Vasoactive intestinal polypeptide requires parallel changes in adenylate cyclase and phospholipase C to entrain circadian rhythms to a predictable phase

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An S, Irwin RP, Allen CN, Tsai C, Herzog ED. Vasoactive intestinal polypeptide requires parallel changes in adenylate cyclase and phospholipase C to entrain circadian rhythms to a predictable phase. J Neurophysiol 105: 2289–2296, 2011. First published March 9, 2011; doi:10.1152/jn.00966.2010.—Circadian oscillations in the suprachiasmatic nucleus (SCN) depend on transcriptional repression by Period (PER) 1 and PER2 proteins within single cells and on vasoactive intestinal polypeptide (VIP) signaling between cells. Because VIP is released by SCN neurons in a circadian pattern, and, after photic stimulation, it has been suggested to play a role in the synchronization to environmental light cycles. It is not known, however, if or how VIP entrains circadian gene expression or behavior. Here, we tested candidate signaling pathways required for VIP-mediated entrainment of SCN rhythms. We found that single applications of VIP reset PER2 rhythms in a time- and dose-dependent manner that differed from light. Unlike VIP-mediated signaling in other cell types, simultaneous antagonism of adenylate cyclase and phospholipase C activities was required to block the VIP-induced phase shifts of SCN rhythms. Consistent with this, VIP rapidly increased intracellular cAMP in most SCN neurons. Critically, daily VIP treatment entrained PER2 rhythms to a predicted phase angle within several days, depending on the concentration of VIP and the interval between VIP applications. We conclude that VIP entrains circadian timing among SCN neurons through rapid and parallel changes in adenylate cyclase and phospholipase C activities.

phase response curve; pacemaker; Period gene; luciferase; Förster resonance energy transfer

COORDINATED RHYTHMS across populations of neurons are believed critical to many behavioral and cognitive functions (Buzsáki 2006). The mechanisms that synchronize the periods of neural oscillators can include gap junctions that produce in-phase rhythms (Mancilla et al. 2007; Schneider et al. 2006), reciprocal inhibition producing either in-phase or anti-phase cycling (Wang and Rinzel 1992), and fast, weighted, excitatory synapses producing a range of phase relationships (Smarandache et al. 2009). Daily, or circadian, rhythms in behavior and physiology, however, depend on the neuropeptide VIP. The mechanisms by which VIP synchronizes circadian rhythms among cells are unknown.

The daily resetting of circadian timing establishes a stable phase relationship (i.e., the phase angle of entrainment) between behavioral and physiological rhythms and environmental cues. VIP is well positioned to reset circadian oscillators in the brain to each other and to exogenous timing cues. VIP and its receptors, Vipr1 and Vipr2, are expressed in the central and peripheral nervous systems (Chaudhury et al. 2008; Dietl et al. 1990; Mohney and Zigmond 1998), including in the suprachiasmatic nucleus (SCN), a master circadian pacemaker (Cagampang et al. 1998; Shinohara et al. 1999). VIP applied to SCN explants in the late subjective night induces the transcription of Period (Per) 1 and Per2, two genes implicated in rhythm generation and entrainment (Nielsen et al. 2002). VIP can shift the daily rhythms in locomotion (Piggins et al. 1995) and in electrical discharge (Reed et al. 2001) and vasopressin release (Watanabe et al. 2000) of SCN explants. These actions of VIP in the SCN have been shown to depend on the activities of phospholipase C (PLC) (Nielsen et al. 2002), adenylate cyclase (AC), or protein kinase A (PKA) (Meyer-Spasche and Piggins 2004), but the signaling underlying entrainment by VIP has not been studied.

A phase-response curve (PRC) plots the steady-state shift in a rhythm as a function of the time of stimulation. A PRC can be used to predict features of entrainment, including the phase angle of entrainment, the range of periods to which the oscillator can entrain, and how long it will take to entrain (Pittendrigh 1960). Importantly, existing PRCs have not been tested for their ability to predict these features of SCN entrainment. This study aimed to generate a PRC to VIP that would predict features of entrainment and could be used to test the underlying molecular mechanisms and kinetics. We combined pharmacology with recordings of bioluminescence from a reporter of PER2 levels and of Förster resonance energy transfer (FRET) from a reporter of cAMP levels. We found that VIP directly entrains the PER2 rhythms of SCN neurons through rapid, parallel changes in AC and PLC signaling.

MATERIALS AND METHODS

Animals. PER2::LUCIFERASE (PER2::LUC) knockin mice (Yoo et al. 2004; founders generously provided by J. S. Takahashi, Univ. of Texas Southwestern Medical Center, Dallas, TX) were housed in a 12:12-h light-dark cycle and bred as homozygous pairs in the Danforth Animal Facility of Washington University. All procedures were approved by the Animal Care and Use Committee of Washington University or Oregon Health Sciences University and followed National Institutes of Health guidelines.

Drugs. VIP was purchased from Bachem (King of Prussia, PA) or Tocris (Ellisville, MO). MDL-12,330a (MDL), 9-(tetrahydro-2-furyl)adenine (THFA), 1-((6-[[17b]-3-methoxyestra-1,3,5(10)-tri-en-17-yl]amino)hexyl)-1H-pyrrole-2,5-dione (U-73122), forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, Missouri).

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Louis, MO), (7R)-4-hydroxy-7-methoxy-N,N,N-trimethyl-3,5,9-trioxo-4-phosphohexatetracon-1-aminium-4-oxide (edelfosine) was from Tocris. Drugs were dissolved in DMSO or deionized water as stock solutions, stored at −20°C, and diluted with culture medium so that the final DMSO concentration was below 0.4% of the total volume. Culture media consisted of DMEM (Sigma) supplemented with 2% B27 (Invitrogen, Carlsbad, CA), 10 mM HEPES (Sigma), and 2.2 mg/ml NaHCO₃ (Invitrogen). VIP was dissolved in culture medium, and vehicle controls consisted of an equal volume of culture medium.

**Bioluminescence recording.** We recorded bioluminescence rhythms from 300-μm coronal SCN slices from PER2::LUC mice (age: 8–20 days) using a photomultiplier tube (model HC135-11, Hamamatsu, Shizuoka, Japan) as previously described (Abe et al. 2002). SCN explants were cultured on 0.4-mm membrane inserts (Millipore, Billerica, MA) in zincate-glutamate-HEPES (Z/G) solution consisting of (in mM) 124 NaCl, 2.5 KCl, 1.2 NaHPO₄, 1.2 MgCl₂, 2.4 CaCl₂, 10 glucose, and 24 NaHCO₃, adjusted to 300 mosM and bubbled with 5% CO₂ and 95% O₂. The recording chamber was located on the stage of an inverted microscope (Nikon TE2000E, Toyko, Japan), illuminated using a xenon arc lamp, and passed through a 436/20-nm filter (Chroma, Technical Corporation, Bellows Fall, VT) within a Lambda 10-3 filterwheel (Sutter Instruments, Novata, CA) and with light reflected by a 455dcxr dichroic filter (Chroma, Technical Corporation). Images were visualized using an ORCA ER charge-coupled device camera (Hamamatsu Photonics) after being passed through a Dual-View beam splitter at 505dxr (Optical Insights), with 535/40- and 480/30-nm emission filters. Data acquisition was controlled by Metafluor software (Molecular Devices, Sunnyvale, CA) with binning and light exposure optimized to minimize photobleaching. The FRET ratio, fluorescence intensity near 500 nM, and saturation of VIP applied to SCN cultures was determined without subsequent removal.

To measure the phase shift, bioluminescence data were detrended by subtracting a 24-h running average (Abe et al. 2002) and the daily peak of expression was determined using an acrophase fitting function with Clocklab software (Actimetrics, Wilmette, IL). Phase shifts were measured as the time difference between linear regressions of the acrophases on the days before a treatment and the 4–5 days after treatment. In some cases, the shift was measured after one to three cycles of transient shifts. The period of the PER2::LUC rhythm was measured as the average time between acrophases from at least 4 days of recording. The induction of PER2 expression was measured by averaging the raw bioluminescence signal of the cycle with the VIP or vehicle treatment. All statistics were performed with Origin 7.0 software (Origin, Northampton, MA).

**ELISA.** We measured VIP concentrations to determine the profile of the neuropeptide from eight mouse SCN explants treated with either 1 μM VIP (n = 4) or vehicle (n = 4). Medium (40 μl) was collected from each culture at 0, 10, 30, 60, and 120 min and 24 h after treatment, immediately frozen at −35°C, and stored at −80°C. A competitive ELISA was performed according to the manufacturer’s protocol (Peninsula Laboratories, San Carlos, CA). Absorbance was read at 450 nm with a microplate spectrophotometer (Molecular Devices, Menlo Park, CA). A standard curve was generated with serially diluted standards ranging from 0 to 10 ng/ml and an IC₅₀ of 0.24 ng/ml.

**cAMP measurement.** SCN cultures were prepared from neonatal Sprague-Dawley rats and transfected with a cAMP reporter using the bioasitic method as previously described (Ikeda et al. 2003). Briefly, neonatal rat pups (3–7 days old) were decapitated, the brains were removed, and 200- to 300-μm-thick coronal slices were cut with a vibrating blade microtome (Camen Instruments, Lafayette, IN). Slices were placed on Millicell-CM membranes (30-mm diameter, 0.4 μm, Millipore) and maintained in an incubator at 37°C with 5% CO₂. Organotypic cultures were grown in culture media consisting of DMEM-High without L-glutamine and with sodium pyruvate (HyClone, Thermo Scientific, Waltham, MA), 2% B27 supplement (GIBCO, Carlsbad, CA), 10 mM HEPES (GIBCO), and 1× GlutaMax (GIBCO).

cAMP activity was measured using a fusion protein consisting of cyan fluorescent protein (CFP), truncated EpaC1 expressing a cAMP-binding site, and yellow fluorescent protein (YFP) (DiPilato et al. 2004; Dunn et al. 2006). The cDNA for ICUE2 was kindly provided by Dr. Jin Zhang and Dr. Marla B. Feller (DiPilato et al. 2004; Dunn et al. 2006; Violin et al. 2008). A Helios Gene Gun (Bio-Rad Laboratories) was used according to the manufacturer’s instructions to transfect the ICUE2 cDNA, driven by a cytomegalovirus promoter, into 1- to 20-day-old cultures. Individual neurons were imaged between 2 and 7 days after transfection. Slice cultures were transferred to a recording chamber (35°C) with a laminar flow (6–8 ml/min) of ACSF solution consisting of (in mM) 124 NaCl, 2.5 KCl, 1.2 NaHPO₄, 1.2 MgCl₂, 2.4 CaCl₂, 10 glucose, and 24 NaHCO₃, adjusted to 300 mosM and bubbled with 5% CO₂ and 95% O₂. The recording chamber was located on the stage of an inverted microscope (Nikon TE2000E, Toyko, Japan), illuminated using a xenon arc lamp, and passed through a 436/20-nm filter (Chroma, Technical Corporation, Bellows Fall, VT) within a Lambda 10-3 filterwheel (Sutter Instruments, Novata, CA) and with light reflected by a 455dcxr dichroic filter (Chroma, Technical Corporation). Images were visualized using an ORCA ER charge-coupled device camera (Hamamatsu Photonics) after being passed through a Dual-View beam splitter at 505dxr (Optical Insights), with 535/40- and 480/30-nm emission filters. Data acquisition was controlled by Metafluor software (Molecular Devices, Sunnyvale, CA) with binning and light exposure optimized to minimize photobleaching. The FRET ratio, fluorescence intensity near 535/480 nm after background subtraction at each wavelength, was normalized using the ratio before the application of VIP. Neurons were identified by morphological appearance. At the end of each experiment, neurons were treated with forskolin (20 μM) and IBMX (75 μM). Cells that did not respond were excluded.

**RESULTS**

VIP shifts of PER2::LUC rhythms depend on phase and dose. We monitored the effects of 10 nM–100 μM VIP on PER2-driven LUC activity in SCN explants. The half-life of VIP applied to SCN cultures was ~2 h based on ELISA measurements (Supplemental Material, Supplemental Fig. S1). VIP application near the peak of PER2 expression (CT12) reduced the subsequent amplitude and delayed the peak of subsequent cycles compared with vehicle-treated cultures (Fig. 1A). This study focused on the phase-shifting effects of VIP, leaving cause and relevance of the amplitude effects for a subsequent analysis. The steady-state phase shift after 4 days (Fig. 1B) was measured as a function of VIP concentration (Fig. 1C). When applied at CT12, VIP induced a dose-dependent delay in the peak of PER2 expression, with a threshold of ~100 nM (P < 0.05 compared with vehicle treatment, one-way ANOVA, Scheffé post hoc test, F₁,₁₅ = 11.15; EC₅₀ near 500 nM, and saturation of ~10 μM). VIP also induced a transient increase in PER2 expression with a similar dose dependence (Fig. 1E). The threshold for VIP-mediated PER2 induction was ~100 nM, similar to the threshold for phase shifting (P < 0.05 compared with vehicle treatment, one-way ANOVA, Scheffé post hoc test, F₁,₁₆ = 7.85; EC₅₀ near 100 nM, and saturation of ~5 μM). Thus, a 10-fold increase in VIP concentration approximately doubled the steady-state phase delay of SCN rhythmicity (r² = 0.98, n = 30 SCN explants).

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1Supplemental Material for this article is available at the Journal of Neurophysiology website.
To measure whether VIP has phase-dependent effects on SCN rhythms, either 100 nM or 10 μM VIP was applied at various circadian time points. The resulting VIP PRCs had a large delay zone from early subjective day to early subjective night (CT3–18) and a small advance zone from late subjective night to early subjective day (CT19–1) with significant differences between the times of advances and delays (P<0.01, F1,63=7.11, n=63, two-way ANOVA, Tukey post hoc test; Fig. 1D). VIP (10 μM) treatments at CT11–12 induced a phase delay that was similar in magnitude at 1, 2, and 3 days after treatment (P=0.77, one-way ANOVA, F1,18=0.09). In contrast, the same VIP treatment at CT19–23 induced advances that were larger when measured the day after treatment than on subsequent days (P=0.02, one-way ANOVA, F1,16=6.17; Supplemental Fig. 2). VIP (10 μM) induced larger delays than 100 nM VIP at most of the time points tested except the early subjective day. Vehicle treatment induced no or little shift (P<0.05, two-way ANOVA, Tukey post hoc test, compared with 10 μM VIP, n=41). Therefore, adjustment of daily PER2 rhythms depended on the time of administration and concentration of VIP.

**Blockade of both AC and PLC activities is required to suppress VIP-induced phase shifts.** Previous findings have indicated that in the SCN, VIP may signal through cAMP- or Ca2+-mediated pathways. We examined the effects of two inhibitors of AC and two inhibitors of PLC on VIP-induced phase shifts. At the time of VIP administration, we included MDL, an irreversible, competitive inhibitor of AC (Lippe and Ardiizone 1991), THFA, a noncompetitive inhibitor of AC (O’Neill et al. 2008), U-73122, an inhibitor of PLC (Smith et al. 1990), or edelfosine, a specific inhibitor of PLC (Powis et al. 1992). VIP-induced phase shifts of the PER2::LUC rhythms at CT12 were significantly reduced by the combined application of MDL (2 μM) or THFA (100 μM) with U-73122 (10 μM) or edelfosine (10 μM) 1 h before VIP application (P<0.05, F1,15=8.47 for MDL + U-73122; P<0.01, F1,12=...
VIP ENTRAINS VIA AC AND PLC

VIP entrains PER2::LUC rhythms to a predicted phase angle. We investigated if and how the SCN circadian pacemaker might entrain to daily VIP stimulation. Our VIP PRC predicted that daily VIP treatment should fall around CT2 to entrain the SCN, ~10 h before the peak of PER2::LUC bioluminescence (CT12). VIP (10 or 25 nM) or vehicle was applied to SCN cultures for 5 consecutive days starting on the fifth day of the bioluminescence recording. The period of SCN cultures was not altered by the vehicle treatment (before: 24.5 ± 0.3 h vs. during: 24.5 ± 0.2 h, n = 7 cultures, P = 0.97, one-way ANOVA; Fig. 4, A and D). In contrast, the circadian period was shortened by daily 25 nM VIP, with the bioluminescence peak occurring 10.1 ± 0.4 h after VIP on the last day of application (period before: 24.7 ± 0.2 h vs. days 2–5 of the treatment: 24.4 ± 0.2 h, n = 7 cultures; Fig. 4, C and D).

Notably, the phases of cultures treated with 25 nM VIP were more synchronized (Rayleigh test, P < 0.05, r = 0.97) than those of vehicle-treated cultures (P > 0.1, r = 0.52; Fig. 4E).
A lower dose of VIP (10 nM) had a smaller effect on the period (before: 25.0 ± 0.3 h vs. during: 24.7 ± 0.2 h) and a less reliable effect on the phase of entrainment (10.0 ± 1.1 h, n = 6 cultures; Fig. 4, B and D). Application of 25 nM VIP on a 25-h cycle also entrained the period of SCN cultures (24.4 ± 0.1 h before and 25.0 ± 0.2 h during the application, n = 4 cultures) to the predicted phase so that the VIP application fell 7.6 ± 0.7 h before the peak of bioluminescence (Fig. 4F). In contrast, vehicle treatments every 25-h failed to change the period (24.4 ± 0.2 h before vs. 24.6 ± 0.1 h during, n = 2 cultures). Therefore, daily VIP applications entrained PER2::LUC rhythms to the predicted phase angles.

**DISCUSSION**

The mechanisms by which circadian rhythms synchronize to daily timing cues have been formally described as a result of rapid changes in either phase or period of the endogenous oscillator (Comas et al. 2006). Rapid phase adjustment, or nonparametric entrainment, has been shown in the eclosion rhythm of flies (Zimmerman et al. 1968), locomotor activity of nocturnal rodents (Pittendrigh and Daan 1976), and in vitro oscillation of cyanobacterial genes (Yoshida et al. 2009) and predicted for the SCN (Best et al. 1999). Our results implicate VIP in rapid phase adjustment of the SCN on a daily basis. Single pulses of VIP shifted the phase, rather than the period, of the SCN (Supplemental Fig. S3) and repeated pulses entrained SCN rhythms. Importantly, VIP doses near the threshold for phase shifts, when applied daily, entrained circadian rhythms of PER2. VIP similarly entrains circadian rhythms in cortical astrocytes (Marpegan et al. 2009). Thus, we postulate that VIP shifts the oscillations of SCN cells through rapid changes in their clock gene expression. In the SCN, VIP release is both circadian (Shinohara et al. 1995) and increased by light (Shinohara et al. 1995; Shinohara et al. 1993). Based on the PRCs measured here, we make two predictions: 1) increases in VIP release due to light during the day delay SCN rhythms; and 2) in the absence of light, circadian release of VIP peaking around midday delays free-running SCN rhythms. These predictions are consistent with the advanced phase angle of entrainment in a light cycle and shortened free-running period in constant darkness reported in VIP-deficient mice (Colwell et al. 2003).

The PRC to VIP described here is the first based on shifts in PER2 expression in the SCN and differs in shape and amplitude from a PRC to light and also differs in some respects from published responses to VIP. The PRCs for VIP-induced shifts in vasopressin and multiunit firing rate rhythms were previously described as light like with advances three to eight times larger than the shifts reported here (Reed et al. 2001; Watanabe et al. 2000). We found that advances were larger when measured on the day after VIP treatment (as was done in previous studies) compared with the steady-state shift (Supplemental Fig. S2). This illustrates that the isolated SCN can exhibit large, transient adjustments in phase similar to what has been described for behavioral shifts to light during the late night. Importantly, PRCs based on the first day or two after a treatment can differ substantially from the steady-state PRC.

By measuring the steady-state PRC to VIP applied at many different circadian times from long-term recordings of SCN, we conclude the VIP PRC differs from the effects of light in vivo or glutamate in vitro on SCN rhythms. The VIP PRC is dominated by a large delay zone. Although this could be unique to PER2, it is consistent with the period-lengthening effects of chronic VIP infusion on locomotor activity (Pantazopoulos et al. 2010).
Interestingly, the PRC to VIP in the SCN shared a similar shape and amplitude as the PRC to the neuropeptide pigment dispersing peptide (PDF) in the cockroach (Petri and Stengl 1997). PDF in flies appears to play roles similar to mammalian VIP in entrainment and synchrony among circadian oscillators (Lin et al. 2004). Thus, the steady-state PRC with a large delay zone and low-amplitude, narrow advance zone may have features that facilitate coordinated rhythmicity in populations of circadian cells.

The rate of entrainment and the phase angle of entrainment both depended on the concentration of VIP and whether VIP was applied on a 24-h cycle or a 25-h cycle. Thus, aging, light intensity, and other events that change VIP levels or time of release would be expected to impact circadian behaviors. This is consistent with, for example, evidence that age-related changes in VIP timing and levels are intimately associated with menopause in rats (Gerhold et al. 2005; Krajnak et al. 1998). It is clear, however, that VIP is not the sole entraining agent of the SCN since, for example, mice lacking VIP or its receptor can still entrain to light cycles. Photic entrainment likely depends on neuropeptides and transmitters including VIP (Piggins et al. 1995; Reed et al. 2001; Watanabe et al. 2000), pituitary adenylate cyclase-activating polypeptide (Hannibal et al. 1997; Harrington et al. 1999), gastrin-releasing peptide (Albers et al. 1995; Kallingal and Mintz 2006; McArthur et al. 2000; Piggins et al. 1995), glutamate (Asai et al. 2001; Ding et al. 1994; Meijer et al. 1988), and nitric oxide (Ding et al. 1994) and intracellular signals including cAMP (Prosser and Gillette 1989) and cGMP (Liu et al. 1997; Prosser et al. 1989).

Although the exact sites of action for VIP-induced entrainment in the SCN are unknown, we found that VIP increased intracellular cAMP in individual SCN neurons (Fig. 3B). The VPAC2 receptor, encoded by the Vipr2 gene, is presumed to be the primary mediator of VIP activity since the loss of the receptor produces a phenotype similar to loss of VIP (Colwell et al. 2003; Harmar et al. 2002). Consistent with our findings, Vipr2 mRNA (Cagampang et al. 1998; Shinohara et al. 1999) and a transgenic reporter using the human Vipr2 promoter (Kallo et al. 2003) have been shown to be widely expressed in the SCN.

We found convergence of VIP signaling to both AC and PLC signaling pathways in the SCN. Here, low doses of antagonists against the two pathways suppressed VIP-induced shifts, whereas blockade of a single pathway had little effect on shifts. This implicates G<sub>i</sub> in VIP-mediated responses and also raises the possibility for the involvement of G<sub>q</sub> or G<sub>o</sub>/o activities (Aton et al. 2006; Gillette and Mitchell 2002; Hains et al. 2004; Stewart et al. 2007; Trimble et al. 1987; Van Rampelbergh et al. 1997). These results are consistent with previous observations that signals like glutamate can shift SCN rhythms while raising cAMP levels at times when cAMP alone does not shift SCN rhythms (Prosser and Gillette 1989; Tischkau et al. 2000) and that the blockade of cAMP production does not prevent these shifts (Tischkau et al. 2000). We postulate that VIP requires concurrent increases in AC and PLC activities to shift SCN circadian timing. It remains an interesting possibility that changes in the contributions of AC and PLC pathways after VIP stimulation shape the response to VIP at different circadian times (Jenkins et al. 2007; O’Neill et al. 2008; Obrietan et al. 1999).

Previous reports have shown that prolonged blockade of cAMP production or Ca<sup>2+</sup> in the rodent SCN or snail retina stop the progression of circadian rhythms (Khalsa et al. 1993; Lundkvist et al. 2005; O’Neill et al. 2008; Ralph et al. 1992). Here, the low concentrations of antagonists that blocked VIP-induced phase shifts had little effect on PER2 rhythms over 5–6 days. We conclude that the mechanisms mediating shifts to VIP are more sensitive to acute changes in cAMP and Ca<sup>2+</sup> levels than the mechanisms involved in rhythm generation.

Taken together, the findings presented here implicate VIP in the synchronization of SCN neurons to each other and environmental cycles via increases in AC and PLC signaling to rapidly shift clock gene rhythms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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