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Light Entrainment of the Mammalian Circadian Clock by a PRKCA-Dependent Posttranslational Mechanism

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SUMMARY

Light is the most potent stimulus for synchronizing endogenous circadian rhythms with external time. Photic clock resetting in mammals involves cAMP-responsive element binding protein (CREB)-mediated transcriptional activation of *Period* clock genes in the suprachiasmatic nuclei (SCN). Here we provide evidence for an additional photic input pathway to the mammalian circadian clock based on Protein Kinase C α (PRKCA). We found that *Prkca*-deficient mice show an impairment of light-mediated clock resetting. In the SCN of wild-type mice, light exposure evokes a transient interaction between PRKCA and PERIOD 2 (PER2) proteins that affects PER2 stability and nucleocytoplasmic distribution. These posttranslational events, together with CREB-mediated transcriptional regulation, are key factors in the molecular mechanism of photic clock resetting.

INTRODUCTION

Endogenous self-sustained clocks with a period of about 24 hr drive the rhythms of all physiological processes that are subject to circadian variation (Panda et al., 2002; Reppert and Weaver, 2002). In mammals, the master circadian pacemaker resides in the suprachiasmatic nuclei (SCN) of the hypothalamus (Ralph et al., 1990). Several key components of the molecular clockwork have been identified through a combination of genetic and biochemical studies (reviewed by Lowrey and Takahashi, 2004). At the basis of the circadian clockwork lies a network of interlocked transcriptional and translational feedback loops (TTLs). Positive regulatory elements are BMAL1 and CLOCK (or a related protein; see Debruyne et al., 2006) that heterodimerize and activate the transcription of *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes through binding to E-box enhancer elements (Gekakis et al., 1998; Jin et al., 1999; Kume et al., 1999). The PER and CRY proteins interact and translocate to the nucleus, where they shut down their own transcription by inhibiting the CLOCK/BMAL1 transcriptional activator complex (Kume et al., 1999). Another facet of the TTL involves the nuclear orphan receptors REV-ERBα (NR1D1) (Preitner et al., 2002) and RAR-related orphan receptor α (RORA, NR1F1) (Sato et al., 2004) that control the expression of the *Bmal1* gene.

A hallmark of the mammalian circadian pacemaker is its ability to be reset by light, thus allowing organisms to entrain to temporal variations of natural light conditions. A light pulse perceived after dusk will delay the pacemaker, while a pulse applied before dawn will advance the clock. Photic stimuli are transmitted from the retina to target neurons in the SCN, where they are transduced to the molecular clockwork through kinase cascades involving activation of type A and G kinases, calcium-calmodulin protein kinase, and mitogen-activated protein kinases (Butcher et al., 2002; Obrietan and van den Pol, 1998; Oster et al., 2003; Tischkau et al., 2000; Yokota et al., 2001). These signaling pathways converge on the cAMP-responsive element binding protein (CREB) that becomes phosphorylated at Ser133 and Ser142. Such phosphorylation activates CREB, which subsequently translocates to the nucleus and binds to cAMP-responsive elements located in the promoters of light-responsive genes including *Per1* and *Per2* (Ding et al., 1997; Gau et al., 2002; Ginty et al., 1993; Obrietan et al., 1999; Travnickova-Bendova et al., 2002). The TTL model predicts that CREB-mediated transcriptional activation of *Per* genes followed by an increase in PER proteins (Yan and Silver, 2004; although see Field et al., 2000) shifts the clock. At the behavioral level altered expression of clock genes is correlated with changes in locomotor activity rhythms.

It is well established that posttranslational mechanisms like phosphorylation and protein interactions are critical

for light-mediated regulation of the core clock. In *Drosophila* light induces binding of activated CRY to TIMELESS (TIM) protein (Busza et al., 2004; Ceriani et al., 1999; Naidoo et al., 1999). TIM is then phosphorylated, enabling JETLAG to target TIM for degradation through the proteasome pathway (Koh et al., 2006). Thus, in dipterans photic entrainment involves posttranscriptional events, while in mammals it is apparently regulated at the transcriptional level via CREB.

The mammalian protein kinase C (PRKC) family comprises three subfamilies: classical, novel, and atypical (Parker and Murray-Rust, 2004). All of the three classical PRKCs (PRKCA, PRKCB1/2, PRKCG; previously known as PKCα, βI/II, and γ) are activated by binding of diacylglycerol and Ca^{2+} to the regulatory domain of the kinase. This results in the release of a pseudosubstrate site from the substrate binding cavity and subsequently allows substrate protein binding and phosphorylation (Nakashima, 2002; Newton, 2003; Parekh et al., 2000). Several PRKCS are expressed in the SCN of mice, including PRKCA (Van der Zee and Bult, 1995). Pharmacological PRKC inhibitors and activators both evoke phase advances of neuronal firing rhythms in hamster SCN slice cultures (Schak and Harrington, 1999). Although these studies do not uncover the mechanism of action of PRKCs in the circadian clock, they raise the possibility that classical PRKCs have a role in the mammalian circadian timing system. We tested this hypothesis by taking advantage of the *Prkca*-deficient mouse and show that PRKCA is a light-controlled posttranslational regulator of PER2 stability and subcellular localization.

RESULTS

Prkca-Deficient Mice Display an Impaired Resetting of the Circadian Clock

As basal PRKC activity in rat suprachiasmatic nucleus-derived SCN2.2 cells follows a rhythmic pattern peaking between CT14 and CT22 (Rivera-Bermudez et al., 2003), and as PRKCA expression is elevated at CT14 in the SCN of rat (Cagampang et al., 1998), we first investigated whether transcription of the *Prkca* gene is under circadian control. To this end, wild-type mice maintained either in a regular 12 hr light/12 hr dark cycle (LD 12:12) or in constant darkness (DD) were sacrificed at different times and the level of *Prkca* expression in the SCN was determined by in situ hybridization (ISH). Quantification of the ISH signal revealed a substantial daily variation of *Prkca* transcript levels, both under LD and DD conditions, with maxima at the beginning of the activity phase (Figures 1A and 1B). The persistence of rhythmic expression in DD demonstrates that *Prkca* transcription is under endogenous clock control, rather than being driven by the environmental light/dark cycle.

Since rhythmic expression of *Prkca* in the SCN is driven by the circadian core oscillator, we examined *Prkca*-deficient mice for defects in the circadian control of locomotor activity. Such mice are devoid of PRKCA protein, but are morphologically normal, viable, and fertile (Leitges et al., 2002). Following entrainment to an LD 12:12 cycle, $Prkca^{-/-}$ mice and congenic wild-type controls were released into DD. In LD, all animals displayed normal entrainment with activity onsets at the beginning of the dark phase. When released into DD, both wild-type and mutant mice showed a persistent endogenous activity rhythm with comparable period lengths (τ) of 23.70 ± 0.07 hr (mean ± SEM) for wild-type animals and 23.76 ± 0.05 hr for $Prkca^{-/-}$ mice (Figures 2A and 2B). In constant light conditions (LL) τ is known to increase in nocturnal animals (Aschoff, 1979). In wild-type controls τ was 25.18 ± 0.11 hr in LL of 100 lux, but the average circadian

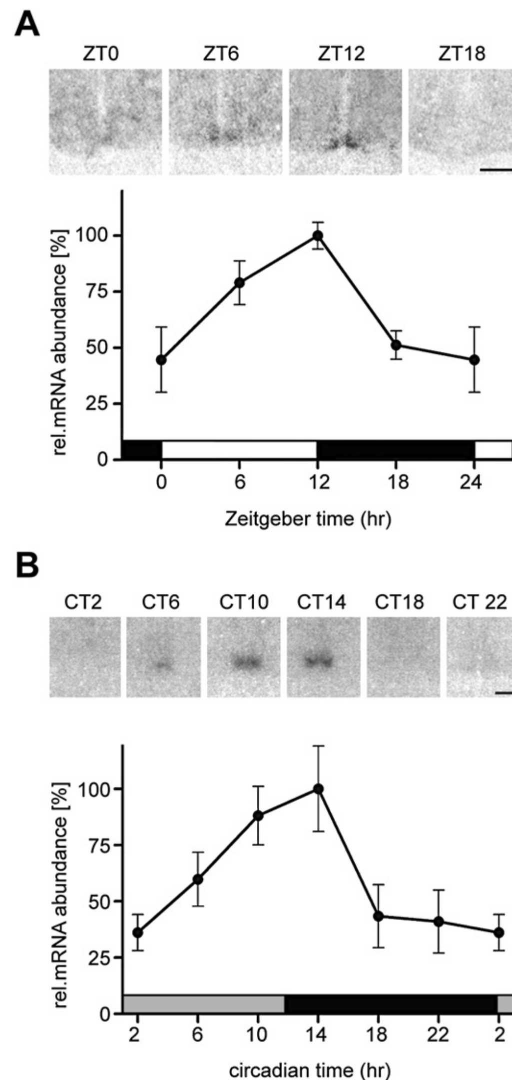


Figure 1. Rhythmic Expression of *Prkca* mRNA in the SCN
Upper panels show autoradiographs of *Prkca* expression in the SCN under LD (A) or DD (B) conditions. Graphs depict densitometric quantification of the ISH signal. White/gray and black horizontal bars indicate (subjective) day and night, respectively. All data are presented as mean ± SEM for three different experiments. Scale bars, 200 μm.

period of *Prkca*-deficient animals was significantly shorter than that of wild-type controls (24.91 ± 0.08 hr) under these conditions (Figures 2C and 2D). The impairment of lengthening of τ in LL provided a first indication that the regulation of the circadian clock by light is abnormal in the absence of PRKCA.

This prompted us to test whether *Prkca*$^{-/-}$ mice exhibit an abnormal response toward short nocturnal light pulses that are capable of resetting the phase of the endogenous clock. Sample actograms (Figures 2E and 2F) and phase response curves (Figure 2G) reveal that wild-type and *Prkca*$^{-/-}$ animals displayed similar phase-shifting responses to light pulses. However, significant quantitative differences were observed in the magnitude of phase delays. Exposure to a light pulse at ZT14 (2 hr after "lights off") caused a delay of 1.01 ± 0.1 hr in wild-type animals; the phase-shifting response of *Prkca*$^{-/-}$ mice was only 0.52 ± 0.09 hr. In contrast, a light pulse given 10 hr after lights off (ZT22, toward the end of the activity phase) led to comparable phase advances in wild-type (0.61 ± 0.11 hr) and *Prkca*$^{-/-}$ mice (0.45 ± 0.03 hr). The restriction of the altered light response to the first half of the activity phase may reflect the fact that *Prkca* expression is downregulated toward the end of the activity period (Figure 1).

Light-Induced Transcriptional Activation of Core Clock Genes Is Not Affected in *Prkca*-Deficient Mice

Since the phase-delay phenotype of *Prkca*-deficient mice is reminiscent of that seen in *CREB*^{S142A} point mutant mice (Gau et al., 2002), the most parsimonious explanation of the *Prkca* mutant data is to assume that the kinase is impinging on the CREB pathway. To investigate this possibility, we examined the induction of the light-inducible clock genes *Per1*, *Per2*, and *Dec1* (Shearman et al., 1997; Albrecht et al., 1997; Honma et al., 2002) in *Prkca*$^{-/-}$ mice. As expected, *Per1*, *Per2*, and *Dec1* gene expressions were strongly induced in the SCN of light-exposed wild-type mice (Figure 3). Surprisingly, this was also the case for *Prkca*-deficient animals, as evident from the absence of differences in magnitude or localization of gene induction between wild-type and mutant animals. This result makes a participation of PRKCA in CREB-mediated clock gene activation less likely, and instead strongly suggests the involvement of an additional, as of yet unknown pathway by which light can regulate the circadian clock.

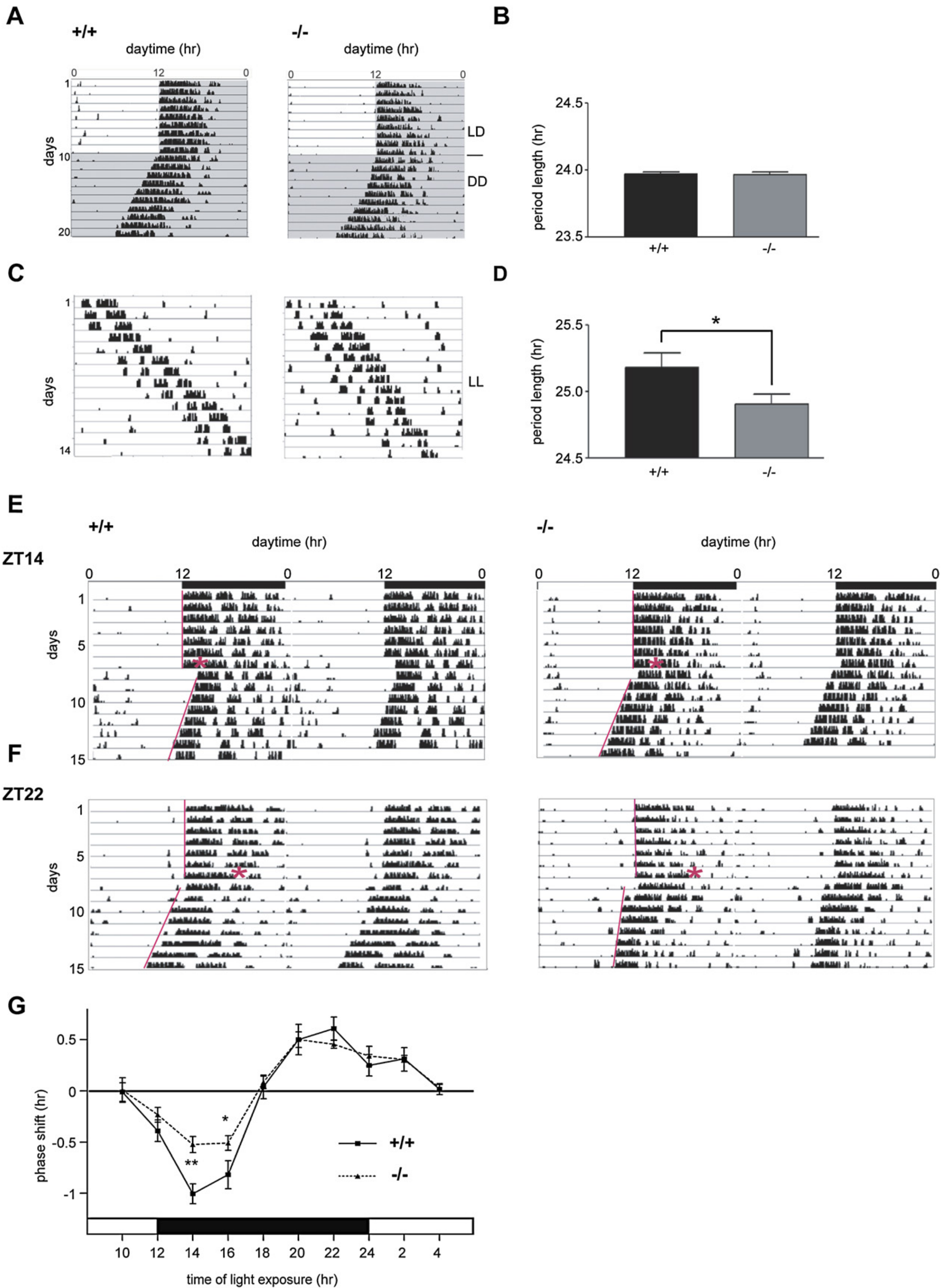
PRKCA Can Bind and Phosphorylate PER2

A number of clock proteins have been shown to interact with kinases, which indicates that phosphorylation plays a key role in the molecular mechanism of the circadian core oscillator (Lowrey et al., 2000; Vanselow et al., 2006; Gallego et al., 2006; Shim et al., 2007; Xu et al., 2007). We therefore aimed at identifying clock protein substrates that bind to, and are phosphorylated by, PRKCA. We thus coexpressed PRKCA with Myc-tagged PER2 (PER2^{Myc}), V5-tagged CRY2 (CRY2^{V5}), or both in COS7 cells (Figure 4A). Notably, PER2 appears an attractive candidate target because it has been implicated in the phase-delay mechanism (Albrecht et al., 2001; Reppert and Weaver, 2001, but also see Bae and Weaver, 2003 for an alternative view), and *Prkca*-deficient mice show a phase-delay phenotype. Immunoprecipitation with PRKCA antibodies, followed by western blot analysis using Myc antiserum, showed that PRKCA precipitates together with PER2^{Myc} protein (Figure 4A, lane 4). In contrast, CRY2^{V5} could not be pulled down with the kinase antiserum when coexpressed with PRKCA alone (lane 5). However, in line with the observation that PER proteins strongly interact with cryptochromes (Chaves et al., 2006; Griffin et al., 1999; Kume et al., 1999; Yagita et al., 2002), a CRY2^{V5}/PER2^{Myc}/PRKCA complex could be coprecipitated when all three proteins were expressed together (lane 7).

We next asked whether PRKCA phosphorylates PER2 and CRY2 in vitro. To this end, PER2^{Myc} and CRY2^{V5} were expressed in COS7 cells, and resulting proteins were pulled down with antibodies against the Myc protein tag. Thereafter, PER2^{Myc} alone or PER2^{Myc}/CRY2^{V5} were incubated with recombinant PRKCA and ^{32}P-ATP, as detailed in the header of Figure 4B. A distinct radioactive protein of $\sim$130 kDa was detected (Figure 4B, lane 1), which was identified as PER2^{Myc} by western blot analysis using an anti-Myc antibody (data not shown). This band was also seen when PER2^{Myc} and CRY2^{V5} were both present in the kinase assay, indicating that CRY2 does not interfere with PER2 phosphorylation (lane 2). Addition of the PRKC inhibitor bisindolylmaleimide resulted in an almost complete inhibition of PER2 phosphorylation (lanes 4 and 5). Phosphorylation of PER2 in the in vitro kinase assay is mediated by added PRKCA rather than by endogenous (copurified) kinases, since without exogenous PRKCA, PER2 was not significantly phosphorylated (Figure 4C). CRY2^{V5}, either alone or in the presence of PER2^{Myc}, appears not to be subject to PRKCA-mediated phosphorylation (lanes 2 and 3; expression of CRY2^{V5} was verified by western blot analysis using V5 antibodies, data not shown). Taken together, these cell-based assays suggest that PRKCA engages in a direct interaction with PER2, but not with CRY2, and that CRY2 was pulled down because of its association with PER2. Moreover, PRKCA phosphorylates PER2, but not CRY2. When both clock proteins were mixed together, presumably resulting in heterodimers (Field et al., 2000; Hogenesch et al., 1998; Kume et al., 1999), PER2 was also phosphorylated.

Photic Control of the Interaction between PRKCA and PER2 in the SCN

We next examined whether the interaction between PRKCA and PER2, as observed in COS7 cells, also occurs in the SCN, and more importantly, whether this interaction is influenced by light, as could be expected from the impaired light-resetting phenotype of *Prkca*$^{-/-}$ mice. To this end, we exposed mice to light pulses at ZT14, the time at which the difference in phase shift responses between wild-type and mutant mice is largest. Protein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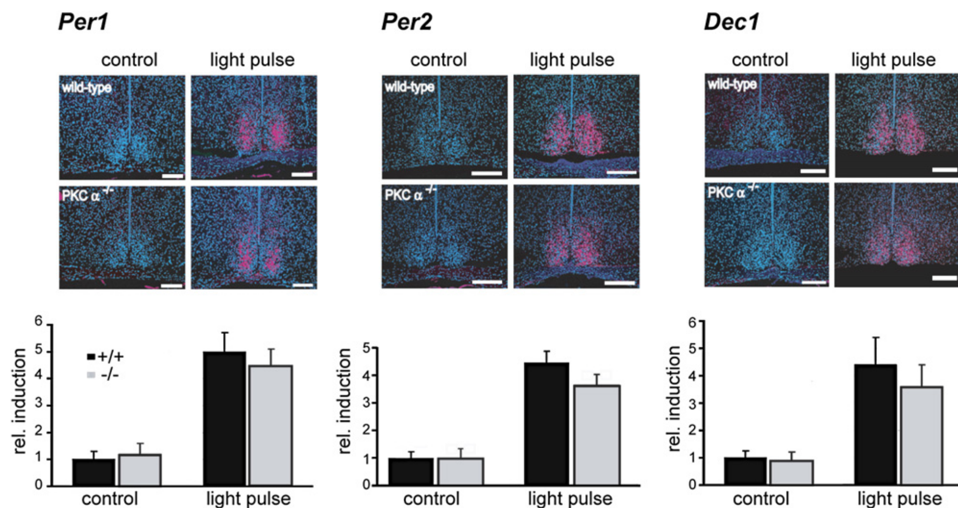

Figure 3. Photic Induction of *Per1*, *Per2*, and *Dec1* Genes Is Comparable in Wild-Type and *Prkca*$^{-/-}$ Animals

LD-entrained wild-type and mutant animals were exposed to a 15 min light pulse at ZT14 and sacrificed 45 min later. Upper panels show representative micrographs of emulsion ISH signal. Bar plots show quantification of mRNA induction by densitometric analysis of X-ray film contact autoradiographs. The intensity of the signal of wild-type controls not exposed to light at ZT15 was set as 1. All data are mean ± SEM of three animals per condition and genotype. Scale bars, 200 μm.

extracts were prepared from the excised SCN, and endogenous PER2 was pulled down with a PER2 antiserum. The resulting immunoprecipitate was evaluated for the presence of, and the interaction between, PER2 and PRKCA by western blot analysis.

As shown in Figure 5A, actin levels were the same in all samples, indicating that the extracts contained comparable amounts of protein. Furthermore, PRKCA protein levels in the SCN extract were not affected by light exposure, indicating that PRKCA is neither induced nor degraded by light-signaling through the retinohypothalamic tract. PER2 levels were also not significantly changed by light exposure of the animal (Figure 7C, top panel and open circles in Figure 7D), which is consistent with previous reports (Beaulé et al., 2003; Field et al., 2000).

Next, PER2 immunoprecipitates were probed with PRKCA antibodies in order to detect PER2/PRKCA complex formation. Prior to light exposure, anti-PRKCA antibodies revealed a weak band at 81 kDa, the predicted size of PRKCA (Figure 5B, lane 1), indicating that a small fraction PRKCA interacted with PER2. With progressively longer light exposure, the intensity of the band representing PRKCA bound to PER2 became substantially augmented, peaking at 40 min after the onset of light exposure and rapidly downregulating thereafter (Figure 5B, lanes 2–5). As expected, immunoprecipitates from SCN extracts of *Prkca*$^{-/-}$ mice were devoid of a PRKCA band (Figure 5B, lane 6). Figure 5C is a quantitative representation of three independent pull-down experiments and illustrates the consistency of the results. When animals were not exposed to light (sham control), the amount of PRKCA-PER2 complex in the SCN (Figures 5D and 5E) was comparable to that seen at ZT14 prior to applying light. When PRKCA antiserum was used to immunoprecipitate proteins from SCN extracts, subsequent western blot analysis with a PER2 antiserum revealed a characteristic 130 kDa PER2 band (Figure 5F).

To address the issue of temporal specificity of PER2/PRKCA complex formation, we exposed mice to light in the late subjective day (CT8), when both PER2 (Figure 5G, lane 1, and Field et al., 2000) and PRKCA (Figure 5G, lane 1) are expressed. Pull-downs with PER2 antiserum failed to produce a band in the subsequent western blot probed with a PRKCA antiserum (Figure 5H, lanes 2 and 3). Sham animals yielded identical results (Figure 5H, lanes 4 and 5). PER2 is neither significantly expressed nor light inducible

Figure 2. Photic Resetting of the Circadian Clock Is Impaired in *Prkca*-Deficient Mice

(A) Actograms (shading indicates darkness) of wild-type and *Prkca*$^{-/-}$ animals.

(B) Average free-running period length (τ) in DD was 23.70 ± 0.07 hr (mean ± SEM) for wild-type animals (n = 19) and 23.76 ± 0.05 hr (n = 24) for *Prkca*$^{-/-}$ mice ($p > 0.05$).

(C) Actograms of wild-type and *Prkca*$^{-/-}$ animals maintained in LL of 100 lux.

(D) In LL, mutants had a significantly shorter τ (24.91 ± 0.08 hr; n = 11) as compared with wild-type animals (25.18 ± 0.11 hr; n = 10; *$p < 0.05$).

(E and F) Double-plotted actograms of wild-type and *Prkca*-deficient mice exposed to a 400 lux/15 min light pulse (red asterisk) either in the early (ZT14, E) or late (ZT22, F) night. Activity onsets are indicated by red lines; white and black horizontal bars on top indicate day and night, respectively.

(G) Photic phase response curves for wild-type and *Prkca*$^{-/-}$ animals. Phase shifts at ZT14 were 1.01 ± 0.1 hr in wild-type animals and 0.52 ± 0.09 hr in *Prkca*$^{-/-}$ mice. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$; Student's t test, n = 6 to 8).

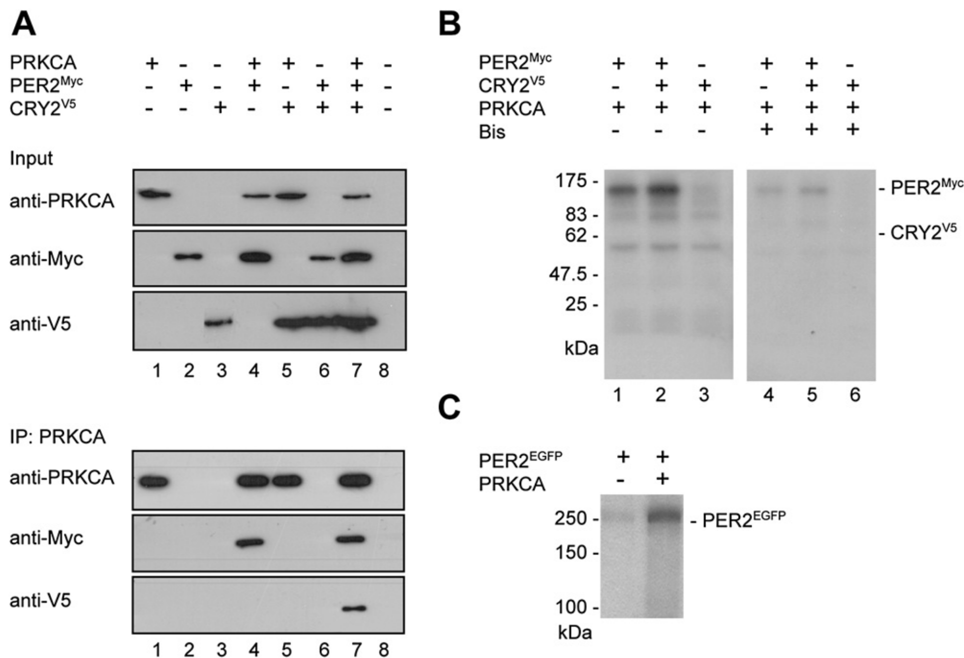

Figure 4. PRKCA and PER2 Proteins Interact in COS7 Cells

(A) PER2^{Myc}, CRY2^{V5}, and PRKCA were coexpressed in COS7 cells in the combinations noted in the header. Upper panel shows protein input; lower panel depicts proteins that were immunoprecipitated followed by western blotting with antisera specified on the left margin.

(B) In vitro phosphorylation of PER2^{Myc} by recombinant PRKCA and the effect of CRY2^{V5} and the PRKC inhibitor bisindolylmaleimide (Bis). Expected positions of PER2^{Myc} and CRY2^{V5} are indicated on the right.

(C) Efficient in vitro phosphorylation of PER2^{EGFP} requires the addition of exogenous PRKCA protein.

in the late subjective night (Field et al., 2000; Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998; Zylka et al., 1998), precluding interaction studies at this time. We conclude that PER2 and PRKCA interact in the SCN. This interaction is transient, light induced, and restricted to the phase-delay period of the circadian cycle. Because neither PER2 nor PRKCA concentrations in the SCN significantly change as a result of light exposure, the formation of PER2/PRKCA complexes is not a consequence of increased transcription or translation, but reflects a posttranslational process.

PRKCA Regulates the Nucleocytoplasmic Distribution of PER2

A reduction in phase delay of locomotor activity, as observed in *Prkca*-deficient mice, could arise from attenuation of the PER/CRY-mediated negative feedback that these proteins exert on transcription of their own genes (see Introduction). We therefore speculated that, following light exposure, cytosolic PRKCA may transiently retain the PER2 protein outside the nucleus, thus transiently retarding its negative feedback on CLOCK/BMAL1-mediated transcription. Cultured cells are widely used to study the dynamics of subcellular distribution of clock proteins (Tamanini et al., 2005). We therefore transiently expressed tagged PER2 (PER2^{Myc} or PER2^{EGFP}) with or without PRKCA in COS7 or U2OS cells. In the absence of PRKCA, tagged PER2 was exclusively nuclear in $\leq$55% of cells, while the remaining cells displayed cytoplasmic (25%–35%) or mixed nuclear and cytoplasmic (20%–25%) distributions (Figures 6A–6C). Coexpression with PRKCA resulted in a marked drop (to as low as 10% in COS7 cells) in the percentage of cells showing an exclusively nuclear PER2 localization. This effect was abolished when PRKCA was replaced by an enzymatically inactive mutant form of the kinase (kdPRKCA). The latter data suggest that kinase activity is required to retain PER2 in the cytoplasm.

The intracellular localization of PER proteins is regulated by a nucleocytoplasmic shuttling mechanism involving homo- and heteromeric complex formation (Chaves et al., 2006; Yagita et al., 2002). To test whether such shuttling is affected by PRKCA, we treated COS7 cells coexpressing PER2^{EGFP} and PRKCA with leptomycin B (LMB), which promotes nuclear accumulation of PER2 by blocking CRM1-mediated nuclear export (Yagita et al., 2002). After LMB treatment, the fraction of PER2^{EGFP}-expressing cells with exclusively nuclear staining became enriched in a time-dependent manner, with ~80% of the cells showing fluorescence in the nucleus 3 hr after initiation of the treatment (Figures 6D and 6E). In the presence of PRKCA, PER2^{EGFP} still entered the nucleus, but this occurred at a reduced efficiency, with only ~45% of the cells exhibiting nuclear green fluorescence 3 hr after LMB application. These data suggest that PRKCA

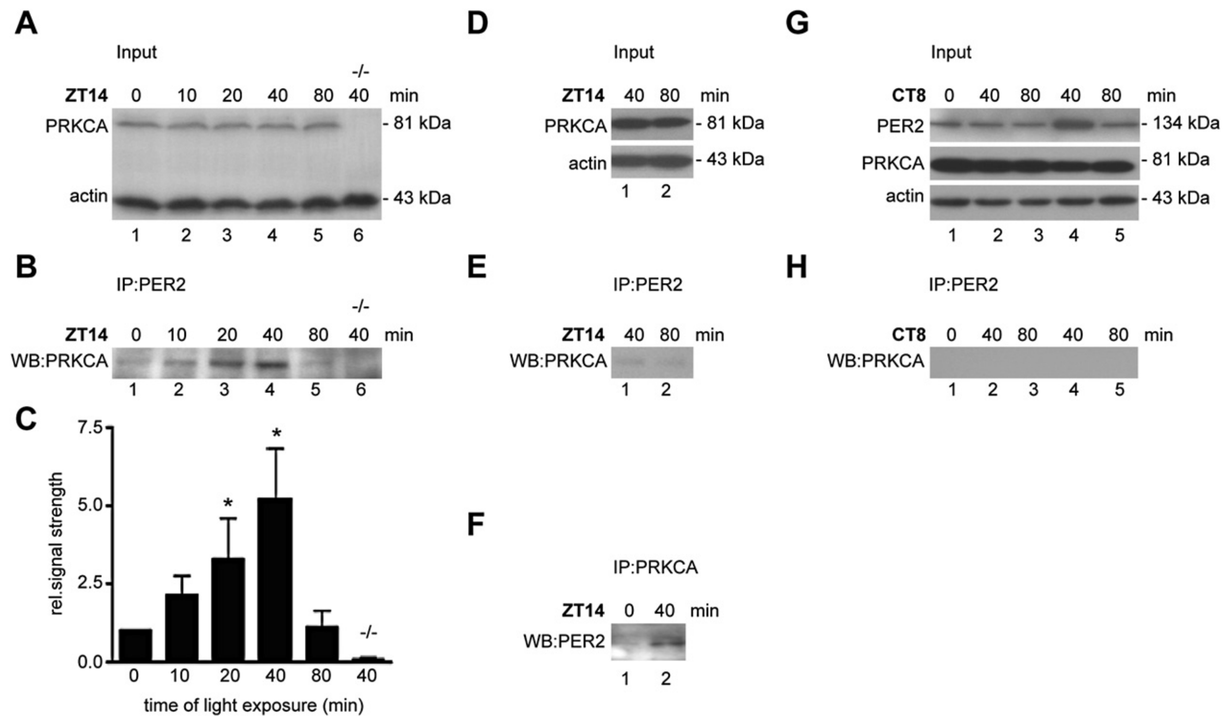

Figure 5. Transient Interaction of PRKCA and PER2 in the SCN Induced by Light Exposure at ZT14

Animals entrained to LD 12:12 were exposed to light pulses (400 lux) of progressively longer duration, starting at ZT14. Sham controls were kept under the same conditions but did not receive any nocturnal light. (A) Western blotting and probing with PRKCA or actin antisera reveals constant levels of these proteins in the SCN of wild-type mice (lanes 1–5). No PRKCA signal was detected in *Prkca*$^{-/-}$ SCN extract (lane 6). (B) Protein extracts were immunoprecipitated with PER2 antibodies, western blotted, and probed for PRKCA. Note a transient interaction of two proteins peaking at 40 min. (C) Densitometric quantifications of three independent experiments of the type shown in (B). For calculation of relative signal strength, see Experimental Procedures. Data are presented as mean ± SEM (*p < 0.05, Student's t test). (D and E) Without light exposure (sham control), immunoprecipitation of SCN extracts with PER2 followed by western blotting with PRKCA antiserum yields a weak signal, indicating that PER2-PRKCA interaction is light evoked. (F) Immunoprecipitation of SCN extracts from light-exposed mice with a PRKCA antiserum followed by western blotting with the PER2 antibody used for immunoprecipitation in the experiments shown in (B)–(E) yields a strong band at t = 40 min (lane 2). Reminiscent of the data shown in Figure 5B (lane 1), a weak band is seen prior to application of a light pulse. (G and H) Mice kept in DD and exposed to light at CT8 show no interaction between PER2 and PRKCA (H). Lane 1 represents SCN prior to light exposure. Lanes 2 and 3 are from SCNs of mice exposed to 40 or 80 min of light. Lanes 4 and 5 are sham controls. The input panel (G) demonstrates that PER2 and PRKCA were both present in the SCN extract.

regulates the kinetics of nuclear import of PER2 protein. A mechanism in which PRKCA enhances cytoplasmic retention of PER2 is consistent with the resetting phenotype of *Prkca*$^{-/-}$ mice. Without PRKCA, the cytoplasmic pool of PER2 would be depleted more rapidly, the negative feedback shortened, and hence, the light-induced phase delay reduced.

PRKCA Controls the Stability of PER2

In addition to its regulation of the kinetics of nuclear import, one can envisage additional mechanisms by which PRKCA may modulate the negative feedback loop of the circadian clockwork. Casein kinase 1ε (CK1ε) is known to phosphorylate PER proteins and influence the period length of the TTL by regulating not only the nuclear import, but also the stability, of PER proteins (Akashi et al., 2002; Eide et al., 2005; Takano et al., 2000; Vanselow et al., 2006; Vielhaber et al., 2000). To test whether PRKCA affects PER2 stability, HEK293 cells were transfected with either PER2^{EGFP} alone or PER2^{EGFP} and PRKCA. After blocking de novo protein synthesis with cycloheximide (CHX), cellular levels of PER2 were determined by western blotting (Figure 7A). This data showed that PRKCA stabilizes PER2. As can be calculated from the decay curves (Figure 7B), the presence of PRKCA extended the half-life of PER2^{EGFP} from 12.8 ± 2.3 hr to 38.6 ± 4 hr. These transient expression studies provide evidence that PRKCA stabilizes PER2 protein in cell cultures.

To test whether PRKCA has a similar stabilizing influence on PER2 in the SCN, we measured PER2 protein levels after light exposure in *Prkca*$^{-/-}$ mice. For this we used the same experimental paradigm as for the analysis of light-evoked PRKCA/PER2 interaction. Western blot analysis revealed comparable amounts of PER2 protein in wild-type and mutant animals at ZT14, prior to exposure to the light pulse (Figures 7C and 7D). Consistent with previous reports (Beaulé et al., 2003; Field et al., 2000), light exposure of wild-type animals did not significantly affect

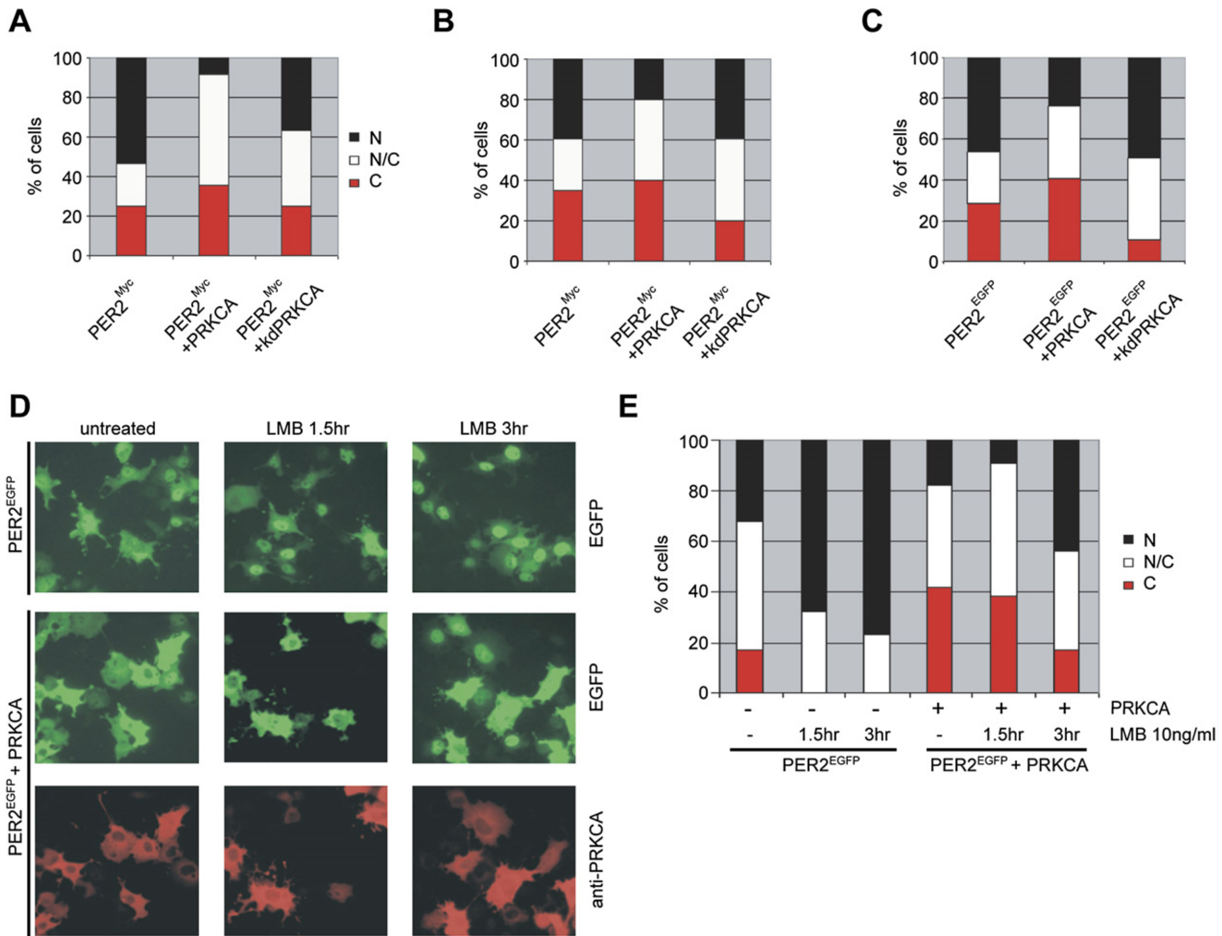

Figure 6. PRKCA Controls Nuclear Import of PER2 Protein

(A–C) Quantitative representation of subcellular distribution of tagged PER2 alone and in the presence of PRKCA or kinase-dead PRKCA (kdPRKCA). Percentage of COS7 (A and C) or U2OS (B) cells showing nuclear (N), nuclear and cytoplasmic (N/C), or cytoplasmic (C) localization of tagged PER2 is shown.

(D and E) Blocking nuclear export in COS7 cells with leptomycin B (LMB) leads to a time-dependent enrichment of $PER2^{EGFP}$ in the nucleus. (D) Representative micrographs of $PER2^{EGFP}$ (green) and PRKCA (red) immunofluorescence before and after LMB treatment, respectively. (E) Bar plots are quantifications of experiments as shown in (D). All bar plots are derived from a field of 100 cells in three independent assays.

the amount of PER2 in the SCN. However, western blot analysis uncovered a marked time-dependent reduction of PER2 protein in the SCN of *Prkca*$^{-/-}$ animals (Figure 7C, quantified in Figure 7D).

Taken together, these data demonstrate that PRKCA stabilizes PER2 after light exposure in vivo. The reduction of such stabilization in the absence of PRKCA is consistent with the resetting phenotype of *Prkca*$^{-/-}$ mice in that having fewer PER2 molecules in SCN neurons of light-exposed mice would attenuate the negative feedback and hence reduce the magnitude of phase delay.

DISCUSSION

In mammals, photic resetting of the circadian oscillator in the SCN involves CREB-mediated transcriptional activation of certain clock genes (Travnickova-Bendova et al., 2002). Our current findings, obtained using mice with a loss-of-function mutation in the *protein kinase C* α (*Prkca*) gene, shed new light on photic entrainment of the mammalian circadian clock. We have shown that *Prkca*$^{-/-}$ animals fail to properly reset their circadian clock in response to a light pulse given at ZT14 and that the underlying defect does not involve light/CREB-mediated transcriptional activation. Instead, as discussed in detail below, we have provided in vitro and in vivo evidence that PRKCA transmits the light signal through posttranslational mechanisms affecting the negative limb of the TTLs that lie at the heart of the circadian clock.

PRKCA and CREB Operate by Different Pathways

The *Prkca*-deficient mice's attenuation of clock resetting in response to light exposure during the early night is reminiscent of that observed in the $CREB^{S142A}$ mutant mouse

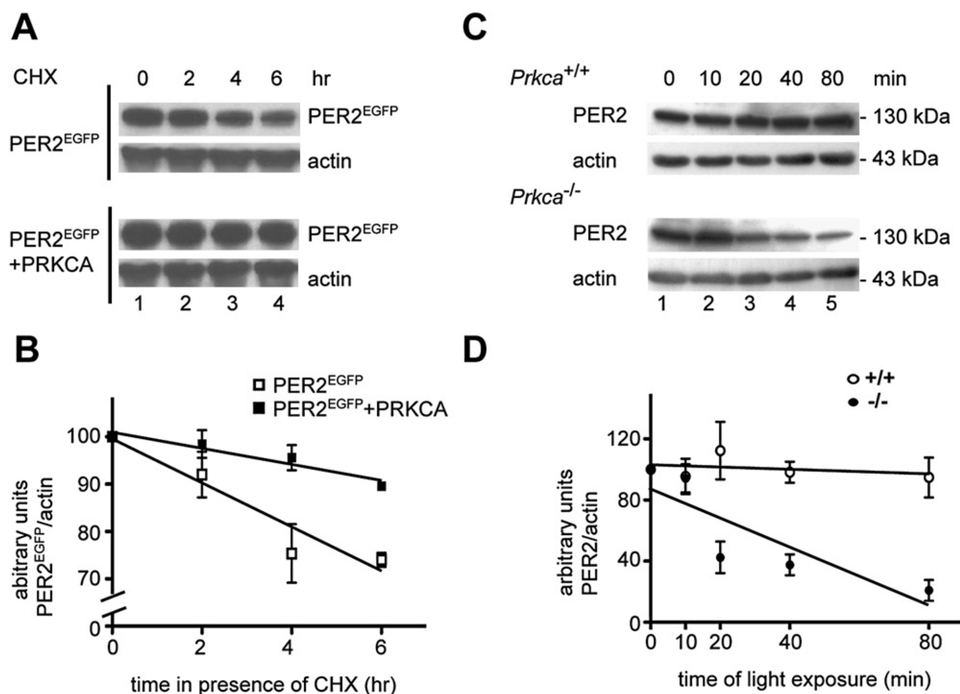

Figure 7. PRKCA Stabilizes PER2

(A) Western blotting of HEK293 cell extracts showing the rate of degradation of PER2^{EGFP} in the absence (top panel) and presence (lower panel) of PRKCA. Duration of cycloheximide (CHX) treatment and antibody used are indicated.

(B) Quantification of western blots (n = 3). PER2^{EGFP} levels were normalized to actin and the amount of PER2^{EGFP} present at the onset of CHX treatment (0 hr) was set to 100%. All data presented are mean ± SEM.

(C) Levels of endogenous PER2 in SCN extracts of wild-type and *Prkca*$^{-/-}$ mice exposed to a progressively longer light pulse beginning at ZT14. The PER2 antiserum used detects a single band at ~130 kDa.

(D) Quantification of western blots (n = 3). PER2 levels were normalized to those of actin and the amount of PER2 detected at ZT14 was set to 100%. All data presented are mean ± SEM.

model, raising the possibility that PRKCA, similar to other kinases, resides in a pathway converging on CREB. In animals homozygous for the CREB^{S142A} point mutation, as well as in animals in which the CREB signaling cascade was compromised by other means, a light pulse decreases the induction of *Per* gene transcription (Dziema et al., 2003; Gau et al., 2002; Oster et al., 2003; Paul et al., 2003; Yokota et al., 2001). By contrast, we found that the extent of light induction of three different clock genes—*Per1*, *Per2*, and *Dec1*—in wild-type and *Prkca*$^{-/-}$ mice was very similar. These data led us to conclude that the CREB pathway is still fully functional in *Prkca*-deficient mice and presumably accounts for the residual phase delay of 30 min seen in *Prkca*$^{-/-}$ animals, and that the photic resetting mechanism involving PRKCA is accordingly different from that mediated by CREB.

PRKCA Functions through Interaction with, and Protection of, PER2

In search of a mechanism of action of PRKCA in photic resetting, we discovered a transient interaction between PRKCA and PER2 in the SCN of light-pulsed mice. It appears that the amount of PER2/PRKCA complex in the SCN parallels the magnitude of behavioral phase shift; the longer the pulse, the more extensive the formation of PER2/PRKCA complexes, the larger the shift. After a very long light pulse, however, the amount of PER2/PRKCA complexes is reduced again. This observation parallels a basic feature of all known circadian clocks, which is that even though initially the amplitude of behavioral phase shifts is directly proportional to the overall quantity of stimulation with light (irradiance), saturation is observed with longer exposure times (Dkhissi-Benyahya et al., 2000; Foster et al., 1991; Nelson and Takahashi, 1991; Yoshimura and Ebihara, 1998).

In the absence of PRKCA, the PER2 protein in the SCN is degraded after exposure of the animal to light. This process occurs on a timescale that is similar to that seen for PER2/PRKCA complex formation, which raises the possibility that the interaction with PRKCA protects PER2 from being degraded by the proteasome. PRKCA-mediated stabilization of PER2 was also observed in cultured cells, albeit the protective effect was less obvious than in the SCN, possibly due to the presence of the EGFP tag. We found no evidence that PRKCA is required for PER2 stabilization during the normal circadian cycle. Thus,

mechanistically, PER2/PRKCA complex formation comes into effect only after phase-delaying light pulsing. There are several examples wherein complex formation of clock proteins assists stabilization of complex constituents. For instance, the stability of PER2 during the circadian cycle is regulated by the formation of a PER2/CRY complex enriched in the nucleus (Kume et al., 1999; Shearman et al., 2000; Yagita et al., 2002). Similarly, the stability and nuclear localization of CLOCK depends on the interaction with BMAL1 (Kondratov et al., 2003). Additional stabilizing components in a PER2/PRKCA complex may be CRY proteins.

A Model of PRKCA Function in Photic Regulation of the Circadian Clock of Mammals

Irrespective of the details of the composition of a PER2/PRKCA complex, our data led us to suggest the following scenario through which PRKCA might affect entrainment of the circadian pacemaker. At the onset of (the subjective) night, SCN cells express high levels of PER and CRY that together represent the negative limb of the circadian feedback loop. Providing a light pulse to a mouse at this time of the day results in the formation of a pool of PER2/PRKCA (and possibly PER2/CRY/PRKCA) in the cytoplasm. In this complex, PRKCA stabilizes PER2 as our biochemical data in *Prkca* mutant mice show. This combination of a transient stabilization and cytoplasmic retention of PER2 has the effect of prolonging the negative feedback. Hence, reactivation of the positive limb of the circadian clock (CLOCK and BMAL1) is delayed. At the behavioral level, this prolongation of the negative feedback causes a phase delay on the day following the light pulse. Our model would predict that if cytoplasmic retention and stabilization of PER2 were reduced—as is the case in *Prkca*$^{-/-}$ mice—the magnitude of light-induced phase delays should be smaller, which is what we observed.

PRKCA is not the sole kinase phosphorylating PER proteins (Xu et al., 2007 and references therein), and conversely, PERs are not the sole PRKC targets (e.g., Shim et al., 2007). The emblematic tau hamster carries a point mutation in the *casein kinase 1 epsilon* gene (*Csnk1e*) and is characterized by a shortened period length (Ralph et al., 1990) and, as shown in an in vitro assay, increased degradation of PER1 and PER2 (Gallego et al., 2006). By contrast, the stabilization of PER2 by PRKCA has no consequences on the period length, but rather plays a role in photic clock resetting. Glycogen synthase kinase-3β also phosporylates PER2 in vitro (Iitaka et al., 2005). Whether this translates into a circadian phenotype is not known. PRKCA and PRKCG can phosphorylate CLOCK, and kinase inhibitor studies in NIH3T3 cells suggest that CLOCK mediates phase resetting in these cells through the activation of *Per1* (Shim et al., 2007).

The clock of *Neurospora crassa* provides a remarkable analogy to our finding of a posttranslational role of a PRKC in photic entrainment (Franchi et al., 2005). During the dark phase the *Neurospora* transcription factor "white-collar-1" (WC1), which regulates expression of the light-inducible clock gene *frequency*, is associated with PRKC. A light pulse evokes a rapid dissociation of the kinase from WC1, followed by the activation of the *frequency* gene (Franchi et al., 2005). Although in mammals, PRKCA binds to the core clock component PER2 as a result of photic stimulation, while in *Neurospora*, PRKC dissociates from WC1, regulation is posttranslational in both species and brings about clock resetting. Taken together, our work affirms the existence of posttranslational mechanisms controlling light entrainment of the circadian clock in mammals, and thus demonstrates the evolutionary conservation of this type of regulation among all organisms that have a circadian clock.

EXPERIMENTAL PROCEDURES

Animals

Behavioral monitoring was performed as described (Jud et al., 2005). For all experiments 129/Sv wild-type mice and *Prkca*-deficient mice of the same genetic background were used. All animals subjected to behavioral experiments were males of at least 3 months of age. Mice were sacrificed by cervical dislocation and decapitated under a 15W safety red light. Animal studies were in compliance with the German Law on Animal Welfare.

Behavioral Recording

Individually housed *Prkca*$^{-/-}$ mutant mice and age-matched wild-type controls were entrained for 8 to 10 days to an LD 12:12 (400 lux) cycle before transfer to DD or LL (100 lux). Locomotor activity was analyzed using ClockLab (Actimetrics). To determine the circadian period of the locomotor activity rhythm, we used χ^2-periodogram analysis. For the photic phase-shifting experiments, entrained mice received a light pulse of 400 lux for the indicated duration at specified time points on the last night in LD or the first day in DD and were subsequently released into DD for 7 to 9 days (Aschoff's type II protocol). Phase shifts were quantified as described in Oster et al. (2002).

In Situ Hybridization

DNA templates for *Prkca* (nts 2318-2847; NM_011101) and *Dec1* (nts 298-1517; NM_011492) were generated by PCR from mouse brain cDNA (Yaylaoglu et al., 2005). Templates for *Per1* and *Per2* were as described (Albrecht et al., 1997). ISH with ^{35}S-labeled antisense RNA probes was carried out on 8 μm paraffin coronal sections through the SCN. Adjacent sections were hybridized with sense probes. Quantification of expression strength was performed using Kodak BioMax MS film (Albrecht et al., 1997; Oster et al., 2002).

Western Blot and Immunoprecipitation from SCN Tissue

Mice were exposed to a 400 lux light pulse of 0, 10, 20, 40, or 80 min at ZT14 or CT8, and brains were removed when the pulse was finished. SCNs were microdissected from 1 mm thick coronal slices. Sham controls were treated identically. SCNs from two mice were pooled and homogenized in lysis buffer (150 mM NaCl, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail [Sigma] [pH 7.4]). After centrifugation supernatants were boiled for 10 min in SDS sample buffer and separated by 8% SDS-PAGE. For immunoprecipitation, protein A agarose beads were preincubated for 1 hr at 4°C with mPER2 antibody (1:50, Alpha Diagnostic International) or with PRKCA antibody (1:50, Cell Signaling). Thereafter, 20 μg SCN lysate protein was added, followed by overnight incubation at 4°C, centrifugation, and washing with PBS. Beads were eluted with Triton X-100-free lysis buffer, and eluates were boiled for 10 min and separated on 8% SDS-PAGE. After transfer to PVDF membranes and blocking

of membranes, proteins were detected with the following antibodies: anti-PER2 antibody, 1:500; anti-PRKCA, 1:1000; anti-actin antibody, 1:2000, Santa Cruz; and secondary goat anti-rabbit antiserum, 1:20,000, Jackson Labs. Detection: Supersignal Westpico or Femto (Pierce). Signal was quantified using computer-assisted densitometry (ImageJ). PER2 and PRKCA bands were normalized to those of actin. Relative strength of PRKCA/PER2 interaction at ZT14 was set as 1.

Western Blots and Immunoprecipitation from Cells

COS7 cells (DMEM, 10% FCS) were seeded in 10 cm dishes at 1.8–2 × 10^6 and transfected (Lipofectamine PLUS, LifeTech or Effectene, Qiagen) 16 hr later with plasmids expressing $PER2^{Myc}$, $CRY2^{V5}$, or PRKCA. Expression vectors were pcDNA3.1/V5-His-TOPO and pcDNA3.1/Myc-His A (Invitrogen). Twenty-four hours later, cells were lysed (lysis buffer: 1% Triton X-100, 50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 10 mM β-mercaptoethanol, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM Na-orthovanadate). For immunoprecipitations 1 mg of total lysate was incubated with 20 μl of protein G agarose beads (Roche) for 30 min at 4°C for removing proteins binding nonspecifically. Samples were centrifuged and supernatants were incubated overnight at 4°C with 1 μg of rabbit polyclonal anti-PRKCA (Santa-Cruz). Forty microliters of protein G beads (preblocked in 2% BSA/PBS) in lysis buffer were added to the samples. This mixture was further incubated for 2 hr at 4°C and centrifuged, and beads were washed four times in lysis buffer. Dry beads were resuspended in 2 × SDS loading buffer, boiled, centrifuged, and analyzed by SDS-PAGE and western blotting using PVDF membranes. Sequential detection of proteins was performed, andfilters were stripped in between with 0.2 N sodium hydroxide. Antibodies were as follows: anti-Myc antibodies, 1:5000; anti-V5 antibodies, 1:3000, both Invitrogen; anti-PRKCA antibodies, 1:1000, Santa Cruz; secondary anti-mouse or rabbit HRP-conjugated, 1:20,000, Jackson Labs. Detection was by chemiluminescence (Supersignal Westpico, Pierce).

For stability studies of PER2, HEK293 cells (DMEM, P/S, 10% FCS) were transfected (Fugene, Boehringer) with $PER2^{EGFP}$ (Yagita et al., 2002) and PRKCA in 60 mm dishes. Cells were trypsinized after 24 hr, equally divided in four 60 mm dishes, and cultured over night. CHX (in ethanol) was added to the cells (100 μg/ml final concentration) and after 2, 4, or 6 hr, cells were lysed with 1 × SDS loading buffer. $PER2^{EGFP}$ and actin were detected on same western blot by chemiluminescence (Renaissance western blot reagent plus, NEN). Antibodies were as follows: anti-EGFP antibodies, 1:1000, Roche; anti-actin antibodies, 1:3000, Chemicon; secondary anti-mouse HRP-conjugated, 1:1000, Dako.

Cell Culture and Immunofluorescence

Immunofluorescence techniques were performed as described (Yagita et al., 2002). Proteins were expressed in COS7 or U2OS cells (grown in McCoy's 5a medium with 1.5 mM L-glutamine, 10% FCS). The subcellular localization of $PER2^{Myc}$ or $PER2^{EGFP}$, singly and double transfected with PRKCA or kdPRKCA, was analyzed 24 hr after transfection and scored in at least two independent experiments. The cellular localizations of the proteins did not vary between 24 hr and 48 hr after transfection. Cells were fixed in 4% paraformaldehyde, washed in PBS, and permeabilized in 0.1% Triton X-100 (10 min each step). Primary antibodies were as follows: mouse anti-Myc (1:650); rabbit anti-PRKCA (1:250 or 1:1000). Secondary antibodies were as follows: FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit IgG (Sigma). In the case of COS7 cells expressing $PER2^{EGFP}$, the secondary antibody was anti-rabbit Alexa 594 (1:1000, Molecular Probes). LMB (Sigma) dissolved in methanol was added to the medium at a final concentration of 10 ng/ml, and cells were incubated for 1.5 hr and 3 hr before fixation.

Kinase Assay

$PER2^{Myc}$, $PER2^{EGFP}$, and $CRY2^{V5}$ proteins alone or in combination were expressed in COS7 cells and isolated by immunoprecipitation with anti-Myc or anti-V5 antibodies (1:650) absorbed to protein G agarose beads. Pull-downs were washed twice in kinase buffer (20 mM Tris [pH 7.5], 2 mM $MgCl_2$, 0.1% NP40, 1 mM Na_2VO_4, 10 μg/ml leupeptin, 0.5 mM DTT). The kinase reaction was performed for 30 min at room temperature with recombinant PRKCA, an aliquot of the pull-down, 100 μM $CaCl_2$, 1 μM ATP, and 5 μCi γ^{32}P-ATP (3000 Ci/mmol, NEN) in presence or absence of 1 μM bisindolylmaleimide. The reaction was stopped by adding loading buffer and boiling for 5 min at 95°C. Twenty-five microliters were loaded into a twelve percent SDS-PAGE and visualized by autoradiography.

Statistical Analysis

In all experiments, statistical significance, with $p < 0.05$ as the criterion of significance, was determined using unpaired two-tailed Student's t tests and GraphPad Prism Software (GraphPad Software, San Diego, CA). (*) and (**) indicate $p < 0.05$ and $p < 0.01$ versus control, respectively.

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