipitated with an antibody against the chicken cone photoreceptor CNGC α-subunit. The 2D-PAGE analysis of a pool of five immunoprecipitates for each group resolved the phosphotyrosine band into two spots (# and *) with slightly different appearances in molecular weight, and both showed different intensities (CT 5 vs. CT 17) for phosphotyrosine. For both spots, the level of phosphotyrosine was much higher from retinal samples collected during the subjective day (CT 5; *top*) than the subjective night (CT 17; *bottom*). (**B**) Retinal membrane proteins from CT 5 were solubilized with RIPA lysis buffer (1% Triton X-100) and stained with Coomassie blue for sample preparation for mass spectrometry. No visible spot was detected (even by silver staining) in the area of the phosphotyrosine spots (*dotted outline*). A significantly higher amount of sample (~100-fold) was loaded compared with the membrane proteins in Figure 2A.