Effects of VPAC$_2$ Receptor Activation on Membrane Excitability and GABAergic Transmission in Subparaventricular Zone Neurons Targeted by Suprachiasmatic Nucleus

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Hermes MLHJ, Kolaj M, Doroshenko P, Coderre E, Renaud LP. Effects of VPAC$_2$ receptor activation on membrane excitability and GABAergic transmission in subparaventricular zone neurons targeted by suprachiasmatic nucleus. J Neurophysiol 102: 1834–1842, 2009. First published July 1, 2009; doi:10.1152/jn.91261.2008. The hypothalamic suprachiasmatic nucleus (SCN) harbors the master circadian pacemaker. SCN neurons produce the amino acid γ-aminobutyric acid (GABA) and several peptide molecules for coordination and communication of their circadian rhythms. A subpopulation of SCN cells synthesizes vasoactive intestinal polypeptide (VIP) and provides a dense innervation of the subparaventricular zone (SPZ), an important CNS target of the circadian pacemaker. In this study, using patch-clamp recording techniques and rat brain slice preparations, the contribution of VIP to SCN efferent signaling to SPZ was evaluated by examining membrane responses of SPZ neurons to exogenous VIP receptor ligands. In ~50% of the SPZ neurons receiving monosynaptic GABA$_A$ receptor–mediated inputs from SCN, bath-applied VIP (0.5–1 μM) resulted in a membrane depolarization caused by tetrodotoxin-resistant inward currents reversing at ~23 mV. These data suggest the existence of postsynaptic receptors that activate a nonselective cationic conductance. In addition, a subset of SPZ neurons showed an increase in the amplitude of SCN-evoked GABAergic inhibitory postsynaptic currents (IPSCs) and a decrease in their paired-pulse ratios. This, together with an increase in frequency of spontaneous and miniature IPSCs, implies the presence of presynaptic receptors that facilitate GABA release from SCN and possibly other synaptic terminals. The effects occurred in separate neurons and could be mimicked by the selective VPAC$_2$ receptor agonist BAY 55-9837 (0.2–0.5 μM) and partially blocked by the VIP receptor antagonist VIP (6-28) (5 μM). The results indicate that VIP acts via both postsynaptic receptors to differentially modulate SCN GABAergic signaling to distinct subpopulations of SPZ neurons.

INTRODUCTION

Many organisms demonstrate the presence of endogenous circadian clocks that coordinate various behavioral activities (e.g., locomotion) and physiological functions (e.g., hormone secretion and body temperature regulation) to the environmental light/dark cycle. In mammals, neurons located in the hypothalamic suprachiasmatic nucleus (SCN) function as a master pacemaker essential for the expression of circadian rhythms (Klein et al. 1991; Moore 1997). Entrained to the solar day by retinohypothalamic afferents, individual SCN neurons display intrinsic circadian oscillations in their electrical and metabolic activity, orchestrated by interacting transcriptional and translational molecular feedback loops involving several “clock” genes (Antle and Silver 2005; Hastings and Herzog 2004; Reppert and Weaver 2002).

Although remarkable progress has been made in uncovering the cellular and molecular machinery underlying the generation and coordination of the circadian rhythm, still elusive is a detailed understanding as to how the rhythm is actually transmitted to target neurons. The observation that, after SCN lesions, recovery of circadian patterns in locomotor and drinking behaviors can be achieved by transplanting encapsulated SCN explants (Silver et al. 1996) has suggested the existence of a diffusible mechanism of communication, possibly fulfilled by molecules such as transforming growth factor α (Kramer et al. 2001), prokineticin 2 (Cheng et al. 2002), cardiotrophin-like cytokine (Kraves and Weitz 2006), or others known to be synthesized by SCN neurons (van den Pol and Tsujimoto 1985). However, although SCN transplants do provide for recovery of locomotor and drinking behaviors (Ralph et al. 1990; Silver et al. 1996), circadian rhythms in hormone release and cyclic gonadal activity are not restored in SCN-grafted animals (de la Iglesia et al. 2003; Lehman et al. 1987; Meyer-Bernstein et al. 1999), implicating a requirement for axonal outgrowth to effector targets where synaptic transmission directs the communication of clock rhythms to the brain (Kalsbeek and Buiks 2002). Consistent with this view are electrophysiological observations in the rat in vitro that document direct amino acid–mediated rapid neurotransmission from SCN to target neurons in the hypothalamus (Cui et al. 2001; Hermes et al. 1996; Sun et al. 2001) and midline thalamus (Zhang et al. 2006).

One of the hypothalamic targets of SCN efferent projections is the subparaventricular zone (SPZ), a region that extends dorsally and caudally from the dorsal border of the SCN to the hypothalamic paraventricular and dorsomedial nuclei, and is defined largely by this SCN terminal field (Watts and Swanson 1987; Watts et al. 1987). In rodents, a substantial area within the SPZ is particularly well outlined by immunolabeling of the projections from vasoactive intestinal polypeptide (VIP) synthesizing neurons located in the ventral segment of the SCN (Card et al. 1981; Moore et al. 2002). Since SPZ neurons project to many of the same regions as SCN it has been suggested that SPZ neurons might serve to amplify SCN output signals (Watts and Swanson 1987; Watts et al. 1987). Indeed, the consequences of discrete lesions in dorsal and ventral segments of SPZ suggest that this area contains subpopulations of neurons specialized in conveying circadian rhythmicity to various functions, notably sleep–waking, body temperature, and hormone release (Lu et al. 2001).
Information is scant on the cellular neurophysiology of SPZ neurons, their input from SCN, and the role of VIP receptors in modulating SCN–SPZ connections. We sought to address these issues using patch-clamp recording techniques in brain slice preparations. Here we report that SPZ neurons are heterogeneous in their intrinsic electrical properties and mostly receive direct γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor-mediated inhibitory innervation from SCN. In SPZ neurons innervated by SCN we detected two distinct responses to VIP mediated by VPAC<sub>2</sub> receptors, comprising a postsynaptic influence to increase cell excitability, and a presynaptic influence to facilitate GABAergic transmission, including that from SCN. A portion of these results was previously reported briefly (Hermes et al. 2007).

METHODS
Preparation of hypothalamic slices
Experiments used Wistar rats (age 21–40 days) maintained in a 12 h light/12 h dark cycle (lights on at 6:00 am). Experimental protocols conformed to the Canadian Council for Animal Care guidelines and were approved by the Ottawa Hospital Research Institute Animal Care and Use Committee. Care was taken to minimize the number of animals and their suffering. Under pentobarbital anesthesia given between 9:00 and 11:00 am, animals were transcardially perfused with an ice-cold, oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>) iced solution containing NaCl 127, KCl 3, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 26, D-glucose 11, MgCl<sub>2</sub> 1.2, and CaCl<sub>2</sub> 1.2 (Ye et al. 2006). After removal of the brain, a block of tissue containing the hypothalamus was placed in a vibrating blade microtome (Leica VT1000S; Leica Microsystems, Nussloch, Germany) and sliced at 350–450 microns in a coronal plane that preserved SCN and its dorsocaudal projections to the SPZ. Slices were maintained for >1 h at room temperature in a bathing solution containing artificial cerebrospinal fluid (ACSF) of the following composition (in mM): glucose 125, KCl 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 26, d-glucose 11, MgCl<sub>2</sub> 1.2, and CaCl<sub>2</sub> 2.4, with osmolality of 310–315 mOsm/kg.

Electrophysiology
For recording, slices were submerged in a custom-built recording chamber and perfused with oxygenated ACSF at room temperature. Patch pipettes were pulled from thin-walled borosilicate glass capillaries (Sutter Instruments, Novato, CA) using a Flaming-Brown P-97 horizontal puller (Sutter Instruments, Novato, CA) using a Flaming-Brown P-97 horizontal puller (Sutter Instruments, Novato, CA) and whole cell current- and voltage-clamp recordings were obtained using the “blind” method of voltage-clamp recordings. The composition of the pipette solution was (in mM): K-glucuronate 135, KCl 10, HEPES 10, EGTA 1, Na<sub>2</sub>-phosphocreatine 10, Mg-ATP 2, and Na<sub>2</sub>-GTP 0.2; the pH adjusted to 7.2–7.4 with 1 N KOH and (if necessary) the osmolality to 305–310 mOsm/kg with sucrose. In selected cases biocytin (0.2%) was added to the pipette solution to label cells for location and morphology. For voltage-clamp recording of reversed inhibitory postsynaptic currents (IPSCs) 40 mM K-glucuronate was substituted with KCl. When filled with the recording solutions pipettes had resistances of 4–7 MΩ. Electrical activity was recorded using an Axopatch 200B amplifier, a Digidata 1200 A/D converter, and pClamp 9.2 software (MDS Analytical Technologies, Sunnyvale, CA). Data were filtered at 2 kHz and sampled at 5 kHz. Series resistance ranged between 13 and 38 MΩ and was usually compensated for >50%, and periodically monitored during the experiment. Recordings were terminated when changes in the series resistance of >20% occurred.

A platinum/iridium bipolar concentric electrode (tip diameter 125 μm: FHC, Bowdoin, ME) was positioned in the SCN to evoke postsynaptic potentials/currents using constant voltage pulses (2–25 V, 0.2 ms) delivered by an isolated stimulation unit (model DS2A Digitimer; Welwyn, Garden City, UK) driven by pClamp software.

Experimental protocols
This analysis focused specifically on those SPZ neurons that were deemed to receive direct innervation from efferent projections of SCN neurons by virtue of their response to electrical stimulation in SCN with predominant inhibitory postsynaptic potentials (IPSPs) that, in 5 μM 6-nitro-7-sulfamobenzo(f)quinoxaline-2,3-dione (NBQX) and 20 μM d-2-amino-5-phosphopentanoic acid (d-AP5) to pharmacologically isolate GABA-mediated innervations, displayed constant latencies over variable stimulus intensities and the ability to follow a train of four stimuli at frequencies of 10–20 Hz (Fig. 1). In the continued presence of NBQX and d-AP5, SPZ neurons were assessed for resting membrane potential, input resistance, frequency of spontaneous action potential (AP) discharge, and rectifying membrane conductances. In current-clamp mode cells were examined for the influence of bath applications of VIP on membrane potentials and membrane depolarizations in response to VIP were considered positive when >3 mV. In voltage-clamp mode, to analyze the membrane and receptor mechanism underlying observed changes in membrane potential, VIP, the selective VIP VPAC<sub>2</sub> receptor agonist BAY 55-9837 and the VIP receptor antagonist VIP(6-28) were applied in bathing media containing NBQX, d-AP5, bicuculline methochloride (BIC: 20 μM), and tetrodotoxin (TTX: 0.5–1 μM). Here VIP responses were considered positive when (at a holding potential of −50 mV) an inward current occurred >5 pA. In experiments to examine SCN-evoked spontaneous and miniature IPSCs (sIPSCs and mIPSCs, respectively), ACSF contained NBQX and d-AP5 (and TTX in the case of mIPSCs). For evoked responses, the stimulation protocol was repeated every 20 s. Paired-pulse experiments used an interstimulus interval of 120 ms. To monitor membrane conductance, the protocols also included a 400 ms 50 mV hyperpolarizing voltage pulse. A response of the SCN-evoked IPSC to VIP was considered positive when its amplitude increased to >120% of control values. Increases in sIPSCs were considered positive when their frequency increased to >120% of control values. Significant changes in amplitude or frequency of mIPSCs were determined with statistics (see Data analysis and statistics).

Drugs
NBQX, d-AP5, BIC, TTX, BAY 55-9837, and VIP(6-28) were purchased from Tocris Bioscience (Ellisville, MO). VIP was purchased from American Peptide (Sunnyvale, CA), Phoenix Pharmaceuticals ( Burlingame, CA), or Tocris Bioscience. All drugs were bath applied and peptide applications usually lasted 2 min with a flow rate of 4 ml/min.

Data analysis and statistics
Intrinsic membrane properties were analyzed using Clampfit 9.2 and criteria previously described (Pennartz et al. 1998). Specifically, resting membrane potential was assessed following stabilization after rupture of the membrane going whole cell. AP characteristics, including spike amplitude and the amplitude of the postspike afterhyperpolarization were measured from the AP threshold indicated by the sharp upward inflection of the voltage trace. Input resistance was determined from the linear part of the current–voltage (I–V) relationship by fitting voltage responses to 1 to 2 s transient hyperpolarizing current pulses with a monoexponential function. Instantaneous inward rectification was assessed by calculating input resistances between −60 and −80 and −90 and −110 mV: an input resistance in the more hyperpolarized range ≤50% of the more depolarized range was considered to be indicative of significant inward rectification. Time-dependent inward rectification was assessed by measuring the ampli-
appearance of low-threshold potentials. To assess heterogeneity among SPZ neurons several of these parameters were tested for normality of distribution using the Shapiro–Wilks test. Peptide-induced changes in membrane potential were assessed by averaging 20 to 60 s sections of traces captured with Axoskope 9.2. Changes in apparent input resistance were measured from averages of at least five consecutive sweeps of the voltage response to repeated injections of hyperpolarizing current. In voltage-clamp, peptide-induced changes in membrane current and membrane conductance were determined at a holding potential of -50 mV and from current responses to voltage-pulse protocols (stepping from -100 to -30 mV in 10 mV steps) generated by Clampex 9.2. Linear regression was applied to membrane current values within the -50 to -70 mV range of the voltage-current (V-I) relationship to obtain slope conductances. Peptide-induced changes in the amplitude of SCN-evoked IPSCs were analyzed by averaging ≥15 sweeps of the current response to repetitive SCN stimulation and measuring the peak amplitude of the mean IPSC. mIPSCs and nIPSCs were detected and measured using MiniAnalysis 6.0.3 software (Synaptosoft, Decatur, GA). Depending on the noise level of the recording, a 5 to 10 pA detection threshold was applied. IPSC frequency was determined by counting the number of events during a 3 min period.

Liquid junction potentials were not taken into account for the data presented. All data are expressed as means ± SE and group statistical significance was assessed using both paired and unpaired Student’s t-tests. Concerning mIPSCs, statistically significant (P < 0.05) differences in frequency and amplitude distributions were determined with the Kolmogorov–Smirnov (K-S) test.

**Histology**

For immunocytochemistry for VIP, three male Wistar rats were transecturally perfused under deep pentobarbital anesthesia with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4); the brains were removed and stored at 4°C in 20% sucrose in PBS for 1–2 days, then sliced at 50 microns, and rinsed in 0.05 M Tris buffer (TBS; pH 7.4). Labeling for VIP was obtained with the primary antibody anti-VIP (dilution 1:1,000; Viper NIBR, kindly provided by Dr. R. M. Bujs) and following an incubation procedure described previously (Kolaj et al. 2007). Instead of fluorescent labeling, chro-mogene labeling was obtained by sequential incubation with biotinylated donkey anti-rabbit (dilution 1:400; Cedarlane, Burlington, ON, Canada), streptavidin–horseradish peroxidase (dilution 1:200; GE Healthcare, Little Chalfont Buckinghamshire, UK), and 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (20 ml acetate buffer containing 500 mg ammonium nickel sulfate, 40 mg β-d-(+) glucose, 8 mg ammonium chloride, 5 mg DAB) to which glucose oxidase was added (1 mg/1 ml). The labeled sections were air-dried, dehydrated through a graded series of ethanol, and coverslipped with Permount (Fisher Scientific, Ottawa, ON, Canada).

To visualize biocytin-filled recorded SPZ neurons, slices were fixed in 4% paraformaldehyde in PBS for 2–12 h and subsequently stored in TBS at 5°C. Biocytin was labeled by incubation for 4 h with streptavidin Alexa Fluor 488 (dilution 1:200; Invitrogen, Burlington, ON, Canada) in TBS containing 1% Triton X-100. After labeling, the sections were mounted in Prolong antifade agent (Invitrogen, Carlsbad, CA). Cells were viewed and photographed using a Biorad MRC1024 confocal laser scanning microscope.

**RESULTS**

**Properties of SPZ neurons in a VIP-immunoreactive field**

Somata immunoreactive for VIP were preferentially localized in the ventral part of the SCN (cf. Card et al. 1981). Axons from these neurons extended into the SPZ in a dorsocaudal direction, branching into a profusion of fine varicose fibers that...
form a terminal field from the dorsal edge of the SCN to the region below, but not contiguous with, the lower border of the hypothalamic paraventricular nucleus (PVN; Fig. 1A). Intracellular biocytin labeling of 23/81 recorded neurons located within this region of the SPZ and innervated by SCN (>95% of the cases) revealed cells with round, ovoid, or fusiform somata with a diameter of 16.2 ± 1.2 microns. From the somata emanated two to four primary dendrites with few secondary branches, extending in all directions up to 415 microns from the cell body (Fig. 1A). SPZ neurons receiving SCN input had a resting membrane potential of −55 ± 1 mV (n = 81) and an input resistance of 1,133 ± 43 MΩ (n = 74). One third of cells discharged spontaneously at 2.1 ± 0.3 Hz (n = 25). Intracellular injections of transient hyperpolarizing current pulses revealed that 38% of SPZ neurons displayed significant instantaneous inward rectification, 18% showed significant time-dependent inward rectification, and 43% exhibited typical rebound low-threshold potentials. Twelve (16%) cells showed both instantaneous inward rectification and rebound low-threshold potentials. These data suggest heterogeneity in the intrinsic membrane properties of SCN-innervated SPZ neurons (Fig. 1A, Table 1).

SCN stimulation evoked predominant IPSPs in 68/81 SPZ neurons, the remaining cells showing predominant excitatory postsynaptic potentials (EPSPs). To focus this analysis on pharmacologically isolated IPSPs, we added a cocktail of NBQX (5 μM) and D-AP5 (20 μM) to block glutamate ionotropic receptors. In seven cells analyzed in detail, SCN-evoked IPSPs displayed a latency of 5.3 ± 0.4 ms and amplitude of 10 ± 2 mV when measured at a membrane potential of −50 ± 1 mV (Fig. 1C, Control). SCN-evoked IPSPs reversed polarity at −72 ± 2 mV (n = 6), approximating the calculated chloride reversal potential of −66.4 mV in this preparation, and were reversibly blocked by the GABA<sub>A</sub> receptor antagonists bicuculline (20 μM) (Fig. 1C, BIC; n = 3) or SR 95531 (10 μM; n = 2). SCN stimulation rarely failed to evoke IPSPs above minimal stimulation intensities, demonstrated constant latency responses to graded stimulus intensities, and followed trains of stimuli at 10 to 20 Hz frequencies (Fig. 1C; n = 5), features suggestive of a monosynaptic response (Berry and Pentreath 1976).

**VIP activates postsynaptic VPAC<sub>2</sub> receptors that open a nonselective cationic conductance**

Current-clamp recordings revealed that 9/23 (39%) cells tested with a 2 min bath application of VIP (0.5–1 μM) responded with a slowly rising 5 ± 1 mV membrane depolarization from a resting membrane potential of −58 ± 1 mV (Fig. 2A; P < 0.01), sufficient to induce AP discharges, with gradual and partial recovery to resting levels after 20 min of washout. This response to VIP was associated with a 4.3 ± 0.2% decrease in apparent input resistance (data not illustrated; n = 6, P < 0.01). When evaluated in voltage-clamp mode in ACSF containing a cocktail of NBQX (5 μM), D-AP5 (20 μM), BIC (20 μM), and TTX (0.5–1 μM), 18/37 (49%) SPZ neurons responded to VIP (0.5–1 μM) with a slowly developing and prolonged inward current of −7.7 ± 0.7 pA (Fig. 2, B and C; P < 0.01), accompanied by a 125.5 ± 5.4% increase in slope conductance (from 1.05 ± 0.08 to 1.32 ± 0.09 nS; Fig. 2B; n = 11, P < 0.01). The inward current had a projected reversal potential of −23 ± 6 mV (Fig. 2C; n = 11), suggesting that the response involved opening of a nonselective cationic conductance.

The SPZ has been shown to contain VIP receptors, mainly of the VPAC<sub>2</sub> subtype (Joo et al. 2004; Kalló et al. 2004). To assess their involvement in the observations described above, neurons were also tested for a response to BAY 55-9837, a selective VPAC<sub>2</sub> receptor agonist (Pan et al. 2007), and VIP(6-28), a VIP receptor blocker (Fishbein et al. 1994). In voltage-clamp experiments, 5/10 (50%) cells responded to BAY 55-9837 (0.2–0.5 μM) with a −4.2 ± 1.1 pA inward current, similar albeit significantly smaller than the VIP-induced inward current (Fig. 2C; P < 0.05). Prior application of BAY 55-9837 occluded the response to VIP when administered at the peak of the BAY 55-9837–induced inward current, whereupon the additional VIP-associated inward current was −2.1 ± 1.8 pA, significantly smaller than that induced by VIP under control conditions (Fig. 2C; n = 4, P < 0.01). V–I relationships revealed that the response to BAY 55-9837 was associated with a significant increase in slope conductance to 115.3 ± 2.0% of control values (n = 5, P < 0.01), with current reversing polarity at a membrane potential of −30 ± 6 mV (Fig. 2C; n = 5), not significantly different from that noted for the VIP response. Additional experiments allowing prolonged (>40 min) washouts following VIP application demonstrated that VIP-induced inward currents were almost completely (>80%) reversible and repeatable. Application of VIP(6-28) (5 μM) for 5 min to VIP-responsive neurons resulted in a small but significant outward current of 3.3 ± 0.8 pA (n = 4, P < 0.05). Subsequent administration of VIP in the presence of VIP(6-28) gave rise to a negligible inward current of −0.7 ± 0.8 pA (n = 4), significantly smaller than the inward current by VIP in control conditions (Fig. 2C; P < 0.01). Postsynaptic responsiveness to VIP was not correlated with a particular set of intrinsic membrane properties of SPZ neurons. Statistical analysis showed no significant differences between control resting membrane potential and input resist-

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**Table 1. Summary of membrane properties of SCN-innervated SPZ neurons**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SE or %</th>
<th>Range</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential, mV</td>
<td>−55 ± 1</td>
<td>−70 to −39</td>
<td>81</td>
<td>0.05</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>1,133 ± 43</td>
<td>621 to 2,566</td>
<td>74</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Action potential amplitude, mV</td>
<td>88 ± 3</td>
<td>61 to 116</td>
<td>25</td>
<td>0.96</td>
</tr>
<tr>
<td>Amplitude of spike after</td>
<td>20 ± 2</td>
<td>8 to 37</td>
<td>25</td>
<td>0.33</td>
</tr>
<tr>
<td>Hyperpolarization, mV</td>
<td>31%</td>
<td>0.4 to 5.7</td>
<td>25/81</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Spontaneous activity, % Hz</td>
<td>38%</td>
<td>28/74</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Instantaneous inward</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rectification, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time-dependent inward</td>
<td>18%</td>
<td>13/74</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>rectification, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rebound low-threshold potentials</td>
<td>43%</td>
<td>32/74</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE, except for descriptive data that are noted as percentage of neurons that exhibited a particular active conductance (see METHODS). The table summarizes data from whole cell recordings from neurons in the region of SPZ, using patch pipettes filled with potassium gluconate–containing media. Of these neurons, biocytin labeling of 23 cells confirmed their localization within the boundaries of SPZ. P values apply to all measurements per category and are according to the Shapiro–Wilk test for normality of distributions.

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Presynaptic VPAC<sub>2</sub> receptors facilitate SCN-evoked GABAergic afferents

We next evaluated the possible influence of VIP receptor activation on SCN-evoked IPSCs. Using chloride-loaded pipettes and ACSF containing NBQX (5 μM) and d-AP5 (20 μM), voltage-clamp recordings of 8/18 (44%) cells revealed that VIP application was followed by a gradual and slowly reversing 145.1 ± 6.3% increase in the amplitude of SCN-evoked IPSCs (from 116.7 ± 27.8 to 163.7 ± 37.4 pA; Fig. 3A; P < 0.01). In 13/23 (57%) cells tested, exposure to VIP was also associated with a 183.0 ± 16.8% increase in the frequency of BIC-sensitive sIPSCs (from 92.8 ± 15.6 to 150.5 ± 16.9 events/min, P < 0.01; data not illustrated). When tested for an influence of VIP on both sIPSCs and SCN-evoked IPSCs, 7/10 cells that showed an increase in sIPSCs also displayed an enhancement of SCN-evoked IPSCs. Also, 7/8 cells that demonstrated an increase in the amplitude of the SCN-evoked IPSCs displayed an increase in sIPSCs.

The observed influences of VIP on GABAergic transmission might arise through an enhanced sensitivity of postsynaptic GABA<sub>A</sub> receptors, an increased excitability of afferent GABAergic neurons, and/or facilitation of GABA release from presynaptic axon terminals. To distinguish between these possibilities, peptide effects on SCN-evoked paired pulses were initially examined. In control, paired-pulse facilitation (PPF) was observed with a P2/P1 ratio of 1.30 ± 0.04. In the presence of VIP, a gradual and selective increase in the amplitude of the initial SCN-evoked IPSC effectively suppressed PPF and reduced the P2/P1 ratio to 1.06 ± 0.05 (Fig. 3B; n = 8, P < 0.01). Next the influence of VIP on mIPSCs was evaluated. On addition of TTX, the frequency of sIPSCs in SPZ neurons was reduced to 43.6 ± 7.6% of control values (n = 12), indicating that the majority of these events were AP dependent. In 8/17 (47%) cells, addition of VIP resulted in a significant change in the frequency distribution of mIPSCs (Fig. 3, C and D; increase to 143.8 ± 10.5% of control), but no significant change in their amplitude distribution (Fig. 3C; change to 100.8 ± 2.3% of control). No significant changes were noted in rise and decay times of mIPSCs. The data indicate that VIP facilitates the release of GABA from presynaptic terminals.

In assessing the VIP receptor mediating the influence on SCN GABAergic transmission, the selective VPAC<sub>2</sub> receptor agonist BAY 55-9837 induced similar increases in the amplitude of SCN-evoked IPSCs, rising to 145.6 ± 6.1% of control values in 5/11 cases (Fig. 3D; P < 0.01), an effect not significantly different from the influence of VIP (Fig. 3D; P = 0.95). Moreover, in 3/11 cells tested, BAY 55-9837 significantly increased the frequency of sIPSCs to 159.8 ± 34.4% of control values (data not shown), in one cell concurrent with the increase of the SCN-evoked IPSC. In the remaining 4 cells that showed a BAY 55-9837–induced increase in SCN-evoked...
IPSC, an insufficient number of mIPSCs precluded reliable measure of the effect of BAY 55-9837.

Application of BAY 55-9837 resulted in a decrease in the P2/P1 ratio from 1.28 ± 0.10 to 1.00 ± 0.11 (n = 5, P = 0.07; data not illustrated), not significantly different from the VIP effect (P = 0.66). BAY 55-9837 also changed the frequency distribution of mIPSCs in 5/9 cells tested (Fig. 3D; increase to 138.8 ± 5.7% of control), again not significantly different from the effect of VIP on this parameter (P = 0.73), and with no significant change in their amplitude distribution (change to 103.8 ± 2.4% of control). Moreover, BAY 55-9837 occluded an increase in frequency of mIPSCs by subsequent application of VIP (Fig. 3D; n = 4, P < 0.05 vs. VIP in control media; change to 101.2 ± 5.6% of control).

Bath application of the VIP receptor antagonist VIP(6-28) consistently resulted in a small but significant reduction in the frequency of mIPSCs, to 94.2 ± 1.6% of control (Fig. 3D; n = 4, P < 0.05), which was undetected by K-S statistics. Subsequent administration of VIP in the presence of VIP(6-28) induced a change in the frequency distribution of mIPSCs in 4/4 cells, to 124.8 ± 4.1% of control values, smaller than but not significantly different from the effect of VIP under control conditions (Fig. 3D; P = 0.25).

With respect to intrinsic membrane properties of SPZ neurons demonstrating increases in GABAergic input, no significant differences were detected in resting membrane potential and input resistance between VIP-responsive neurons (n = 9) and the total cell population (n = 81, P = 0.43 and 0.88, respectively). Similarly, the occurrence of instantaneous inward rectification (22%), time-dependent inward rectification (33%), and rebound low-threshold potentials (33%) does not indicate a specific cell population in SPZ-expressing increases in GABAergic inhibition.

**Post- and presynaptic VPAC2 receptor-mediated effects occur in separate SPZ neurons**

Neurons that showed an increase in the amplitude of SCN-evoked IPSCs or an increase in the frequency of mIPSCs showed negligible inward currents in response to VIP of −1.1 ± 0.6 pA (n = 15), whereas their membrane conductance remained unchanged (100.0 ± 3.4% of control values; n = 6). SPZ neurons that did not demonstrate a change in the amplitude of SCN-evoked IPSCs (n = 10; 97.3 ± 3.6% of control values) or an increase in the frequency of mIPSCs (n = 9; change to 104.5 ± 3.6% of control values) showed an inward current of −4.4 ± 1.1 pA (n = 19) and an overall small increase in membrane conductance to 106.2 ± 2.7% of control values (n = 6). Statistical analysis revealed that the inward current in SPZ neurons that did not show a VIP-induced presynaptic effect was significantly larger than that in cells that did display a presynaptic response to VIP (P < 0.05). In fact, the inward current in the former group of cells was not significantly different from the VIP-induced inward current measured in the total population of cells tested for VIP-induced inward currents with K-gluconate electrodes (−4.5 ± 0.7 pA; n = 37, P = 0.90). These data suggest that pre- and postsynaptic effects of VIP occur on separate SPZ neuronal populations.
DISCUSSION

This analysis addressed the cellular physiology of the projection of the SCN to the SPZ, a region that receives a dense innervation from SCN VIP-immunoreactive neurons (cf. Card et al. 1981; Moore et al. 2002). Whereas the sampled SPZ neurons displayed heterogeneity in their morphological and intrinsic membrane properties, features of their postsynaptic response to electrical stimulation in SCN indicate a prevalent direct inhibitory innervation from the circadian pacemaker, mediated by GABA_A receptors. These results extend earlier observations in describing rapid neurotransmission between the SCN and hypothalamic and thalamic areas targeted by SCN efferents (Cui et al. 2001; Hermes et al. 1996; Zhang et al. 2006).

Evaluation of the responses of these SPZ neurons to exogenous application of VIP, a selective VPAC_2 receptor agonist BAY 55-9837, and a VIP receptor antagonist VIP(6-28) indicate that VIP derived from SCN neurons acts at VPAC receptors to have two distinct influences and that endogenous VIP may be tonically released to exert these actions. In a subpopulation of SPZ neurons VIP increased neuronal excitability by inducing a modest, long-acting, and partially reversible membrane depolarization. Voltage-clamp analyses revealed an action through postsynaptic VPAC_2 receptors that induced inward currents with a reversal potential suggestive of activation of a nonselective cationic conductance. In addition, a slowly rising and reversible increase of the amplitude of SCN-evoked iPSCs and suppression of their PPF was observed, implying that VIP can facilitate GABAergic neurotransmission. The observation that VIP and BAY 55-9837 significantly enhanced the frequency of sIPSCs and the frequency, but not the amplitude, of mIPSCs in a population of tested neurons indicates the presence of presynaptic VPAC_2 receptors on GABAergic afferents to SPZ neurons that originate in SCN and possibly other unknown hypothalamic nuclei. Since the VIP receptor antagonist VIP(6-28) resulted in small but significant opposite effects to those of VIP, both post- and presynaptic mechanisms may be tonically activated by endogenously released peptide. Finally, detailed analysis of the data revealed that the presynaptic effect occurs in SPZ neurons that are distinct from those displaying the postsynaptic action of VIP.

The postsynaptic consequence of VPAC_2 receptor activation in SPZ neurons is consistent with membrane actions of VIP observed elsewhere in the brain, such as in locus coeruleus, medial pontine reticular formation, and thalamus, where response to VIP is characterized by membrane depolarization and inward current, increase in membrane conductance, and reversal of polarity of the inward current at depolarized membrane potentials ranging from −44 to 0 mV (Kohlmeier and Reiner 1999; Lee and Cox 2003; Sun et al. 2003; Wang and Aghajanian 1989). Whereas the reversal potential of the VIP-activated current in the present report is more negative than usually observed with nonselective cationic currents (i.e., transient receptor potential channels that reverse around 0 mV), it is not inconsistent with particular subtypes in the large diversity of nonselective cationic channels, i.e., the cyclic nucleotide-gated channels (Kaupp and Seifert 2002). In the thalamus, VIP appears to selectively activate I_h, the hyperpolarization-activated nonselective cationic current (Lee and Cox 2003; Sun et al. 2003). An involvement of I_h in the action of VIP on SPZ neurons seems unlikely in view of the absence of significant time-dependent inward rectification (the expression of I_h in current-clamp recording mode) in most SPZ neurons.

The presynaptic effect of VPAC_2 receptor activation on GABAergic neurotransmission extends earlier observations demonstrating modulation of synaptic inhibition by VIP (Itri and Colwell 2003; Veruki and Yeh 1992; Wang et al. 1997). Specifically, it is comparable with the presynaptic influence of VIP reported within SCN, in which the peptide exerted its effect through activation of adenyl cyclase and stimulation of cAMP synthesis and protein kinase A (PKA) activity (Itri and Colwell 2003). Consistent with the VIP influence in SCN it was also noted that antagonism of the receptor resulted in a decrease of presynaptic GABA release, suggesting active endogenous release of VIP. Last, the pharmacological data support previous results indicating that VPAC_2 receptors are the main type of VIP receptor involved in cellular interactions within the circadian system (Cutler et al. 2003; Harmar et al. 2002; Itri and Colwell 2003).

Somewhat intriguing is the finding of an excitatory action of VIP, since the peptide is likely to coexist with GABA present in most SCN neurons (Moore and Speh 1993). Should GABA and VIP be coreleased from synapses on targeted neurons, how their contrasting actions might modulate neuronal excitability remains to be defined. The answer may reside in the time course of their actions: for GABA, the response would likely be a transient reduction in excitability; for VIP, which operates via G-protein–coupled VPAC_2 receptors, any opposing influence on neuronal excitability would probably occur over a longer time frame, perhaps lasting minutes. An alternative scenario may be derived from indications that GABA and VIP, despite the predominant GABAergic nature of SCN neurons, can be transmitter molecules in separate SCN cell populations and their axon terminals. One investigation specifically aimed at addressing the issue of colocalization reported that GABA coexisted in only about 40% of VIP-positive SCN terminals within the SCN and in the dorsomedial hypothalasmus, a site targeted by VIPergic SCN efferents (Buiks et al. 1995). Others have demonstrated that not all SCN neurons are GABAergic and that GABA may not be present in all peptidergic neurons (Castel and Morris 2000; Decavel and van den Pol 1990). The present results—showing that VPAC_2 receptor activation results in distinct membrane consequences that occur in separate SPZ neuronal populations—add support to the notion that SCN VIPergic neurons may be heterogeneous and have distinct roles in SCN–SPZ communication.

Within the SCN VIP neurotransmission has been implicated in synchronizing and maintaining circadian rhythms in subsets of SCN neurons, possibly through modulation of GABA_A receptor-mediated synaptic transmission (Atontat et al. 2005; Cutler et al. 2003; Harmar et al. 2002; Itri and Colwell 2003). VIP’s role in areas targeted by SCN efferents is likely to assist in the integration of pacemaker activity in CNS central circuits. Interestingly, VIP, its precursor mRNA, and VPAC_2 receptor mRNA demonstrate diurnal (but not circadian) variations in SCN, with levels peaking in the early hours of the projected dark portion of the 24-h light/dark cycle (Albers et al. 1990; Shinohara et al. 1993, 1999). This is perhaps surprising since the overall electrical activity in SCN in this period has been reported to be at its lowest (Inouye and Kawamura 1979;
Vasoactive intestinal polypeptide microinjections into the oral sleep-induction zone.


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