Effects of VPAC₂ Receptor Activation on Membrane Excitability
and GABAergic Transmission in Subparaventricular Zone Neurons Targeted
by Suprachiasmatic Nucleus

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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. 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 GABAₐ⁰ 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 VPAC₂ 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 post- and presynaptic VPAC₂ receptors to differentially modulate SCN GABAergic signaling to distinct subpopulations of SPZ neurons.

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 (Kalbeek 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).

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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>) glycerol-based solution of the following composition (in mM): glycerol 250, 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 (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 bath containing oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl 127, KCl 3, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 26, d-glucose 10, MgCl<sub>2</sub> 1.3, 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), and whole cell current- and voltage-clamp recordings were obtained using the “blind” method of patch-clamp recording. The composition of the pipette solution was (in mM): K-gluconate 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. In selected cases biocytin (0.2%) was added to the pipette solution to label cells for location and morphology. For voltage-clamp recording. The composition of the pipette solution to the pipette solution to label cells for location and morphology. For voltage-clamp recording. The composition of the pipette solution to the pipette solution to label cells for location and morphology. For voltage-clamp recording. The composition of the pipette solution was applied and peptide applications usually lasted 2 min with a flow rate of 4 ml/min.

**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-
Following addition of a GABAA receptor antagonist bicuculline (BIC) to the mean current density map, sparse VIP-containing fibers arising from neurons in the ventral SCN. Branching of immunocytochemical labeling reveals a prominent innervation of the SPZ by dense. Same scale as in cells in the SPZ showing cell somata and dendritic trees. Bar is 25 μm. A, top: 2 current–voltage (I–V) relationships of SPZ neurons showing the presence of significant instantaneous inward rectification (both I–V relationships), time-dependent inward rectification (right I–V), and rebound low-threshold potentials (left I–V). According to the criteria used (see methods) the rebound potential in the right I–V was not classified as a typical low-threshold potential. Inset: current pulse protocol. Bottom: plots of the I–V relationships above demonstrating instantaneous and time-dependent inward rectification. C, left: typical example of an IPSP (trace is an average of 10) following electrical stimulation (* of SCN in artificial cerebrospinal fluid (ACSF) containing NBQX (5 μM) and d-AP5 (20 μM) (Control), and complete blockade following addition of a GABAA receptor antagonist bicuculline (BIC) to the ACSF. Right: traces of constant latency response to increasing intensities of electrical stimulation to SCN (5, 10, and 20 V) and ability of IPSPs to follow a train of 10 Hz SCN stimuli.

tude of the depolarizing sag during transient hyperpolarization to −100 mV; a depolarization ≥5 mV was considered as significant time-dependent inward rectification. Rebound potentials were assessed by measuring their amplitude following transient hyperpolarization to −100 mV; amplitudes ≥15 mV confirmed the typical 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 mIPSCs 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 transcardially 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. Buiks) 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/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. 1B, 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 neurons, the remaining cells showing predominant excitatory (Fig. 1, Table 1).

VIP activates postsynaptic VPAC2 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 VPAC2 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 VPAC2 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 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 resis-

**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>Spontaneously active, %, Hz</td>
<td>38%</td>
<td>28/74</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Instantaneous inward rectification%</td>
<td>18%</td>
<td>13/74</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Time-dependent inward rectification%</td>
<td>43%</td>
<td>32/74</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Rebound low-threshold potentials%</td>
<td></td>
<td></td>
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</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–Wilks test for normality of distributions.
Presynaptic VPAC₂ 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 of sIPSCs.

The observed influences of VIP on GABAergic transmission might arise through an enhanced sensitivity of postsynaptic GABA_A 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 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 of sIPSCs.

In assessing the VIP receptor mediating the influence on SCN GABAergic transmission, the selective VPAC₂ receptor agonist BAY 55-9837 induced similar increases of 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...
Application of BAY 55-9837 resulted in a decrease 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 ± 1.0 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-glutamate 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.

FIG. 3. Presynaptic VPAC₂ receptor activation enhances SCN-evoked IPSCs by facilitating GABA transmitter release. A: left: example of an increase in the amplitude of SCN-evoked IPSCs by VIP (0.5 μM; traces are an average of 15). Right: collective data of 8 cells indicating that the effect was readily apparent immediately following VIP-application but that a slow further increase in amplitude of SCN-evoked IPSC could be observed up until 18 min after, followed by a partial recovery. B: SCN paired-pulse stimulation paradigm showing that VIP (0.5 μM) selectively enhances the amplitude of the first IPSC (average of 15 traces). The bar histogram illustrates that VIP resulted in a significant reduction (**, P < 0.01) of the P2/P1 paired-pulse ratio (PPratio). C: left: example of miniature (m)IPSCs recorded in tetrodotoxin (TTX) demonstrating a reversible increase in their frequency following VIP application. Right, top graph: a significant leftward shift of the cumulative plot of the distribution of interevent intervals illustrating the increase in frequency of mIPSCs occurring in the example to the left. Inset: collective data of 8 cells showing that VIP increased the frequency of mIPSCs to 143.8 ± 10.5% of control values. Bottom graph: no shift in the cumulative plot of the distribution of amplitudes was observed. Inset: collective of 8 cells demonstrating no significant change in the amplitude of mIPSCs following VIP application (to 100.8 ± 2.3% of control). D: 2 sets of data showing that the presynaptic effect of VIP on GABAergic transmission is mediated by VPAC₂ receptors. Left: the increases in amplitude of SCN-evoked IPSCs by VIP and the VPAC₂ receptor agonist BAY 55-9837 are not significantly different from each other. Right: the increase in frequency of mIPSCs by VIP can be fully replicated (BAY 55-9837) and occluded (VIP/BAY) by BAY 55-9837. The VIP receptor antagonist VIP (6-28) alone results in a small reduction in the frequency of mIPSCs and reduces their increase following a subsequent VIP application. **Significantly different from control VIP-induced increases in mIPSCs, P < 0.01. †Significantly different from predrug mIPSC frequency, P < 0.05. IPSC, an insufficient number of mIPSCs precluded reliable measure of the effect of BAY 55-9837.
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\textsubscript{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\textsubscript{2} receptor agonist BAY 55-9837, and a VIP receptor antagonist VIP(6-28) indicate that VIP derived from SCN neurons acts at VPAC\textsubscript{2} 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-acti, and partially reversible membrane depolarization. Voltage-clamp analyses revealed an action through postsynaptic VPAC\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 co-released 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\textsubscript{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 hypothalamus, a site targeted by VIPergic SCN efferents (Buijs 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\textsubscript{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\textsubscript{A} receptor-mediated synaptic transmission (Aton 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\textsubscript{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; Shinozaka 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;
Prosper and Gillette (1989) and is thus not conducive to peptide release requiring prolonged periods of increased AP discharge (Croppet et al. 1990; Shakiryanova et al. 2005). However, recent observations of separately tuned rhythms in SCN (Jagota et al. 2000; Schapet al. 2003) suggest that particular subsets of SCN neurons do have peak activities during the early projected dark phase and thus may be responsible for the liberation of VIP in this period. When released, the described influences of VIP may signal environmental darkness to SCN-targeted cells and associated processes. Several studies have suggested an implication of VIP in the regulation of rapid eye-movement sleep, by acting on neurons located in the brain stem and thalamus (Bourgin et al. 1997; Sun et al. 2003). Lesions of the SPZ result in significant disturbances of the circadian rhythm in sleep and locomotor activity (Lu et al. 2001). We speculate that the release of VIP from SCN to modulate the excitability of neurons located in SPZ may, at least in part, play a role in processes controlling sleeping and waking.

To summarize, neurons in the SPZ region that receive a dense terminal innervation from VIPergic neurons in SCN predominantly respond to SCN stimulation with a rapid GABA<sub>α</sub> receptor-mediated inhibition that is deemed to be monosynaptic. Subpopulations of these SPZ neurons display two mutually exclusive patterns of response to exogenous VIP receptor activation: 1) a slowly rising and prolonged membrane depolarization and inwards current mediated by postsynaptic VPAC<sub>2</sub> receptors that engage a nonselective cationic type of conductance, thus enhancing membrane excitability; and 2) a slow progressive enhancement in SCN-evoked, spontaneous and miniature IPSCs, mediated by presynaptic VPAC<sub>2</sub> receptors and resulting in reduced excitability by increasing GABAergic tone. Further definition of conditions governing synaptic release of neuropeptides, in particular VIP, can be expected to contribute to a better understanding of the role of this peptide in SCN efferent neurotransmission.

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