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Melatonin Action and Signal Transduction in the Rat Suprachiasmatic Circadian Clock: Activation of Protein Kinase C at Dusk and Dawn*

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ABSTRACT

Nocturnal synthesis of the pineal hormone melatonin (MEL) is regulated by the circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. We examined the hypothesis that MEL can feed back to regulate the SCN using a brain slice preparation from rat. We monitored the SCN ensemble firing rate and found that MEL advanced the time of peak firing rate by more than 3 h at restricted circadian times (CTs) near subjective dusk [CT 10–14 (10–14 h after lights on)] and dawn (CT 23–0) on days 2 and 3 after treatment. The effect of MEL at CT 10 was blocked by pertussis toxin. The protein kinase C (PKC) activator, 12-*O*-tetradecanoylphorbol 13-acetate, reset the SCN firing rate rhythm with a profile of temporal sensitivity congruent with that of MEL. Two specific PKC inhibitors, calphostin C and chelerythrine chloride, independently blocked MEL-induced phase advances at each sensitive period. Furthermore, MEL administration increased PKC phosphotransferase activity transiently to 200% at CT 10 and CT 23, but not at CT 6. These data demonstrate that 1) MEL can directly modulate the circadian timing of the SCN within two windows of sensitivity corresponding to dusk and dawn; and 2) MEL alters SCN cellular function via a pertussis toxin-sensitive G protein pathway that activates PKC. (*Endocrinology* **138:** 627–634, 1997)

A CENTRAL component for regulating daily, estrual, and seasonal changes in the endocrine state of mammals is the circadian pacemaker in the suprachiasmatic nuclei (SCN) of the hypothalamus (1–3). This biological clock generates approximate 24-h rhythms in the behavior, physiology, and metabolism of the animal. It is subject to external entrainment signals that adaptively synchronize the organism to environmental change. In addition to integrating signals that produce circadian adjustments, the SCN has been implicated in the regulation of seasonal adaptations. SCN-lesioned hamsters are incapable of adjusting their reproductive axis in response to changing photoperiod (3, 4). This loss of functional response has been hypothesized to be due to the loss of SCN-directed synthesis of the pineal hormone, melatonin (MEL) (3).

MEL is a neuroendocrine signal of darkness. The hormone is small (mol wt, 232.3) and lipophilic, and is rapidly carried by blood and cerebrospinal fluid to all tissues of the organism. Its production is tightly restricted to night, or the dark phase of the lighting cycle (3). The rise in plasma MEL levels signals the nocturnal state throughout the internal milieu, and the duration of elevated MEL levels is proportional to night length. As night length changes over the annual cycle, the alteration in MEL profile induces profound changes in the reproductive state of seasonally breeding animals.

Converging evidence suggests that MEL can modulate the mammalian circadian system as well. Although pinealectomy has little effect on circadian activity rhythms in mammals (5), pinealectomized rodents entrain to a reversed photoperiod regimen more quickly than intact animals (6). Also, pinealectomy dampens the amplitude of the firing rate rhythm in the SCN (7). Daily MEL administration can entrain activity rhythms in free running rats, independent of pinealectomy. This effect is temporally restricted: MEL at circadian time 10 (CT 10 = 10 h after lights on in the entraining 24-h lighting schedule), significantly advances behavioral rhythms (8). Armstrong also reported a single incident of phase advance at CT 22 during late subjective night (9). When the SCN of a rat is ablated, MEL has no entraining effect (8). These behavioral studies suggest that MEL can entrain circadian rhythms, and that it acts at the level of the SCN.

The hypothesis that MEL affects the SCN directly can be developed from anatomical and physiological evidence. First, the SCN is a major site of MEL binding in the rodent brain (10, 11). The SCN also exhibits circadian variation in the density of MEL-binding sites. Maximum [^{125}I]MEL binding peaks late in the day (12). Second, MEL alters the metabolic activity of rat SCN in a bimodal fashion (*i.e.* the response is a function of the CT of administration) (13). MEL at late subjective day inhibits 2-deoxyglucose uptake, whereas treatment before lights on increases the metabolic rate in the SCN. Fos-like immunoreactivity is induced in the SCN in response to MEL treatment during late subjective night only (14). Third, single unit activity in rat and hamster SCN brain

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slices is acutely depressed when MEL is applied near the transition to subjective night (15, 16).

In the present report, a brain slice preparation from the rat is used to examine the interaction between MEL and SCN function. When the SCN is maintained *in vitro*, it continues to generate a near 24-h rhythm of the population firing rate (17). This rhythm matches that recorded *in vivo* with multiunit electrodes (18). Furthermore, this preparation achieves virtual isolation of the SCN pacemaker from signals integrated at other brain sites, permitting direct evaluation of responses at the SCN (19). In preliminary studies, we reported that MEL acts directly on the SCN clock *in vitro*, resetting the phase of the neuronal activity rhythm at late subjective day (20). Here we fully characterize the parameters of this effect and delineate more thoroughly the phase-dependent modulation of the SCN pacemaker by MEL. These studies reveal a second period of sensitivity at late subjective night. We also report experiments probing the transduction pathway(s) that transmits the MEL signal within SCN cells and provide evidence for protein kinase C (PKC) activation in this hormone's mechanism of resetting the clock.

Materials and Methods

Animals

Eight- to 10-week-old male Long-Evans rats born into our inbred colony and maintained on a 12-h light, 12-h dark cycle were selected as donors for these studies. Males were selected for these studies to eliminate possible experimental variation due to the influence of the estrous cycle.

Brain slice preparation

The procedure for preparation, maintenance, and recording from SCN hypothalamic slices was detailed previously (21, 22). Briefly, animals were removed from the colony and quickly decapitated, and 500-μm coronal slices containing the SCN were prepared during the donor's subjective lights on period only. Slices were maintained at the interface of the brain slice chamber, where they were continuously perifused with Earle's Balanced Salt Solution (EBSS; Life Technologies, Grand Island, NY) supplemented to 24.6 mM glucose and 26.2 mM sodium bicarbonate. Antibiotic (0.005% gentamicin) was included in incubations longer than 24 h. The medium, adjusted to pH 7.2, was warmed to 37 C and saturated with 95% O_2-5% CO_2. The slices were prepared within 5 min of decapitation and allowed to equilibrate for at least 1 h before experimental paradigms were initiated.

Electrophysiological recording and data analysis

Extracellular activity from single units was sampled with a glass microelectrode filled with 5 M NaCl and positioned by a hydraulic microdrive. A signal to noise ratio of 2:1 was the minimum requirement for discerning individual cells. Each cell was monitored for two or three sequential, 120-sec intervals. Spikes were counted in 10-sec bins, and the mean firing rate of the cell was calculated. On the average, four to six cells were sampled throughout the SCN each hour during a recording period of 8–14 h. The mean firing rate values were then grouped into 2-h bins, and the pattern was smoothed by plotting the data as 15-min running averages. The time of peak in the electrical rhythm (based upon 28–61 U/experiment) was visually discerned. Phase shifts in peak activity were evaluated as the difference between the experimental result and the mean time of peak electrical activity recorded in vehicle-treated controls (0.001% ethanol for 1 h at CT 10, peak = CT 7.0 ± 0, n = 3; 0.05% dimethylsulfoxide (DMSO) for 40 min at CT 10, peak = CT 7.0, n = 1), which overlap with data from untreated slices.

Experimental treatments

Pharmacological treatments were administered by static bath application of reagents in warmed oxygenated EBSS, pH 7.2. At specified times, perifusion of the SCN slice was stopped, and the medium in the dish was quickly removed and manually replaced with the experimental treatment. Treatments were terminated by manual exchange for normal EBSS, after which perifusion was resumed. Phase-shifting treatments were 1 h in duration, unless otherwise indicated. 1) MEL was dissolved at 1 mM in 95% ethanol and serially diluted in EBSS to 1 nM MEL (ethanol equivalent 0.001%). 2) Treatments with 2-iodo-MEL (2-I-MEL; RBI, Natick, MA), a specific MEL agonist, followed the same protocol. 3) Treatments with pertussis toxin (PTX; 1 μg/ml, 6 h), which selectively inhibits a subclass of G proteins including G_i, G_o, and G_q, and control incubations (EBSS exchange, 6 h) were achieved by two sequential 3-h static pulses. In some experiments, MEL was added for the last 10 min of PTX or EBSS static treatment. 4) 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), a phorbol ester that directly activates PKC, was bath-applied in a 10-min pulse (10 nM, ethanol equivalent 0.06%). 5) Calphostin C (CAL C), a specific PKC inhibitor, was dissolved at 1 mM in DMSO and serially diluted in EBSS to 0.1 μM (DMSO equivalent 0.05%), except for the PKC assays, in which CAL C was used at 1 μM. CAL C incubations lasted 40 min, and in some experiments, treatments were followed by a 15-min pulse of 1 nM MEL in CAL C (CAL C/MEL). All experiments with CAL C were carried out after direct exposure to normal laboratory fluorescent lighting conditions. 6) Chelerythrine chloride (CC; Calbiochem, La Jolla, CA), another specific inhibitor of PKC, was dissolved to 50 mM in aqueous DMSO and serially diluted in EBSS to 0.25 mM (0.05% DMSO equivalent). CC incubations lasted 20 min, and in some experiments, treatments were followed by a 15-min pulse of 1 nM MEL in CC (CC/MEL). 7) Staurosporine (STP; 10 μM STP and 0.9% ethanol equivalent), a general kinase inhibitor, was used alone for 1 h. In some experiments, STP treatments were followed by a 10-min pulse of MEL (STP/MEL) or TPA (STP/TPA). All chemicals not specifically noted were purchased from Sigma Chemical Co. (St. Louis, MO).

PKC activity assays

Procedures for slice preparation and bath application follow those stated in the *Experimental treatments* section, with the exception that the duration of MEL treatments lasted only 1 min after the time course of activation had been established. Reduced SCN slices cut in half through the optic chiasm were treated with 1 μM MEL or control medium (EBSS) in the brain slice chamber, and the reaction was stopped by rapid freezing of the tissue on dry ice. In some cases, the inhibitors were added before MEL in the brain slice chamber to evaluate the specificity of the PKC activation. Slices were thawed in assay dilution buffer and briefly sonicated. Total PKC phosphotransferase activity was assayed on this broken tissue preparation using a PKC assay kit (Upstate Biotechnology, Lake Placid, NY). Activity was based on incorporation of [γ-^{32}P]ATP (DuPont-New England Nuclear, Boston, MA) into a synthetic substrate peptide (QKRPSQRSKYL) corresponding to amino acid residues 4–14 of myelin basic protein in the absence of lipids. This assay has been fully validated for the selective assay of PKC in crude tissue extracts (23). A cocktail of protease inhibitors and phosphatase inhibitors was added to the reaction mixture after slices had been thawed and sonicated (0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 mM phenylmethylsulfonylfluoride, 1 mM EGTA, 5 μM microcystin, and 1 mM NaF). The protein content of each slice was determined using the Bradford assay (24), and activity is expressed as counts per min/μg protein.

Statistical analysis

Data are presented as the mean ± SEM or SD where indicated. Statistical significance between or among groups was tested using Student's *t* test (unpaired) or one-way ANOVA followed by preplanned comparisons or the Tukey *post-hoc* test.

Results

MEL and its agonist, 2-I-MEL, permanently reset the phase of the SCN circadian clock at dusk and dawn

The effect of MEL on the biological clock in the SCN was assessed by evaluating the time of peak in the endogenous circadian rhythm of neuronal firing (Fig. 1A, replotted from Ref. 25). In untreated or vehicle-treated controls, the peak occurred near CT 7 (CT 6.9 ± 0.1; n = 8). The experiments contributing to this mean were performed for this study by the authors. The time of peak is consistent with that of the control peaks reported in earlier studies on these inbred rats obtained by our recording methods (26).

When the medium was replaced with 1 nM MEL for 1 h, the timing of the peak was significantly altered. The magnitude of MEL's effect was dependent upon the phase of the circadian cycle at which treatment was applied. After MEL treatment at CT 10 (subjective dusk), the oscillation peaked at CT 3 on days 2 (Fig. 1B) and 3 *in vitro* (Fig. 1B; separate experiment). This phase advance is significant on both day 2 (ϕ_A = 3.8 ± 0.1 h; n = 6; $P < 0.0001$, by Student's *t* test) and day 3 (ϕ_A = 3.8 ± 0.4 h; n = 4; $P < 0.0001$). The new phase of the rhythm is stable; that is, the two subsequent peaks following treatment occur at the same circadian time. Thus, the clock is fully reset during the interval between the treatment and the time the next peak appears. A similar effect was observed when MEL was applied at CT 23 (subjective dawn; Fig. 1C). The mean phase advance was 3.7 ± 0.1 h (n = 3) on day 2 and = 3.6 h (n = 2) on day 3.

When the period of exposure to MEL either at CT 10 or CT 23 was reduced from 1 h to 15 min, the phase advances were 4.25 h (n = 2) at CT 10 and 4.00 ± 0.00 h (n = 3) at CT 23 (data not shown), which overlap those seen with 1-h incubations. This indicates that the processes underlying MEL induction of phase shifts are fully stimulated within 15 min of SCN exposure to MEL.

SCN sensitivity to the MEL agonist, 2-I-MEL, was temporally similar to that of MEL. 2-I-MEL treatment (1 nM) at CT 10 induced a robust phase advance of 6.8 ± 0.1 h (n = 3; Fig. 1D). 2-I-MEL treatment early in the subjective day at CT 2, a MEL-insensitive time, did not affect the electrical activity rhythm (ϕ_A = 0.3 ± 0.2 h; n = 3). The means of the results of these experiments at CT 2 and CT 10 with 2-I-MEL have previously been reported (20).

Ten different time points across the circadian cycle were evaluated for MEL sensitivity (CT 0, 2, 6, 7, 10, 11, 14, 18, 22, and 23). A total of 38 experiments were performed to construct the MEL phase response curve (Fig. 2); five of the points in this curve originally appeared in Ref. 20. A phase response curve is obtained by plotting the magnitude of the phase shift in hours *vs.* the circadian time of treatment. These experiments reveal two separate periods of SCN sensitivity to MEL that correlate with points of transition in the entrained lighting cycle. The first, a relatively broad window, appears to surround the subjective dusk period (CT 10–14). A second period of sensitivity appears near subjective dawn (CT 23–0). Although this morning window of sensitivity is

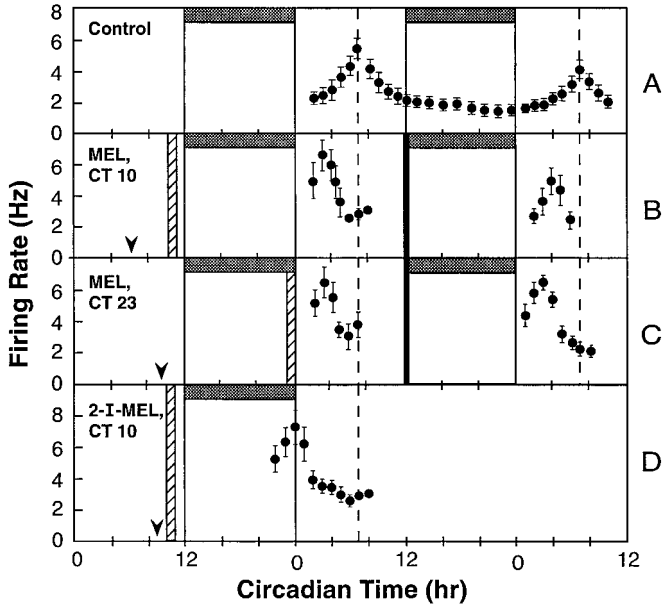

FIG. 1. MEL application to the SCN *in vitro* at subjective dusk or dawn induces stable phase advances in the circadian rhythm of neuronal activity. A, In untreated brain slices, the peak SCN neuronal firing rate occurs near CT 7 each day (A was originally published in Ref. 25). B, MEL treatment (1 nM for 1 h) at CT 10 induced a near 4-h phase advance of the peak on day 2 *in vitro* (ϕ_A = 3.8 ± 0.1 h; n = 6). In a separate experiment, a similar phase advance was seen on day 3 (ϕ_A = 3.8 ± 0.4 h; n = 4), indicating that MEL induced a stable, permanent phase shift. C, At CT 23, MEL induced similar phase advances (day 2: ϕ_A = 3.7 ± 0.1 h, n = 3; day 3: ϕ_A = 3.6 h, n = 2). D, The MEL agonist, 2-iodo-MEL, also advanced SCN electrical activity rhythm. Agonist treatment at CT 10 resulted in a near 7-h ϕ_A on day 2 *in vitro* (ϕ_A = 6.8 ± 0.1 h; n = 3), whereas treatment at CT 2 had virtually no effect on the time of peak compared to vehicle controls (data contributed to the mean reported in Ref. 20). The *dashed line* indicates the mean time of peak in vehicle controls. The *dark horizontal bar* indicates subjective night. The *vertical striped bar* indicates the time of treatment. *Arrow* indicates the time of slice preparation.

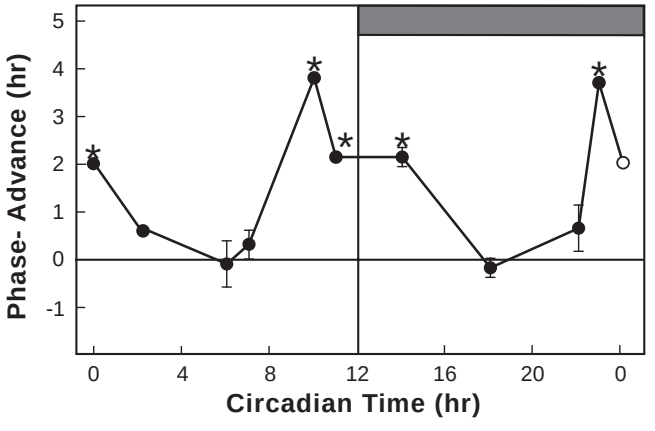

FIG. 2. Phase-response curve for MEL shows two windows of sensitivity. The relationship between the time of MEL treatment and the magnitude of its effect (±SEM) on the SCN electrical activity rhythm is plotted. Treatments at either point of transition in the environmental lighting cycle (CT 10–14 and CT 23–0) elicited near 4-h phase advances, whereas those at other times were without significant effect. Three replications were performed at each CT, except for CT 10, where n = 6. Some data at CTs 2, 6, 10, 14, 18, and 22 are replotted from Ref. 20. The *open circle* indicates double plotting of CT 0 data. *Asterisks* indicate statistically significant phase shifts ($P < 0.01$, by ANOVA, Tukey's comparison).

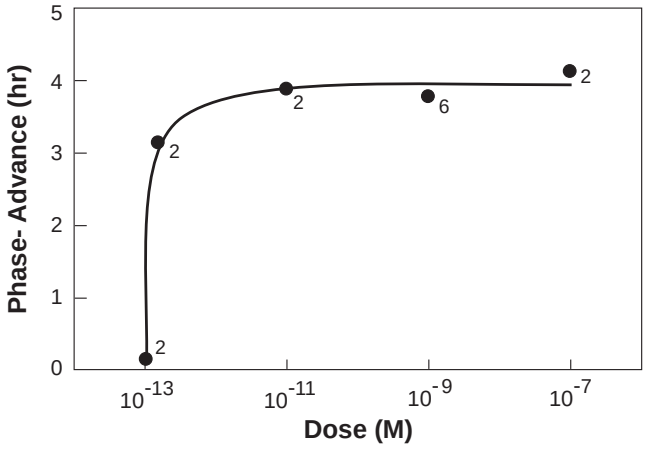

FIG. 3. The dose-response relationship reveals that the SCN is highly sensitive to phase resetting by MEL. Plotted are the mean phase advances of the electrical activity rhythms induced by various concentrations of MEL applied at CT 10. At 2×10^{-13} M, MEL elicited a phase advance of 3 h, whereas at 1×10^{-13} M MEL was ineffective. Data at the 10^{-9}-M concentration used for the CT 10 experiments in Fig. 2 were reused in this figure. *Number subscripts* indicate the number of replications at each dose.

narrower than the one at dusk, the magnitudes of the phase advances are equivalent (~4 h).

The dose-response curve (Fig. 3) demonstrates that the 1-nM concentration used in these experiments elicits the maximal response and is well within the plateau range. Remarkably, a robust phase advance in electrical activity was induced at 0.2 pM MEL ($\phi_A = 3.15$ h; n = 2), whereas no effect was found with 0.1 pM MEL ($\phi_A = 0.15$ h; n = 2). This sharp transition is indicative of an "all or none" physiological response to ligand binding.

PTX blocks the MEL phase advance at dusk

PTX, which ADP-ribosylates certain guanine nucleotide-binding regulatory proteins (G proteins), was used to probe the signal transduction pathway(s) involved in the MEL response. A 6-h preincubation of the slice in PTX (1 μg/ml) blocked the MEL phase shift. PTX alone (Fig. 4A) did not alter the time of peak ($\phi_A = 0.1 \pm 0.1$ h; n = 3), indicating that this treatment is neither toxic to the SCN tissue nor are the G proteins that it affects involved in time-keeping during subjective day (CT 4–10). The SCN tissue was still responsive to exogenous treatment of MEL after a 6-h static bath of normal EBSS (Fig. 4B). PTX incubation before a 10-min PTX/MEL pulse at CT 10 blocked the phase-shifting effects of MEL (Fig. 4C). This indicates that MEL acts through a PTX-sensitive G protein pathway in the SCN. The time of peak in these experiments occurred at CT 6.6 ± 0.1 h (n = 5), which is comparable to the time of peak in vehicle controls and untreated slices.

TPA mimics MEL-induced phase shifts, whereas inhibitors of PKC block MEL and TPA action

A well defined PTX-sensitive signaling pathway involves G protein stimulation of phospholipase C (PLC), producing lipids that activate PKC. To probe this pathway, the phorbol

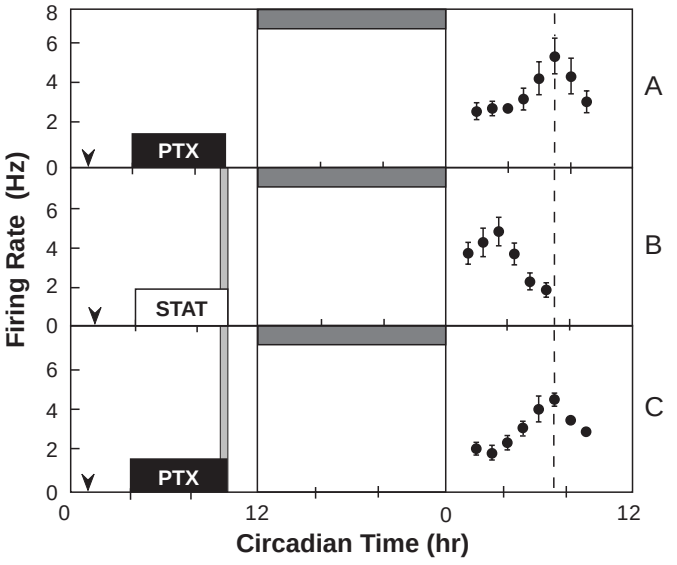

FIG. 4. PTX blocks MEL's ability to reset the SCN clock at dusk. A, PTX exposure (two sequential 3-h periods = 6 h in static bath) did not alter the time of peak in electrical activity (CT 6.8 ± 0.1; n = 3) compared to untreated slices (CT 6.9 ± 0.1; n = 8). B, A 6-h static bath of EBSS (same protocol as for PTX) did not affect SCN sensitivity to MEL treatment (10 min), as a 4.15-h phase advance was observed. C, A 6-h PTX incubation followed by 10 min of MEL/PTX at CT 10 resulted in blockade of the MEL phase advance, with time of peak occurring at CT 6.6 ± 0.1 (n = 5). The *horizontal bar* indicates static incubation. Other symbols are explained in Fig. 1.

ester TPA was applied to the SCN in a 10-min pulse. TPA reset the SCN clock with a phase-dependent profile similar to that seen with MEL (Fig. 5). Near midsubjective day, a MEL-insensitive time, TPA was relatively ineffective (CT 6; mean $\phi_A = 1.0$ h; n = 2). Treatment at CT 10 or CT 23 resulted in significant phase shifts. When applied at CT 10, TPA produced a $\phi_A = 4.8 \pm 0.4$ h (n = 3); treatment at CT 23, subjective dawn, induced a $\phi_A = 4.0 \pm 0.4$ h (n = 3). These phase advances are not significantly different from those seen with MEL.

TPA is a specific and direct activator of PKC. Therefore, we tested the efficacy of two specific PKC inhibitors, CAL C and CC, in blocking the effect of MEL. The inhibitors themselves did not alter the time of peak when administered alone for 40 or 20 min before CT 10 or 23 (Fig. 5), which indicates that these treatments do not affect ongoing pacemaker function at those times (CT 10: Cal C $\phi_A = 0.1 \pm 0.4$ h, n = 3; CC $\phi_A = 0.0 \pm 0.3$ h, n = 3; CT 23: Cal C $\phi_A = -0.1 \pm 0.1$ h, n = 4; CC $\phi_A = 0.4 \pm 0.1$ h, n = 3). However, when a 40-min Cal C treatment was followed by a 15-min pulse of CAL C/MEL, at dusk or dawn, the phase-advancing effect of MEL was completely blocked (CT 10 $\phi_A = 0.2 \pm 0.2$ h, n = 3; CT 23 $\phi_A = 0.1 \pm 0.1$ h, n = 3). CC, when applied 20 min before a 15-min CC/MEL pulse, also completely blocked the effect of MEL (CT 10 $\phi_A = 0.1 \pm 0.1$ h, n = 3; CT 23 $\phi_A = 0.1 \pm 0.1$ h, n = 3). The nonspecific kinase inhibitor STP produced the same results as those seen with CAL C and CC as well as blocking the phase advances seen with TPA at dusk and dawn (data not shown). These results support a role for PKC in transduction of the MEL signal for phase resetting in the SCN.

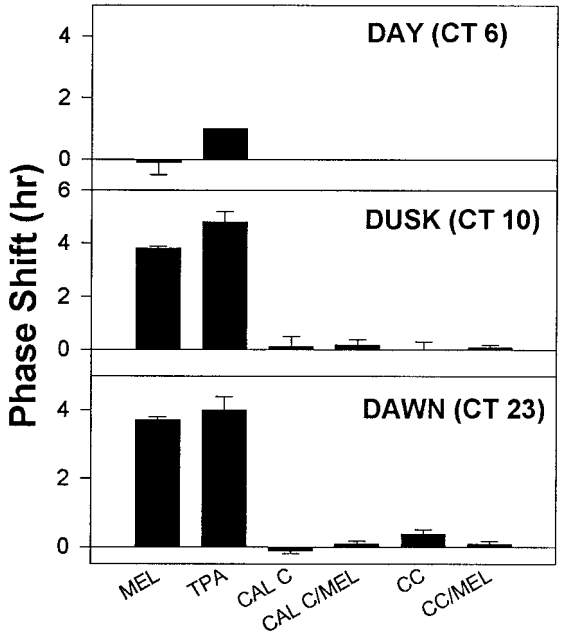

FIG. 5. Phorbol ester treatment induces MEL-like phase advances at dusk and dawn, whereas the PKC inhibitors, CAL C and CC, blocks the MEL-induced phase advances at both windows of sensitivity. Plotted are the mean phase shifts ± SEM (n = 3–6, except TPA at CT 6, where n = 2). MEL or TPA treatment at midday, CT 6, did not significantly alter the time of peak in electrical activity rhythm ($P > 0.05$, by Student's t test). At dusk and dawn, MEL and TPA induced similar, near 4-h phase advances. Administration of PKC inhibitors alone did not alter time of peak ($P > 0.05$), whereas these inhibitors completely blocked the phase-advancing effect of MEL alone. MEL, 1 nM MEL; TPA, 1 μM TPA, 10 min; CAL C, 0.1 μM CAL C, 40 min; CC, 0.25 mM CC, 20 min.

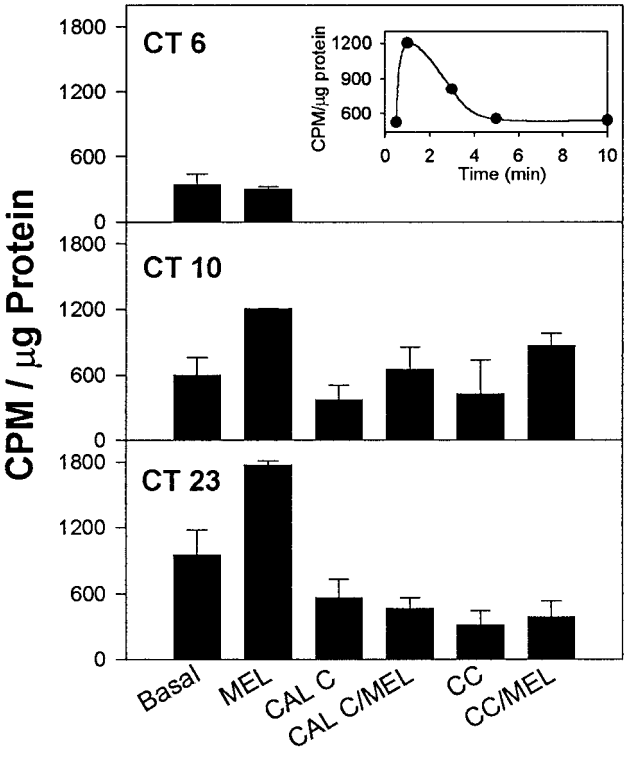

FIG. 6. MEL increases PKC phosphotransferase in the SCN at dusk and dawn. Results are plotted as the mean ± SD (n = 3 in all cases). During midday, basal PKC activity was low, and MEL application induced no significant change in activity ($P > 0.5$, by Student's t test). At dusk, MEL induced a 2-fold increase in PKC activity ($P < 0.003$). Addition of inhibitors or inhibitors plus MEL showed no significant difference in activity from basal levels ($P > 0.05$, by one-way ANOVA). At CT 23, PKC activity was increased 2-fold by stimulation with MEL ($P < 0.004$, by Student's t test). Addition of inhibitors alone or inhibitors plus MEL showed significant suppression of basal activity, but no significant difference between treatments ($P < 0.05$, by one-way ANOVA, preplanned contrasts). A time course of MEL treatment at CT 10 (*inset*) shows that 1 min of MEL exposure produces maximal stimulation of PKC. Abbreviations are explained in Fig. 5.

MEL increases PKC phosphotransferase activity

To directly test the hypothesis that MEL activates PKC in the SCN, PKC enzyme activity was measured at CT 6, 10, and 23. An assay was used that is based on the transfer of radiophosphate [^{32}P]ATP from [γ-^{32}P]ATP into a substrate peptide that corresponds to a fragment of myelin basic protein that is specifically phosphorylated by PKC. During midday (CT 6), PKC activity was low and not altered by 1-min MEL treatment (Fig. 6; basal activity, 341.8 ± 98.13 cpm/μg protein; MEL, 300.49 ± 23.35 cpm/μg). However, at dusk (CT 10), MEL nearly doubled PKC phosphotransferase activity (basal activity, 598.93 ± 160.29 cpm/μg; MEL, 1204.65 ± 7.23 cpm/μg). The same effect was seen at CT 23 (basal, 948.34 ± 226.33 cpm/μg; MEL, 1769.43 ± 40.82 cpm/μg). Results are plotted as the mean ± SD and n = 3 in all cases.

Careful evaluation of the time course of MEL-induced PKC activation at CT 10 revealed that activation is rapid and transient. Maximal stimulation was detected after 1 min, and activity had returned to basal levels by 10 min (time course *inset* in Fig. 6). When SCN slices were incubated with the specific PKC inhibitors, CAL C and CC, alone or before a 1-min pulse of MEL, activity was either not significantly different from basal levels (CT 10; CAL C = 371.67 ± 132.50 cpm/μg; CAL C/MEL, 647.00 ± 206.00 cpm/μg; CC, 414.33 ± 319.66 cpm/μg; CC/MEL, 865.67 ± 111.73 cpm/μg) or lower than them (CT 23; CAL C, 563.78 ± 167.14 cpm/μg; CAL C/MEL, 465.23 ± 97.33 cpm/μg; CC, 314.47 ± 130.59 cpm/μg; CC/MEL, 390.24 ± 143.90 cpm/μg) These results demonstrate that MEL activates PKC in the SCN at both dusk and dawn, but not at midday.

Discussion

The present study demonstrates that MEL can induce robust changes in the phase of the SCN circadian clock *in vitro* and that this regulation is limited to the crepuscular periods in the lighting cycle. The brain slice preparation provides an isolated environment; therefore, circadian variation in SCN sensitivity to MEL is gated by the clock itself. Similar temporal sensitivity to the agonist, 2-iodo-MEL, supports the specificity of this effect. Treatments that affect PTX-sensitive G proteins or that inhibit PKC block this effect, whereas a phorbol ester activator of PKC mimics it. Furthermore, MEL can activate PKC in the SCN selectively, at the circadian times that it induces phase shifts. Together these data support the hypothesis that MEL activation of G protein-linked receptors

in the SCN leads to phase resetting through a PKC mechanism.

The response of the SCN *in vitro* to MEL correlates in several respects with responses of animals *in vivo* to MEL treatment. Firstly, rats free running in constant darkness entrain to daily MEL injections during late subjective day (CT 8–12) by phase advancing their wheel-running activity (27). To date, little evidence has been shown for a late night entraining effect of MEL *in vivo*, although Armstrong reports one incident of a phase advance with MEL application at dawn (9).

Secondly, behavioral entrainment to MEL is dose dependent (ED_{50}, 5 μg/kg) and quantal (28), with rats either fully responding or not at all. A similar all or none response describes the relationship between MEL concentration and its effects on SCN phase resetting *in vitro*. This supports the idea that MEL's action on behavioral entrainment is at the level of the SCN.

Thirdly, the threshold level for maximal response to MEL's action *in vitro* (4.64 pg/ml) is near daytime levels of plasma MEL in rats (4.00 pg/ml) (29), which do not shift circadian rhythms. It is possible that the absence of MEL in the brain slice perfusion medium may make the SCN *in vitro* hypersensitive to MEL. *In vivo*, the sensitivity of rats to MEL entrainment is increased by lack of exposure to MEL. Rats that have been pinealectomized and are free running in constant darkness can be entrained by daily MEL injections of 100 ng/kg, which is one third the amount needed to entrain sham-operated controls (30). Both groups responded to single MEL treatments.

Our results also demonstrate that recent exposure to endogenous MEL is not a prerequisite for SCN sensitivity to exogenous MEL treatment. Brain slices are prepared during the subjective day, when MEL production is inhibited in the animal, and there is no available source of endogenous MEL to the brain slice. In our hands, the SCN can survive for up to 3 days (without exposure to MEL), and maintain stable rhythms. Experiments with MEL treatment at CT 23 require slices to be without MEL for 23 h or more, and yet the dawn window of sensitivity appears. Dusk sensitivity, followed by a long period of MEL insensitivity through the night, then the reappearance of sensitivity at dawn, indicate that the response to MEL is a clock-regulated phenomenon and is not induced by the hormone itself. Margraf and Lynch (16) have reported that the circadian profile of MEL sensitivity in hamster SCN single unit activity is also independent of prior exposure to endogenous MEL. MEL-induced suppression of the firing rate in SCN neurons disappeared during night 1 *in vitro*, but reappeared in their preparation at subjective dusk on the second day *in vitro*.

It is curious that the MEL agonist induces a greater change than MEL itself. A similar effect, however, is seen with serotonin and its agonists, 5-carboxamidotryptamine and 8-hydroxy-dipropylaminotetralin. When serotonin is applied to an SCN slice at CT 9, it produces a phase advance of 4.6 $\pm$ 0.5 h, whereas 5-carboxamidotryptamine advances the rhythm by 6.0 $\pm$ 0.1 h, and 8-hydroxy-dipropylaminotetralin advances it by 7.0 $\pm$ 0.0 h (22). However, no difference in the maximal amplitude of the phase-shifting response is seen with a wide range of cholinergic agonists (31). This suggests an unusual feature of 2-I-MEL action. The difference may be due to 2-I-MEL's greater binding affinity at the receptor (32, 33), or the agonist could be coactivating an additional pathway, due to its differential structure, and producing an additive response.

Mechanisms of MEL signal transduction show both species and tissue specificity. Many studies have shown that MEL alters cellular function by modifying cyclic nucleotide levels. The cloned mammalian MEL receptor, in fact, has been shown to be coupled to inhibition of adenylate cyclase in COS-7 cells (33). However, our investigations into MEL signal transduction mechanisms in the rat SCN *in vitro* did not demonstrate cyclic nucleotide involvement. The phase-response curves for reagents stimulating cAMP and cGMP are out of phase with each other, and their periods of sensitivity are temporally distinct from the MEL phase-response curve (19, 26, 34). Further, we found no convincing evidence that MEL treatment at late subjective day altered basal levels of cAMP or cGMP, or that forskolin-stimulated cAMP levels in the SCN were affected by MEL treatment (35). As the SCN has a low level of MEL receptors (36), it is possible that local changes in cAMP levels may occur in some SCN cells, but these changes may be undetectable when the whole SCN is assayed. Thus, we cannot exclude the possibility that MEL acts to inhibit adenylate cyclase locally within the SCN.

Studies in two systems have now shown that the MEL-receptor complex uses a PTX-sensitive G protein pathway; PTX disrupts signaling pathways in COS-7 cells transfected with the cloned mammalian MEL receptor (33), and PTX completely blocks the MEL-induced phase advance at CT 10 in our system. PTX uncouples receptor-ligand complexes from their usual G protein interactions by inducing ADP-ribosylation of the regulatory subunit of multiple G proteins. PTX-sensitive G proteins include G_i, which inhibits adenylate cyclase and modulates potassium and calcium channels; G_o, which activates PLC and regulates ion channels; and G_q, which activates PLC. Recent reports suggest that activation of PLC by PTX-sensitive G proteins occurs via the $\beta\gamma$-subunit, and not the α-subunit, as is usually the case (37, 38).

Diacylglycerol (DAG) and PKC are key elements in the PLC pathway. PLC has been measured in moderate amounts in rat SCN (39). Activation of PLC by PTX-sensitive G proteins leads to the production of DAG. DAG, in combination with calcium, can activate PKC. The SCN contains significant amounts of PKC (Hunt, A., unpublished results). Thus, should MEL act at a PTX-sensitive G protein-coupled receptor to activate PLC in the SCN, it could stimulate PKC. Alternatively, should the MEL-activated receptor act primarily via a G protein-linked ion channel, the resulting ionic fluxes could lead to phospholipase A_2 activation. Phospholipase A_2 can subsequently activate PKC via ecosanoids such as arachidonic acid. The elements of these linkages mediating MEL activation of PKC in the SCN are yet to be determined.

The phorbol ester, TPA, directly activates PKC by mimicking unsaturated DAG (40). Our electrophysiological experiments examining the ability of TPA to phase shift the ensemble firing rate rhythm revealed that the SCN has a profile of sensitivity to TPA comparable to that of MEL with regard to time of day and magnitude of response. These experiments demonstrate that the clock regulates a permissive element in the PKC pathway at an intracellular level (41)

downstream from membrane phospholipids. This gate to PKC activation and phase resetting is closed at CT 6, but opens at both dusk and dawn. MEL or TPA exposure at these times can access the clock mechanism via a PKC-mediated cascade and adjust clock timing.

PKC inhibitors have been developed that can interact with the substrate-binding site (ATP or protein) or with the regulatory domain binding sites (DAG or phorbol ester). CAL C, a compound isolated from the bacterium *Cladosporium cladosporides*, binds to the DAG-binding site of PKC, thereby inactivating the enzyme. CAL C is highly specific for PKC, with an IC_{50} value of 0.05 μM, and shows virtually no inactivation of other protein kinases (42). A benzophenanthridine alkaloid, CC, binds and blocks the ATP-binding site of the PKC molecule and is again a highly specific inhibitor of PKC, with an IC_{50} of 0.66 μM (43). STP, a *Streptomyces*-derived indole alkaloid, binds directly to the regulatory subunit of PKC, thereby preventing activation of this enzyme (44), but is nonspecific for PKC, interacting with other protein kinases. When treatment with any of these inhibitors was coupled with a subsequent pulse of MEL at either window of SCN sensitivity, blockade of both PKC activation and the phase advance was observed in all cases. Thus, activation of PKC must be a primary path by which MEL resets the SCN pacemaker.

Finally, PKC activity assays showed that MEL increases kinase activity in the SCN at dusk and dawn, but not at midday. In both cases, PKC phosphotransferase activity was doubled upon the addition of MEL. The response to MEL was very rapid and transient; it could be maximally measured within 1 min of treatment, and at that time activation was significant and robust. PKC activity after the addition of inhibitors was not significantly different from basal levels of PKC activity at CT 10. This may be related to the fact that inhibition of PKC at CT 10 has no effect on circadian rhythmicity, suggesting that the clock itself is not using PKC at this time. It is through MEL exposure that PKC has an effect on clock timing. At CT 23, inhibitors suppressed PKC activity below basal levels, suggesting a subtle difference in the PKC interaction with the inhibitors at this time. It is possible that different isoforms or regulators of PKC may be present at dusk and dawn.

It is noteworthy that the recently cloned mammalian MEL 1a receptor is a G protein-linked receptor bearing two consensus sequences for PKC phosphorylation on its cytoplasmic domain (33). This observation coupled with our results demonstrating a role for PKC in MEL signal transduction suggest an intracellular feedback role of this pathway in the receptor function, possibly through regulation of receptor desensitization.

In conclusion, the results of the present study support a new signal transduction mechanism for MEL that involves PTX-sensitive activation of PKC. The sensitivity of the SCN to MEL is clock-driven, rather than a response to previous exposure to the hormone. Circadian clock-controlled sensitivity results in precise temporal ordering of the MEL-sensitive periods so that they fall at the crepuscular transitions (19). MEL activation of the PKC pathway alters the clock function at subjective dawn and dusk, periods when seasonal changes in night length alter the endogenous MEL profile. MEL acts rapidly on the SCN at these times, advancing the clock and thus facilitating physiological changes in anticipation of environmental demands and maintaining adaptive synchrony with nature.

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