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Protein kinase C modulates the phase-delaying effects of light in the mammalian circadian clock

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Abstract

The mammalian circadian pacemaker located in the suprachiasmatic nuclei (SCN) drives a vast array of biochemical and physiological processes with 24-h periodicity. The phasing of SCN pacemaker activity is tightly regulated by photic input from the retina. Recent work has implicated protein kinase C (PKC) as a regulator of photic input, although stimulus-induced PKC activity has not been examined. Here we used a combination of biochemical, immunohistochemical and behavioral techniques to examine both the regulation and role of PKC in light-induced clock entrainment in mice. We report that photic stimulation during the subjective night, but not during the subjective day, stimulates PKC activity within the SCN. To assess the role of PKC in clock entrainment, we employed an *in-vivo* infusion approach to deliver the PKC inhibitor bisindolylmaleimide I to the SCN. The disruption of PKC activity significantly enhanced the phase-shifting effects of light, indicating that PKC functions as a negative regulator of light entrainment. Importantly, bisindolylmaleimide I infusion in the absence of light treatment did not phase shift the clock, demonstrating that transient disruption of basal PKC activity does not affect inherent pacemaker activity. The capacity of light to stimulate immediate early gene expression in the SCN was not substantively altered by PKC inhibition, suggesting that PKC does not couple light to rapid transcriptional activation. Rather, a combination of *in-vivo* and cell culture assays indicates that PKC functions as an inhibitor of PERIOD1 degradation. Thus, PKC may influence clock entrainment via a post-translational mechanism that influences clock protein stability.

Introduction

The suprachiasmatic nuclei (SCN) located in the anterior hypothalamus function as the major biological clock. The inherent pacemaker activity of the SCN is driven by interlocking genetic feedback loops derived from a limited subset of 'clock genes' (Sassone-Corsi, 1994; Harms *et al.*, 2004). A number of external stimuli, such as light, function as potent entrainment cues by regulating the expression of clock genes. Photic information is relayed from the eyes to the SCN via the retinohypothalamic tract, a branch of the optic nerve. In response to photic stimulation, retinohypothalamic tract nerve terminals secrete the excitatory neurotransmitter glutamate (Ebling, 1996; Hannibal, 2002) and the modulatory neurohormone pituitary adenylate cyclase-activating polypeptide (Harrington *et al.*, 1999; Hannibal, 2002). These two transmitters work in a coordinated manner to activate a set of second messenger signaling events that couple light to the core clock timing mechanism.

Several intracellular signaling pathways have been implicated in light-induced clock entrainment. For example, the p42/44 mitogen-activated protein kinase (MAPK) pathway is required for both the phase-advancing and phase-delaying effects of light (Obrietan *et al.*, 1998; Butcher *et al.*, 2002; Coogan & Piggins, 2003). The MAPK pathway appears to regulate the clock via induction of gene expression (Akiyama *et al.*, 2003; Dziema *et al.*, 2003; Coogan & Piggins, 2004). Likewise, both calcium/calmodulin kinases and nitric oxide/guanosine 3′,5′ monophosphate/guanosine 3′,5′ monophosphate-dependent protein kinase have been implicated in transcriptionally dependent entrainment of the clock (Golombek & Ralph, 1994; Weber *et al.*, 1995; Mathur *et al.*, 1996; Fukushima *et al.*, 1997; Ding *et al.*, 1998; Yokota *et al.*, 2001; Agostino *et al.*, 2004). In addition to these pathways, several studies have identified protein kinase C (PKC) as a potential regulator of photic input (McArthur *et al.*, 1997; Schak & Harrington, 1999; Motzkus *et al.*, 2000; Bult *et al.*, 2001). PKC is a member of the AGC class of serine/threonine kinases. PKC comprises a large family (10 at present) of isozymes that fall into three classification groups depending on structure, function and essential cofactors. Conventional PKC isoforms (α, β and γ) require calcium and diacylglycerol as cofactors, novel PKC isoforms (δ, ϵ, η and θ) require diacylglycerol, and atypical isoforms (ζ, ι and λ) do not require diacylglycerol or calcium for activation (Newton, 1997; Dempsey *et al.*, 2000). In addition to their regulation by cofactors, a series of phosphorylation events are required for catalytic activity, stability and subcellular localization of PKC (Newton, 2001, 2003).

As a receptor-regulated signaling pathway, PKC has been shown to exhibit diverse, context-specific physiological effects, including transcription activation, alterations in cell morphology and receptor sensitization (Newton, 1995). In the central nervous system, PKC has been implicated in neuronal plasticity-dependent processes, including long-term potentiation and long-term depression (Zhuo & Hawkins, 1995; Hussain & Carpenter, 2005). With respect to the circadian clock, an array of PKC isozymes has been found in functionally discrete regions of the SCN (Cagampang *et al.*, 1998; Bult & Smale, 1999). Furthermore, disruption of PKC activity has been shown to entrain the circadian firing rhythms of the SCN slices in a phase-dependent manner (Schak & Harrington, 1999). Although these data indicate a

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functional role for PKC in the SCN clock, as yet an examination of PKC activation by light and its contribution to clock entrainment has not been performed.

Here we show that photic stimulation during the subjective night triggers PKC activity in the SCN and that the disruption of PKC activity enhances light-induced entrainment of the circadian clock. These effects of PKC do not appear to be mediated at the level of transcription. Rather, these data indicate that PKC regulates the stability of the clock gene product PERIOD1, thus providing a potential post-translational mechanism by which PKC affects clock entrainment.

Materials and methods

Drug infusion and behavior analysis

Adult (6–10-week-old) C57BL/6 mice were anesthetized with ketamine/xylazine and stereotaxically implanted with a guide cannula piercing the roof of the third ventricle. The stereotaxic coordinates and surgical procedures are described in Butcher *et al.* (2002). After cannulation, mice were individually housed and allowed 2 weeks to recover. Cannulated mice were then transferred to cages equipped with running wheels (15 cm diameter); wheel rotation was automatically recorded to a PC running VITAL VIEW (Minimitter Corp, Bend, OR, USA) data-acquisition software. For circadian locomotor activity analysis, mice were entrained to a 12 h/12 h light/dark cycle for 14 days and then dark-adapted. During the day, luminescence was provided with a fluorescent white light (~100 lx at mid-cage level). Animals received a once-weekly food and water replenishment, and a change of bedding. Cage maintenance occurred at varying times during the subjective night. During cage maintenance, mice were exposed to a dim red light (< 3 lx). To disrupt PKC activity, dark-adapted mice were infused under dim red light with the PKC inhibitor (bisindolylmaleimide I) (3 μL, 1 mM, Calbiochem, La Jolla, CA, USA) at circadian time (CT) 14.5. Control animals were infused with drug vehicle (dimethylsulfoxide) (3 μL). At 30 min after infusion, animals were exposed to light (50 lx) for 10 min and then returned to darkness. Control mice not exposed to light were handled in a similar manner as the light-treated animals. After infusion, mice were returned to their home cages and locomotor behavior was monitored. Each mouse was exposed to at least two of the four stimulus paradigms (1, vehicle/light; 2, vehicle/no light; 3, bisindolylmaleimide I/light; 4, bisindolylmaleimide I/no light). A minimum of 10 days separated each stimulus. All animal experiments were approved by the Ohio State University animal care and use committee.

To assess the effects of PKC on light entrainment of the circadian clock, we used the linear regression method described in Daan & Pittendrigh (1976). To this end, a regression line was drawn through activity onset for the 6 days preceding light treatment. This line was used to project when activity onset should occur following drug infusion and/or light treatment. A second regression line was fitted through the actual activity onset following drug infusion and/or light treatment. Days 3–10 after light treatment were used to generate this line. The difference between the projected and the actual activity onset was the light-induced phase shift. Significance was assessed using the two-tailed Student's *t*-test and data are expressed as the mean phase shift ± SEM.

Immunohistochemistry

For phospho-PKC (pPKC) and PKC phospho-substrate motif immunostaining, mice were dark-adapted for 2 days, exposed to light (100 lx for 15 min) and then immediately killed by cervical dislocation and decapitation. For immediate early gene analysis, mice were killed 90 min after light stimulation. Mice were also killed under a standard 12 h L/D cycle. Brains were then isolated and immersed in oxygenated physiological saline and thick-sectioned (600 μm) with a vibratome. Tissue was then fixed in 4% paraformaldehyde, incubated in 30% sucrose overnight and then thin cut (40 μm) using a freezing microtome. For diaminobenzidine staining, sections were initially washed (3×) in phosphate-buffered saline supplemented with 0.1% Triton X-100 (PBST) for 10 min and then incubated in 0.3% H_2O_2 in PBST (15 min). Next, tissue was blocked (1 h) in 5% goat serum/phosphate-buffered saline and incubated (overnight, 4 °C) in a rabbit phospho-Ser 660 PKC (pPKC) antibody (1 : 1000 final dilution, Cell Signaling Technology, Beverly, MA, USA), or a rabbit anti-pPKC substrate antibody (1 : 1000, Cell Signaling Technology). Sections were then incubated (2 h) at room temperature (23 °C) in biotinylated anti-rabbit IgG (1 : 300, Vector Laboratories, Burlingame, CA, USA) and then placed in an avidin/biotin horseradish peroxidase complex for 1 h (prepared according to the manufacturer's instructions, Vector Laboratories). The signal was visualized by the addition of diaminobenzidine-nickel-intensified substrate (Vector Laboratories), mounted on gelatin-coated slides and coverslipped with Permount media (Fisher Scientific, Houston, TX, USA). For fluorescent immunolabeling against neuronal nuclear protein (NeuN) and pPKC, tissue sections were incubated (overnight, 4 °C) with mouse monoclonal NeuN (1 : 1000, Chemicon, Temecula, CA, USA) and rabbit polyclonal pPKC (1 : 1000) antibodies. The sections were then washed and incubated (4 h at room temperature) with an Alexa 488-conjugated goat anti-rabbit IgG antibody (1 : 500, Molecular Probes, Eugene, OR, USA) and 594-conjugated anti-mouse IgG antibody (1 : 500, Molecular Probes). Sections were mounted with Gelmount (Biomedia, Foster City, CA, USA).

Images of diaminobenzidine-labeled sections were captured using a 16-bit digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ, USA) connected to a Leica DM IRB microscope (Nussloch, Germany). For fluorescent immunolabeling, a Zeiss 510 Meta confocal microscope (Oberkochen, Germany) was used to capture 2-μm-thick optical sections.

Immunohistochemistry quantification

Cell counting

All data were quantified using METAMORPH software (Universal Imaging Corporation, West Chester, PA, USA). For cell counts from diaminobenzidine-labeled tissue, an intensity threshold filter was initially applied to the SCN image. The filter eliminated non-specific background labeling from analysis. The threshold value was equal to the mean pixel intensity within a non-immunoreactive region of the lateral hypothalamus. Digital circles were then overlaid on regions with a detectable signal above threshold (defined as positive cells) and counted. Cell counts were averaged over three consecutive central SCN sections for each experimental animal. Average values for each animal were used to generate mean values for each condition. Significance was determined using the two-tailed Student's *t*-test and was defined as $P < 0.05$. All data are expressed as the mean ± SEM.

Densitometry

To quantify image intensity, coronal brain sections containing the central SCN were captured using a 10× objective. To quantify pPKC expression, a digital oval (150 pixels, *x*-axis; 200 pixels, *y*-axis) was placed over each SCN and the adjacent hypothalamic area, just lateral to the SCN, and the mean intensities were determined. The SCN

intensity measurement was then divided by the lateral hypothalamus intensity value. The mean value for the control (no-light) group was set equal to 1 and the light-flash group was scaled accordingly. Student's *t*-tests were performed to determine significance.

Immunoprecipitation and western blotting

For both immunoprecipitation and western blotting, SCN-containing coronal brain sections were cut using a vibratome and then tissue was immediately frozen on dry ice. SCN and piriform cortical tissue were isolated using a razor blade and stored at −80 °C until use. For western blotting, the SCN were pooled from two mice for each condition; for immunoprecipitation, SCN were pooled from six mice. SCN tissue was initially lysed in RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM sodium vanadate, 1 mM NaF and 1× protease inhibitor cocktail (Roche)]. For immunoprecipitation, 500 μg protein lysate samples were pre-cleared with protein-A-agarose, incubated (60 min) with 2 μg of PKCα antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then 30 μL of protein-A-agarose was added to the protein lysates and rotated overnight at 4 °C. After centrifugation, the agarose–protein complex was washed in RIPA buffer (3×) to remove non-specific protein binding and then 3× sodium dodecyl sulfate sample buffer was directly added to the agarose–protein complex. Samples were heated (95 °C, 5 min) and then processed via western blotting (described below).

For western blotting, 25 μg of protein lysates from the SCN and piriform cortex was loaded into 8% sodium dodecyl sulfate polyacrylamide gels and transblotted onto polyvinylidene fluoride (Immobilon P, Millipore, Bedford, MA, USA). Next, membranes were washed with PBST (1×), blocked with 5% (w/v) powdered milk dissolved in PBST and then incubated (overnight at 4 °C) with primary antibody diluted in PBST supplemented with 5% bovine serum albumin. The following primary antibodies were used in this study: rabbit polyclonal anti-phospho-Ser 660 PKC (1 : 1000 final dilution, Cell Signaling Technology), rabbit polyclonal anti-pPKC substrate (1 : 1000, Cell Signaling Technology), rabbit polyclonal anti-pPKC ζ/λ (1 : 1000, Cell Signaling Technology), rabbit polyclonal anti-phospho-protein kinase D/PKCμ (1 : 1000, Cell Signaling Technology), rabbit polyclonal anti-extracellular signal-regulated kinase (ERK)1 and anti-ERK2 (1 : 2000, Santa Cruz Biotechnology), and mouse monoclonal anti-Ras (1 : 2000, Upstate Biotechnology, Billerica, MA, USA). Next, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies directed against the IgG domains of primary antibodies (1 : 2000, Perkin Elmer Life Sciences, Norwalk, CT, USA). Renaissance chemiluminescent horseradish peroxidase substrate (Perkin Elmer Life Sciences) was used to visualize the signal. Data were quantified via densitometric band intensity measurements. Initially, the intensity value for the band of interest was normalized to the protein loading control; in most cases this was ERK1. This ratio was then averaged across a minimum of three independent experiments and expressed as the mean ± SEM normalized value.

PERIOD protein stability assay

Dark-adapted cannulated mice were infused with bisindolylmaleimide I (3 μL, 1 mM) or dimethylsulfoxide (3 μL, vehicle) 30 min prior to photic stimulation (100 lx, 10 min) during the early night (Circadian time, CT 15). Mice were then returned to the dark and killed 90 min later (CT 16.5). Tissue was harvested as described above and fixed in 4% paraformaldehyde. Sections were immunolabeled with polyclonal mPER1 antibody (1 : 10 000 dilution; provided by Dr Steven Reppert, University of Massachusetts) and polyclonal mPER2 antibody (1 : 500 dilution, Alpha Diagnostic Intl. Inc., San Antonio, TX, USA). Sections were immunohistochemically processed as described above.

Human embryonic kidney (HEK) 293T cells were transfected with *mperiod1* (*mper1*)-V5-His or *mperiod2* (*mper2*)-V5-His in pcDNA3.1. The *mper* expression vectors were a generous gift of Dr Steven Reppert. At 20 h after transfection, cells were incubated with cycloheximide (25 μg/mL; Sigma, St Louis, MO, USA) 60 min prior to phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (1 μM) stimulation. To block PKC activation, bisindolylmaleimide I (1 μM) was added 30 min prior to TPA stimulation. After 90 min of TPA stimulation, cells were harvested with ice-cold RIPA buffer. Lysates were then centrifuged and supernatant protein levels quantified. Protein (30 μg) was loaded into 8% polyacrylamide gels and processed via western analysis as described above. The following primary antibodies were used: mouse monoclonal anti-V5 (1 : 5000, Invitrogen, Carlsbad, CA, USA), rabbit polyclonal anti-pPKC substrate (1 : 1000, Cell Signaling Technology) and rabbit polyclonal anti-ERK1 and anti-ERK2 (1 : 2000, Santa Cruz Biotechnology).

Results

As a starting point to examine the role of PKC in light entrainment of the circadian clock, we monitored its activation state following photic stimulation. To this end, mice were dark-adapted for 2 days and then exposed to light (15 min, 100 lx) during either the subjective night (CT 15) or the subjective day (CT 6), and the tissue was processed using an antibody that detects the Ser 660 (for the PKCβII isoform) phosphorylated form of PKC. PKC is autophosphorylated at Ser 660 and its phosphorylation is part of a series of sequential phosphorylation events that lead to its activation. Importantly, the Ser 660 motif is conserved in many conventional and novel (but not atypical) PKC isoforms (Keranen *et al.*, 1995), and thus this antibody can be used to examine broadly reflected changes in PKC activity. Figure 1A reveals that exposure to light during the subjective night led to a marked increase in pPKC levels. Consistent with the molecular weight of most PKC isozymes, two major bands running from 80 to 85 kDa were detected. To test whether the increase in PKC phosphorylation was consistent with the activation of other signaling events implicated in entrainment, the blot was also probed for the expression of the activated, Thr 202/Tyr 204-phosphorylated, forms of ERK1 and ERK2. As with PKC, photic stimulation during the subjective night triggered an increase in ERK1 and ERK2 phosphorylation. As a protein loading control, the blot was also probed for total ERK expression. The effects of light appear to be specific to the SCN, as photic stimulation did not increase PKC phosphorylation (or ERK activation) in tissue isolated from the piriform cortex (Fig. 1A). Consistent with the western blotting data, immunohistochemical analysis revealed that light triggered an increase in the pPKC in the SCN (Fig. 1B). Light-induced PKC phosphorylation was observed throughout the SCN, roughly corresponding with the retinohypothalamic tract projection pattern recently characterized in the mouse (Hattar *et al.*, 2006; Morin *et al.*, 2006). Double immunofluorescent labeling against pPKC and the neuronal nuclear marker NeuN was used to show that light stimulated PKC activation in SCN neurons (Fig. 1C). The non-nuclear pPKC expression pattern is consistent with other studies showing a cytoplasmic distribution of activated PKC (Saito *et al.*, 1989; Tanaka & Saito, 1992; Buchner *et al.*, 1999). In contrast to the effects of light during the subjective night, photic

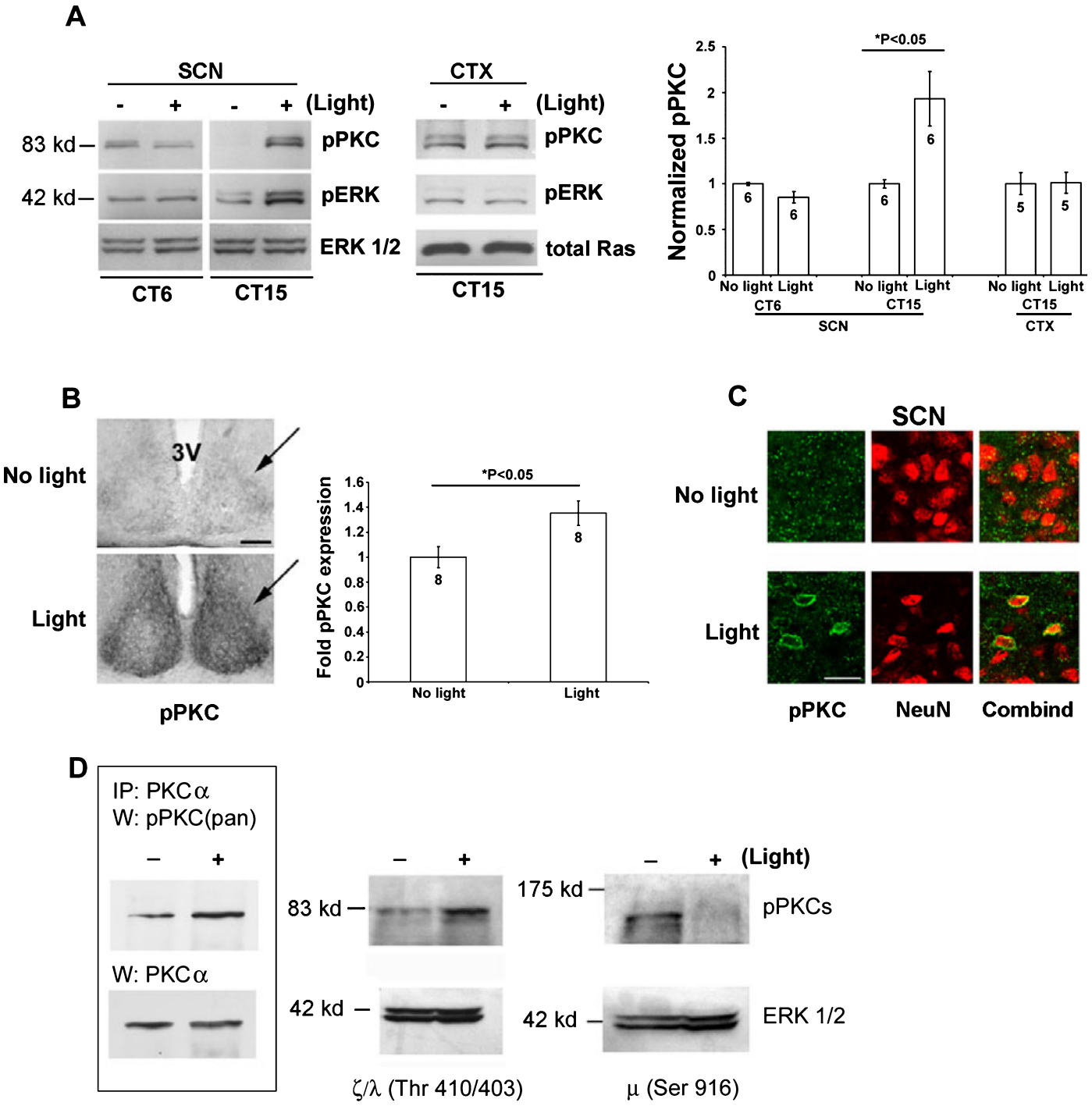

FIG. 1. Light-induced protein kinase C (PKC) phosphorylation in the suprachiasmatic nuclei (SCN). (A) Western analysis of SCN tissue from animals exposed to light (15 min, 100 lx) during the subjective night [circadian time (CT) 15] and subjective day (CT 6). Light exposure during the subjective night triggered an increase in the Ser 660 phosphorylated form of PKC (pPKC) in the SCN. Light also induced extracellular signal-regulated kinase (ERK)1 and ERK2 phosphorylation (pERK). In contrast, light did not induce PKC phosphorylation in the piriform cortex (CTX). As a protein-loading control, blots were stripped and probed for total ERK or Ras expression. Mean densitometric band analysis is shown to the right. Numbers inside bars indicate the number of experiments performed. Error bars denote SEM. (B) Immunohistochemical labeling detected a light-induced (CT 15, 15 min, 100 lx) increase in PKC phosphorylation in the SCN. Arrows denote approximate lateral SCN boundary. Bar, 125 μm. Quantification of pPKC-immunoreactive cells is shown to the right. Please refer to Materials and methods for a description of the quantification. Numbers within a bar denote the number of animals analysed. Error bars denote SEM. (C) SCN tissue from control (no light) and light-treated (15 min, 100 lux, CT15) animals was immunolabeled with antibodies against pPKC (green) and NeuN (red). A representative high-magnification image of the SCN shows the localized expression of pPKC and NeuN, indicating that light triggers PKC activity in neurons. Bar, 25 μm. (D) Light (15 min, 100 lux, CT15) regulation of PKC isozymes. (Boxed) SCN tissue was immunoprecipitated using a PKCα antibody and probed using the phospho-Ser 660 PKC antibody. Light increased PKCα phosphorylation relative to tissue from animals not exposed to light. Light triggered PKCζ/λ phosphorylation at Thr 410/403, respectively, in the SCN, whereas PKCμ autophosphorylation at Ser 916 was decreased by photic stimulation. As a protein-loading control, blots were stripped and probed for total ERK levels. Data are representative of three independent experiments. For all western blotting, SCN were pooled from two animals for each experiment. 3V, third ventricle; IP, immunoprecipitate; W, western blot. *$P < 0.05$.

stimulation during the subjective day (CT 6) did not lead to an increase in the activated form of PKC relative to control animals not exposed to light (Fig. 1A). Interestingly, this result parallels the phase-restricted capacity of light to activate the MAPK pathway (Obrietan *et al.*, 1998).

To identify specific PKC isotypes that are regulated by light, we probed for PKC activity using isoform-specific antibodies. For PKCα, SCN tissue from control and light-stimulated (100 lx, 15 min, CT 15) animals was immunoprecipitated with a PKCα antibody, and PKCα activation was probed using the phospho-Ser 660 antibody. Consistent with the SCN lysate results, a light-induced increase in PKCα activation was observed (Fig. 1D). The activation states of novel and atypical forms of PKC were examined. Along these lines, we found that the phosphorylation of PKCζ/λ on Thr 410/413 (activation loop site) was increased after light stimulation. Interestingly, phosphorylation of protein kinase D/PKCμ at Ser 916 (autophosphorylation site) was reduced with light stimulation (Fig. 1D). These results reveal that photic stimulation triggers complex and PKC isoform-specific responses within the SCN.

Next, we addressed the role of PKC in SCN light entrainment. As a starting point for this analysis, we employed a PKC substrate-specific antibody to test for light-induced changes in PKC-dependent phosphorylation patterns in the SCN. As noted by the manufacturer, the PKC substrate antibody detects phosphorylated Ser surrounded by Arg or Lys at the −2 and +2 positions, and a hydrophobic residue at the +1 position. This motif is specifically targeted by PKC and its phosphorylation is associated with activation of PKC. For these experiments, dark-adapted animals were exposed to light (100 lx, 5 or 15 min) at CT 15 and then immediately killed. SCN lysates were probed via western blotting for both pPKC and PKC target motif phosphorylation levels. Relative to control animals, photic stimulation triggered an increase in the phosphorylation of a number of unidentified proteins (Fig. 2A). Consistent with the pPKC levels, the levels of pPKC substrate labeling were dependent on light duration; thus, relative to a 5 min light stimulus, a 15 min light pulse elicited a higher level of substrate phosphorylation. As a protein loading control, the membrane was also probed for total ERK levels. Light pulses of 10 and 15 min elicited equivalent levels of pPKC (data not shown). Next, we used immunohistochemical labeling to detect changes in PKC substrate phosphorylation levels in the SCN. Under control conditions, little immunoreactivity was observed in the SCN. However, following photic stimulation (100 lx, 15 min) at CT 15, an increase in immunoreactivity was observed specifically in the SCN (Fig. 2C).

To assess the functional role of PKC in the SCN, we devised a ventricular infusion approach to deliver the broad-spectrum PKC inhibitor bisindolylmaleimide I to the SCN. To assess the efficacy of bisindolylmaleimide I infusion, tissue sections were probed for PKC substrate phosphorylation levels. Representative data in Fig. 2B reveal that bisindolylmaleimide I infusion (1 mM, 3 μL, CT 14.5) 30 min prior to killing triggered a reduction in PKC activity along the ventricular border region, which includes the SCN. Importantly, this infusion paradigm potently repressed the light-induced enzymatic activity of PKC (Fig. 2C). Thus, bisindolylmaleimide I effectively uncoupled light from PKC activation in the SCN. In a control set of experiments we found that bisindolylmaleimide I led to a decrease in PKC activity that lasted from 90 min to ∼3 h post-infusion (data not shown).

Next, the potential role of PKC as a regulator of light-induced clock entrainment was analysed via wheel-running behavior. To this end, mice were entrained to a light/dark cycle for 2 weeks and then released into total darkness. After 10 days in total darkness, animals were either infused with bisindolylmaleimide I (1 mM, 3 μL) or drug vehicle and exposed to light (50 lx, 10 min) 30 min later (CT 15). In vehicle-infused mice, photic stimulation led to a significant phase delay (−110 min) in wheel-running activity onset. Interestingly, relative to vehicle-infused mice, the disruption of PKC activity led to a significant lengthening (−144 min) of the phase-delaying effect of light (Fig. 3). Importantly, in the absence of photic stimulation, bisindolylmaleimide I infusion did not significantly alter clock phase, indicating that light-induced PKC activity modulates the phase-delaying effects of light.

We then examined the potential mechanism by which PKC regulates light entrainment of the clock. Given a number of studies showing that PKC stimulates gene transcription, we examined whether disruption of light-induced PKC activation affects immediate early gene expression. For these experiments mice were infused with bisindolylmaleimide I at CT 14.5 and exposed to light (15 min, 100 lx) 30 min later. Animals were killed 90 min after light exposure and the tissue was processed for the expression of Jun-B or c-Fos. As expected, in vehicle-infused mice, photic stimulation led to a dramatic increase in the expression of both immediate early genes (Fig. 4A and B). Interestingly, the infusion of bisindolylmaleimide I did not have a significant effect on light-induced Jun-B or c-Fos expression in the SCN, although the number of immunopositive cells was reduced. Furthermore, in control (no-light) mice PKC inhibition did not effect basal immediate early gene expression (Fig. 4). Together these data suggest that PKC is not a principal regulator of rapid light-dependent transcriptional activation in the SCN.

Finally, we examined whether PKC could affect the clock via a post-translational mechanism (Fig. 5). For this analysis, mice were infused with bisindolylmaleimide I and the effects on PERIOD1 (PER1) and PERIOD2 (PER2) protein expression were analysed. Initially, we found that photic stimulation (100 lx, 10 min) during the early night (CT 15) led to an increase in the number of PER1-immunoreactive SCN cells (Fig. 5A) in mice killed 90 min later (CT 16.5). Given the short interval between photic stimulation and killing (90 min), the increase in PER1 expression is not likely to result from a transcriptionally dependent mechanism but rather may be the result of an alteration in protein stability. Interestingly, the infusion of bisindolylmaleimide I prior to photic stimulation led to a significant decline in PER1 expression (Fig. 5A), suggesting that PKC influences the stability of PER1. The effects were specific to PER1; neither light nor bisindolylmaleimide I had an effect on PER2 expression (Fig. 5B). In the absence of photic stimulation, pre-treatment (CT 14.5) with bisindolylmaleimide I did not significantly alter PER1 or PER2 levels.

To test whether PKC alters PER1 expression via a post-translational mechanism, HEK 293T cells were transfected with mPER1-V5 or mPER2-V5 expression constructs and mPER stability was examined following PKC activation via administration of TPA (1 μM). Initially, translation was blocked by pre-treatment (60 min) with cycloheximide (25 μg/mL) and cells were harvested 90 min after TPA stimulation. Cell extracts were then examined via western blotting for PERIOD expression using a V5 antibody. Treatment with TPA led to a marked increase in the expression of mPER1 (Fig. 5C). In contrast, the expression of mPER2 was not altered by TPA administration (Fig. 5D). Given that translation was blocked with cycloheximide, these results suggest that TPA treatment increased mPER1 stability. To determine whether this effect was mediated by PKC, cells were treated with bisindolylmaleimide I (1 μM) prior to TPA administration. Abrogation of PKC activity blocked the increase in mPER1 expression, indicating that the effects of TPA were mediated by PKC. The efficacy of bisindolylmaleimide I was validated by probing the blot with the PKC phospho-substrate antibody. Thus, TPA treatment

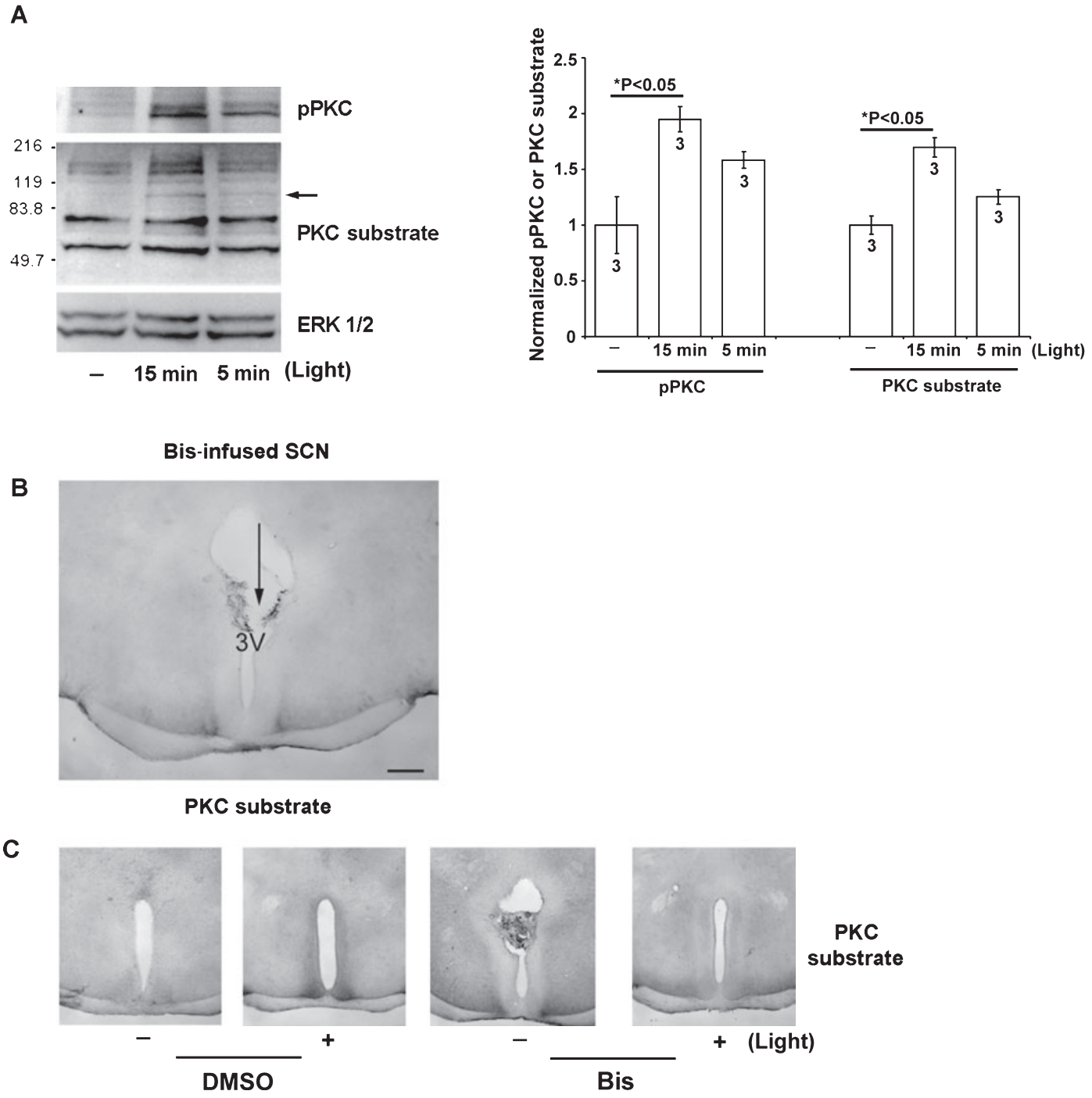

FIG. 2. Light-induced protein kinase C (PKC) activity in the suprachiasmatic nuclei (SCN). (A) SCN tissue from control mice (no light) and animals exposed to light (100 lx) were probed using a PKC phospho-substrate-specific antibody (PKC substrate). Relative to control animals, both 5 and 15 min of light triggered an increase in PKC substrate phosphorylation, indicating that light stimulates PKC enzymatic activity in the SCN. Paralleling this effect, levels of phospho-PKC (pPKC) increased with increasing light duration. As a protein loading control, the blot was also probed for total extracellular signal-regulated kinase (ERK) expression. Mean ± SEM densitometric band analysis is shown is shown to the left. For PKC substrate densitometric analysis, a representative band running at ~100 kDa (arrow) was analysed. Numbers inside bars indicate the number of experiments performed. Please refer to Materials and methods for a description of the quantification. (B) Disruption of PKC activity in the SCN. Cannulated mice were infused in the third ventricle with the PKC inhibitor bisindolylmaleimide I at circadian time (CT) 14.5. Mice were killed 30 min later and tissue was immunolabeled using the PKC phospho-substrate-specific antibody. This low-magnification representative image of the hypothalamus reveals the location of the infusion (arrow). 3V, third ventricle. Bar, 250 μm. (C) Cannulated mice were infused with dimethylsulfoxide (DMSO) (vehicle) or bisindolylmaleimide I (Bis) 30 min before light (15 min, 100 lx) exposure at CT 15. Coronal tissue sections were immunolabeled with the PKC phospho-substrate-specific antibody. Light triggered an increase in PKC substrate phosphorylation in animals infused with drug vehicle. In contrast, the infusion of bisindolylmaleimide I effectively blocked PKC enzymatic activity. *$P < 0.05$.

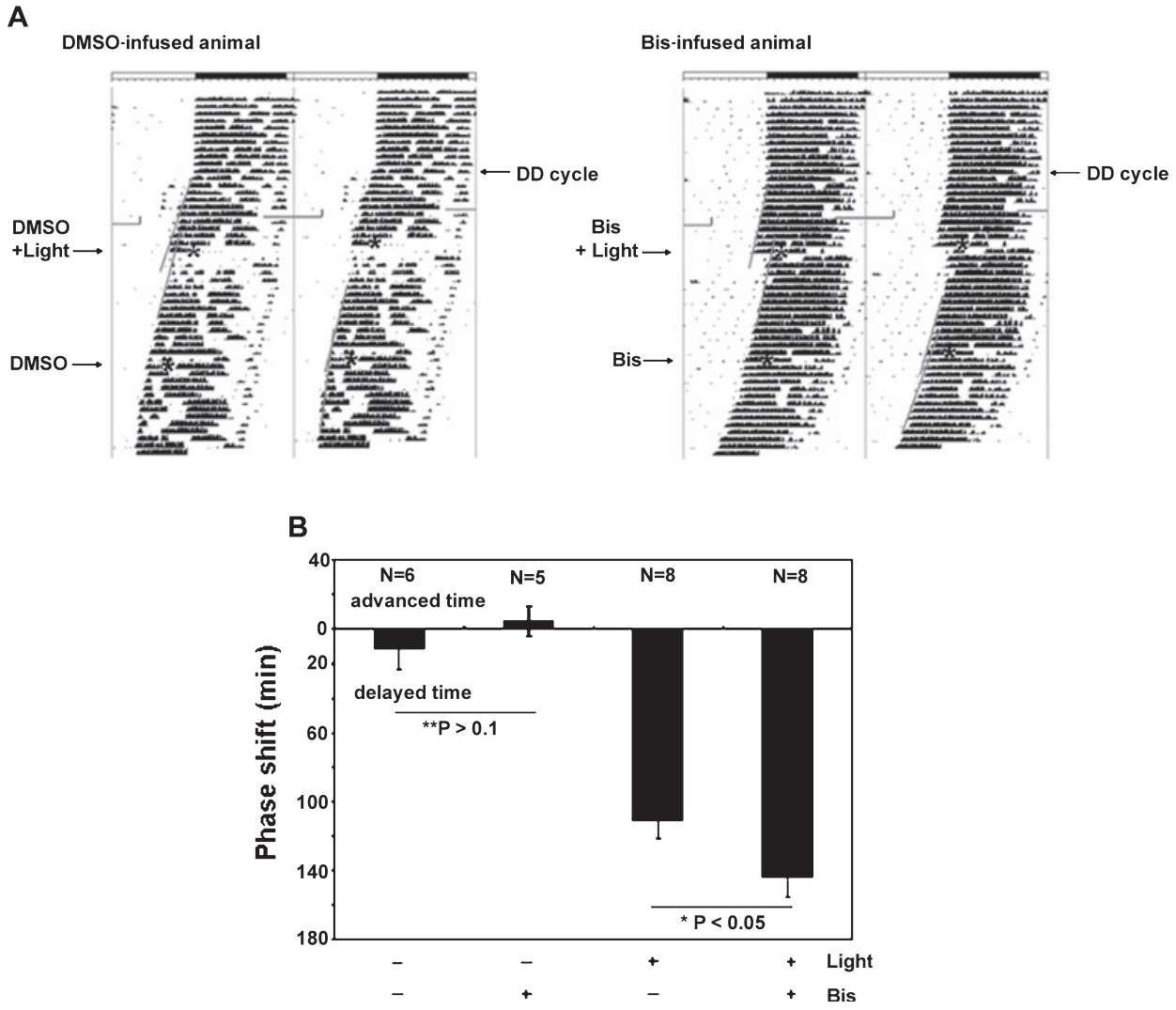

FIG. 3. Disruption of light-induced protein kinase C (PKC) activity augments the phase-shifting effect of light. (A) Representative double-plotted actographs. Initially, mice were maintained on a 12 h light/dark cycle, then dark adapted (DD). After 10 days of free running, mice were infused with either drug vehicle [dimethylsulfoxide (DMSO), left side] or the PKC inhibitor bisindolylmaleimide I (Bis) (right side) 30 min before light (50 lx, 10 min) exposure at circadian time (CT) 15 (*). Relative to the DMSO-infused animal, bisindolylmaleimide I administration enhanced the light-induced phase delay. To determine whether drug infusion altered timing in the absence of photic stimulation, mice were allowed to free run for an additional 14 days and then infused with DMSO or bisindolylmaleimide I at CT 14.5 (*). Regression lines approximate stimulus-induced phase shift. Please refer to Materials and methods for a description of the regression analysis. Horizontal bars in the activity record denote an 'off-line' period when wheel-running activity was not recorded. (B) Quantification of the phase-delaying effects of light. Mean ± SEM of the phase-delaying effects of light. $*P < 0.05$ (significant), $**P > 0.1$ (not significant), two-tailed Student's *t*-test. Numbers above bars denote sample size for each condition.

triggered an increase in the number of immunoreactive bands and bisindolylmaleimide I pre-treatment effectively blocked this increase (Fig. 5C and D). Together, these data indicate that PKC regulates the stability of the clock protein PER1, thus providing a potential mechanism by which light-induced PKC activity influences circadian clock entrainment.

Discussion

The goal of this study was to determine whether PKC plays a role in light entrainment of the SCN clock. Here we have shown that photic stimulation triggers the phase-specific activation of PKC in the SCN and that the disruption of PKC activity augments the phase-shifting effect of light. Data also suggest that PKC may be affecting the entrainment process by altering the stability of the core clock protein PER1. Together these data identify a physiological role for PKC in light entrainment and also provide a potential mechanism by which PKC affects the clock.

The activation of PKC is driven by three related processes: subcellular localization, conformation and phosphorylation (Dekker & Parker, 1994; Newton, 1997; Parekh *et al.*, 2000). As with other member of the AGC family of kinases, the primary structure of PKC consists of a C-terminus kinase domain and an N-terminus regulatory domain. The regulatory domain contains a pseudosubstrate motif, which blocks PKC activity by interacting with the catalytic domain, and either a single or tandem C domain. The ligands diacylglycerol and Ca^{2+} target PKC to the membrane by binding to the regulatory domain. Membrane localization disrupts the association of the pseudosubstrate domain with the catalytic domain, thus facilitating PKC activation (Hofmann, 1997; Newton, 1997; Parker & Murray-

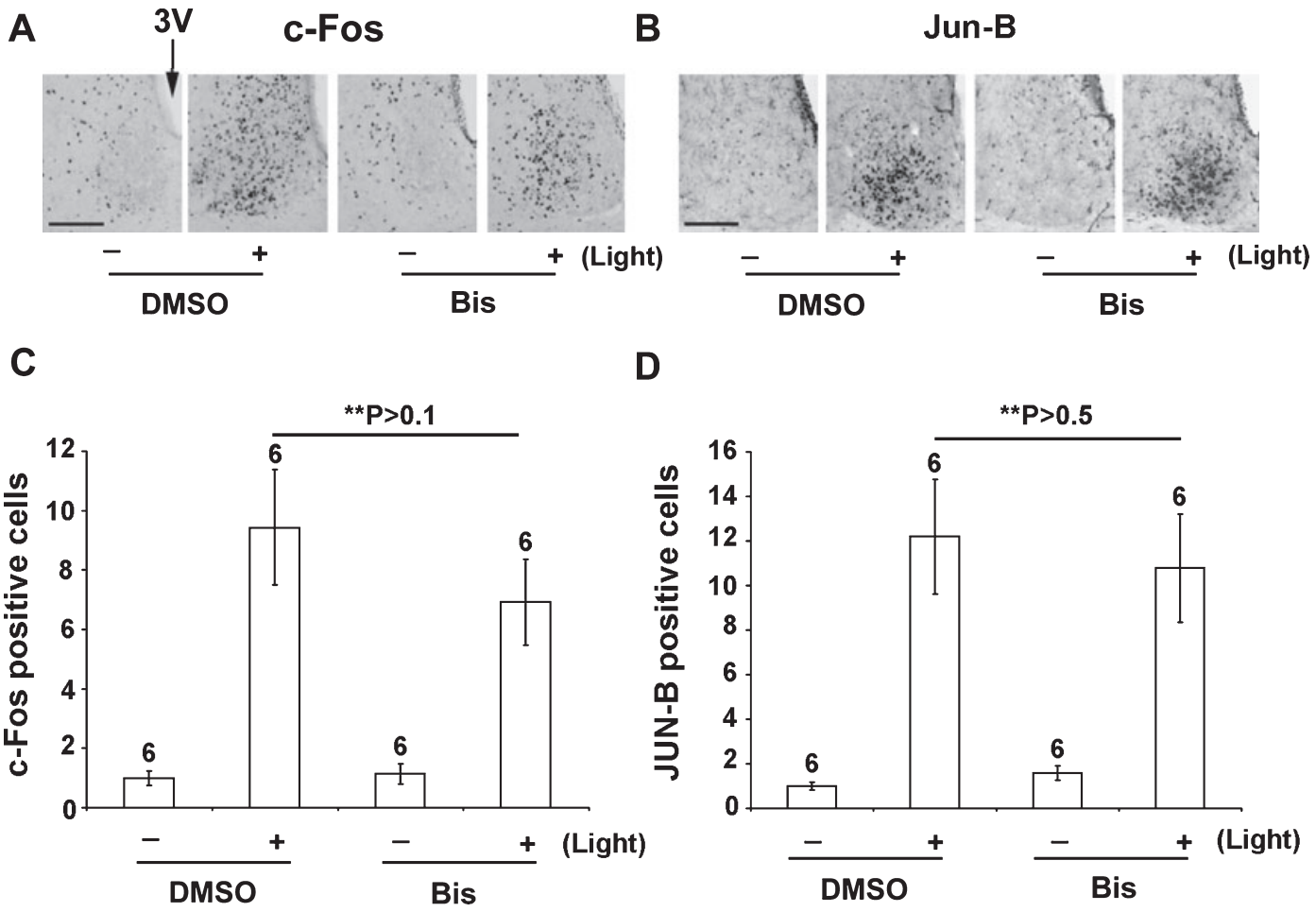

FIG. 4. Protein kinase C is not essential for light-induced light to immediate early gene expression in the suprachiasmatic nuclei. Drug vehicle [dimethylsulfoxide (DMSO)] or bisindolylmaleimide I (Bis) was infused 30 min before light exposure (10 min, 100 lx) at circadian time 15. After light exposure, mice were returned to darkness for 90 min and then killed. Immunolabeling revealed that light triggered robust c-Fos (A) and Jun-B (B) expression in mice infused with either DMSO or bisindolylmaleimide I. 3V, third ventricle. Bar, 125 μm. (C and D) Quantification of light-induced immediate early gene expression. Error bars denote SEM. Numbers above bars indicate the number of animals analysed for each condition. $^{**}P > 0.1$ (not significant). Arrow indicates 3V.

Rust, 2004). With respect to phosphorylation, three sequential phosphate modifications are required for full PKC catalytic activity. Initially PDK-1-dependent phosphorylation within the catalytic domain 'activation loop' triggers a conformational change that exposes the substrate binding motif (Le Good *et al.*, 1998). Next, PKC phosphorylates within the 'turn motif' of the catalytic subunit. This, in turn, triggers PKC autophosphorylation within the catalytic subunit 'hydrophobic motif'. This last phosphorylation event facilitates enzymatic activity, stability and subcellular localization of PKC (Newton, 2001, 2003). The phospho-specific PKC antibody employed in this study monitored phosphorylation at this hydrophobic motif and thus could be used to infer the activation state of PKC. It should be noted that atypical PKCs do not contain a phospho-acceptor Ser or Thr residue within the hydrophobic motif and thus their activation state was not assessed in this study. Importantly, throughout this study, we employed a PKC motif-specific antibody to confirm that an increase in hydrophobic motif phosphorylation was associated with an increase in PKC enzymatic activity.

Western analysis detected a robust light-induced pPKC signal. This approach was complemented by immunohistochemical and immunoprecipitation assays that also detected a light-induced increase in PKC phosphorylation. Of interest was the finding that photic stimulation during the subjective day did not stimulate PKC activity. This phase-restricted capacity of light to activate PKC parallels the phase-restricted responsiveness of the MAPK pathway to light (Obrietan *et al.*, 1998), thus raising the possibility that a common phase-regulated upstream event, such as receptor coupling, determines whether these signaling pathways are activated by light.

To assess the role of PKC in clock entrainment, we developed an *in-vivo* infusion approach to uncouple light from PKC activation. This method employed the PKC inhibitor bisindolylmaleimide I to block PKC enzymatic activity. The efficacy of the inhibitor was monitored using a pPKC substrate-specific antibody. With this assay we were able to detect inhibition of PKC activity within the periventricular region that includes the SCN. This approach is analogous to the ventricular infusion method used to deliver the MAP kinase kinase inhibitor U0126 and, in turn, implicate the MAPK pathway in transcription regulation and light entrainment of the clock (Butcher *et al.*, 2002; Dziema *et al.*, 2003).

Interestingly, the disruption of PKC activity significantly enhanced the phase-shifting effects of light, indicating that PKC functions as a negative regulator of light entrainment. These data are, in some respects, consistent with a study showing that the phase-delaying effects of light were inversely correlated with the level of PKCβI in

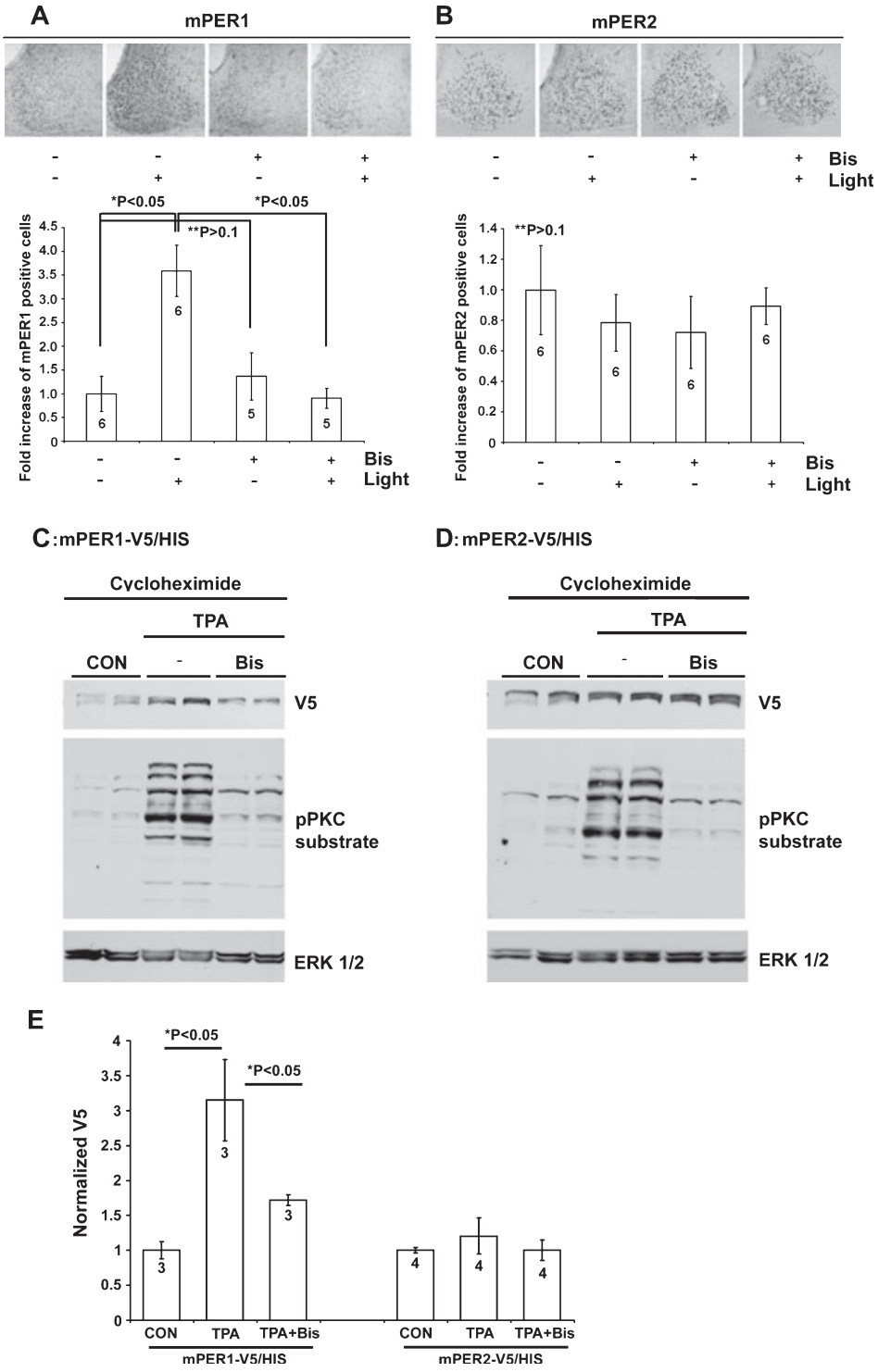

FIG. 5. Protein kinase C (PKC) regulates PER1 protein stability. (A and B) Mice were infused with drug vehicle (dimethylsulfoxide) or bisindolylmaleimide I (Bis) 30 min before light exposure (10 min, 100 lx) at circadian time 15. After light exposure, mice were returned to darkness for 90 min and then killed. Representative immunolabeling revealed that light triggered an increase in PER1 expression (A) that was attenuated by bisindolylmaleimide I. PER2 expression (B) was not affected by light. Quantification of PER1 and PER2 immunolabeling is shown below each data set. Error bars denote SEM. Numbers inside bars indicate the number of animals examined for each condition. For PER2, no significant difference was observed for any of the conditions ($P > 0.1$). HEK 293T cells were transfected with mPER1-His-V5 (C) or mPER2-His-V5 (D) constructs. At 20 h after transfection, cells were pre-treated with cycloheximide (25 μg/mL, 60 min) and then stimulated with phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (1 μM). After 90 min of stimulation, cells were harvested and probed via western analysis. The V5 antibody was used to detect PER1 or PER2 fusion protein expression, PKC activity was assessed using the PKC substrate motif antibody and protein loading was examined using a total extracellular signal-regulated kinase (ERK) antibody. TPA administration led to an increase in PKC activity and PER1 stability. In contrast, TPA did not dramatically alter PER2 stability. Pre-treatment (30 min) with bisindolylmaleimide I (1 μM) blocked TPA-induced PKC activity and reversed the stabilization of PER1. Representative samples were run in duplicate. These data indicate that PKC regulates PER1, but not PER2, stability. (E) Mean ± SEM densitometric band analysis. Numbers inside bars indicate the number of experiments performed. Please refer to Materials and methods for a description of the western blotting quantification method. CON, control; pPKC, phospho-PKC. *$P < 0.05$, **$P > 0.01$.

the SCN (Bult *et al.*, 2001). Thus, higher levels of PKCβI were associated with smaller phase shifts, whereas lower levels of PKCβI were associated with a relatively larger light-induced phase shift. Importantly, disruption of PKC in the absence of light treatment did not significantly phase shift the clock, indicating that transient disruption of basal PKC activity does not affect inherent pacemaker activity. Rather, it is light-actuated PKC that regulates entrainment. This lack of an effect of PKC inhibition in the absence of photic stimulation is consistent with work showing that PKC inhibition during the early night does not alter the circadian firing properties of SCN slices (Schak & Harrington, 1999). However, it should be noted that PKC inhibition during the late subjective night and early subjective day was found to phase advance clock firing rhythms (Schak & Harrington, 1999).

Protein kinase C has been implicated as a signaling intermediate that couples neuropeptide Yergic input from the thalamic intergeniculate leaflet to the circadian clock (Biello *et al.*, 1997). The intergeniculate leaflet is a proposed site for the integration of photic and non-photic inputs to the circadian system (Janik *et al.*, 1995; Moore *et al.*, 2000; Vrang *et al.*, 2003; Thankachan & Rusak, 2005) Interestingly, neuropeptide Y has been shown to attenuate the phase-shifting effects of light (Lall & Biello, 2002, 2003a,b; Yannielli *et al.*, 2004). This finding, coupled with the aforementioned study showing that neuropeptide Y influences the clock via a PKC-dependent mechanism (Biello *et al.*, 1997), provides one potential mechanistic explanation for how disruption of PKC augments light-induced phase shifting. However, the two principal transmitters of the retinohypothalamic tract, glutamate and pituitary adenylate cyclase-activating polypeptide, stimulate robust PKC activation (Gillette & Tischkau, 1999; Nowak *et al.*, 2001; Dziema & Obrietan, 2002). Thus, additional work will be required to identify the transmitter and receptor-mediated signaling events that couple light to PKC activation in the SCN. Lastly, each light-activated PKC isoform may have a distinct effect on clock entrainment; our use of a broad-spectrum inhibitor would average these effects and thus mask potentially unique contributions of each isoform. In line with this idea, a recent report using cell culture-based assays showed that PKCα and PKCγ influence the transactivation potential of CLOCK (Shim *et al.*, 2007).

The role of PKC as a negative regulator of clock entrainment is counter to the roles of other light-actuated pathways, which function as positive regulators of clock entrainment. Along these lines, the MAPK pathway is necessary for robust light-induced phase shifting (Butcher *et al.*, 2002; Coogan & Piggins, 2003). Likewise, disruption of calcium/calmodulin kinase signaling suppresses light-induced phase shifting, as does inhibition of nitric oxide-dependent signaling (Tischkau *et al.*, 2003; Golombek *et al.*, 2004). The effects of PKC on clock entrainment suggest that it modulates the clock timing process via a mechanism that is distinct from that of the MAPK cascade, calcium/calmodulin kinases and nitric oxide/guanosine 3′,5′ monophosphate-dependent protein kinase. In line with this supposition, we found that PKC inhibition did not significantly alter light-induced immediate early gene expression. However, it should be noted that light-evoked immediate early gene expression does not necessarily tightly correlate with the phase-shifting effect of light (Lin *et al.*, 1997). Rather, these assays were used as a 'read-out' to examine whether PKC regulates gene expression in the SCN.

Given the lack of a marked effect on light-induced gene expression, we examined whether PKC regulates the clock via a post-transcriptional mechanism. Initially, we tested whether the *in-vivo* disruption of PKC altered expression of the clock proteins PER1 and PER2. Interestingly, as part of this study, we found that PER1 immunoreactivity was increased 90 min after light stimulation. This increase was in part dependent on PKC, as the disruption of PKC activity significantly attenuated light-induced PER1. Both light induction and PKC modulation were restricted to PER1; neither light nor PKC affected PER2. Given that light-induced PER1 expression requires 4 h or longer (Field *et al.*, 2000; von Gall *et al.*, 2003; Yan & Silver, 2004) to be detected, we were surprised to find that light triggered an increase in PER1 immunoreactivity 90 min after photic stimulation This relatively rapid increase in immunoreactivity suggested that the change in expression that we detected at 90 min post-light treatment was not likely to occur via a transcriptionally dependent mechanism.

To our knowledge, this rapid light-induced increase in PER1 has not been previously characterized. It is unclear why this increase in immunoreactivity has not been documented previously. Possible explanations include differences in stimulus paradigms, counting criteria and immunolabeling conditions. Along these lines, the ABC-based immunohistochemical labeling technique that we, and many other laboratories, employ is extremely sensitive and slight modifications in labeling and development conditions can lead to dramatic differences or, conversely, an absence of a difference in target protein expression. Together, these data suggested that PKC may be modulating PERIOD expression via a post-translational mechanism that leads to protein stability. However, it is important to note that our *in-vivo* data do not rule out other possible mechanisms, including a role for PKC in regulating the rate of *mper1* mRNA translation.

To directly test whether PER1 stability is regulated by PKC, we employed a cell culture-based over-expression system. Importantly, to eliminate the potentially complicating effects of inducible PERIOD expression, translation was blocked by pre-treatment with cycloheximide. As with photic stimulation, PKC activity was required for the stimulus-mediated increase in PER1 levels. This effect was specific to PER1; PKC did not alter the stability of PER2 or PER3 (data not shown). These data raise the possibility that PKC may affect the clock timing process via the regulation of clock protein stability. Hence, PKC appears to counteract an active PER1 degradation pathway. The precise route by which PKC affects PER1 stability is not known. Interestingly, recent work has shown that PKC can abrogate protein degradation via the ubiquitin-proteasome pathway (Leung *et al.*, 2001; Hernandez-Pigeon *et al.*, 2005). This mechanism could offset the effects of casein kinase I, which targets PERs for ubiquitin-mediated degradation (Akashi *et al.*, 2002; Eide *et al.*, 2005). Another possibility is that PKC could stimulate phosphatase activity, which in turn would reduce phosphorylation-targeted degradation. In line with this model, several studies have shown that PKC can stimulate protein phosphatase 1 and protein phosphatase 2A activity (Layne *et al.*, 2001; Li *et al.*, 2006), and that phosphatases play a key role in regulating clock protein stability (Gallego *et al.*, 2006). In conclusion, these data provide a new framework to begin to examine the role of PKC as a regulator of the mammalian circadian clock.

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Abbreviations

CT, circadian time; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NeuN, neuronal nuclear protein; PBST, phosphate-buffered saline supplemented with 0.1% Triton X-100; PKC, protein kinase C; pPKC, phosphorylated protein kinase C; SCN, suprachiasmatic nuclei; TPA, phorbol ester 12-O-tetradecanoylphorbol-13-acetate.

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