Cluster I neurons of the suprachiasmatic nucleus (SCN), which are thought to be pacemakers supporting circadian activity, fire spontaneous action potentials that are followed by a monophasic afterhyperpolarization (AHP). Using a brain slice preparation, we have found that the AHP has a shorter duration in cells firing at higher frequency, consistent with circadian modulation of the AHP. The AHP is supported by at least three subtypes of K Ca channels, including apamin-sensitive channels, iberiotoxin-sensitive channels, and channels that are insensitive to both of these antagonists. The latter K Ca channel subtype is involved in rate-dependent regulation of the AHP.

Key words: suprachiasmatic nucleus; calcium channel; calcium-activated potassium channel; action potential; spontaneous activity; afterhyperpolarization; circadian rhythm

Introduction

The suprachiasmatic nuclei (SCN) of the hypothalamus contain the primary circadian clock that controls various physiological and behavioral rhythms in mammals, including rhythms in body temperature and sleep-wake behavior (Moore and Eichler, 1972; Klein et al., 1991). Single SCN neurons are autonomous pacemaker cells that harbor the fundamental molecular workings of the circadian clock, an interconnected set of transcriptional/translational negative and positive feedback loops that produce coordinated, rhythmic changes in expression of clock genes (Dunlap, 1999; Reppert and Weaver, 2001). In keeping with the role of a 24 hr pacemaker, firing of SCN neurons follows the circadian clock, with higher spike activity during the day and lower activity at night (Inouye and Kawamura, 1979; Gillette, 1991; Jagota et al., 2000). Even an isolated SCN neuron in culture, lacking any synaptic input, will sustain spontaneous and rhythmic firing (Welsh et al., 1995). Such circadian oscillation in firing frequency is known to be critical for transmittal of time information because suppression of action potentials via selective application of tetrodotoxin to the SCN abolishes the circadian rhythm of many organismal behaviors (Schwartz et al., 1987; Earnest et al., 1991; Schwartz, 1991; Newman et al., 1992). Little is known, however, regarding the mechanism that links the molecular clockworks to rhythmic electrophysiological output.

A prerequisite for understanding how the rhythm of the core of the clock is transduced into a rhythm in spike rate is the identification of ion channels responsible for spontaneous, rhythmic firing. Some of these have been identified. Thus it is known that a slowly inactivating Na + current generates an interspike depolarization that brings the membrane potential to firing threshold (Pennartz et al., 1997). Additional ion channels, including T-type Ca 2+ channels, L-type Ca 2+ channels, and hyperpolarization-activated cation channels (Ih), may assist in generating the interspike depolarization (Aksu et al., 1993; Jiang et al., 1995; Pennartz et al., 2002).

Other channels involved in spontaneous, rhythmic firing have remained uncharacterized. After action potential repolarization, SCN neurons exhibit an intermediate-duration afterhyperpolarization (AHP) that may contribute to regulation of firing rate. Because the AHP in other spontaneously active central neurons is a key determinant of cellular excitability (Aizenman and Linden, 1999; Bevan and Wilson, 1999) and is subject to modulation by intracellular messengers (Sah, 1996), the AHP represents a potential target for regulation by the circadian clock.

In this study, we have found that the duration of the AHP in SCN neurons firing at higher frequencies is significantly shorter than in those firing at lower frequencies, consistent with circadian modulation. To better understand the nature of the changes in the AHP, we undertook a pharmacological analysis of the ion channels supporting the AHP in cluster I SCN neurons, which are the most abundant neuronal type in the SCN and are thought to be important in the output of these nuclei (Pennartz et al., 1998). This analysis classifies K Ca channels that underlie the AHP, identifies the Ca 2+ channels that trigger the AHP, and highlights particular subtypes of K Ca and Ca 2+ channels as candidates for circadian regulation.

Materials and Methods

Preparation of brain slices. Hypothalamic slices containing the SCN were obtained from Fisher 344 or Sprague Dawley rats (17–25 d postnatal) that had been maintained on a 12 hr light/dark cycle for at least 1 week before they were killed. Animals were anesthetized with halothane and
decapitated, and the brain was quickly removed and placed in ice-cold artificial CSF (ACSF) of the following composition (in mM): 126 NaCl, 3 KCl, 1.5 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, 11 glucose, 25.9 NaHCO₃, continuously gassed with 95% O₂–5% CO₂, pH 7.4. Coronal hypothalamic slices (250 μm) were cut on a vibratome and incubated in ACSF at room temperature (–23–25°C) for at least 45 min before recording. Experiments were performed primarily on neurons taken from rats killed during their subjective day, although a subset of experiments was performed with neurons from subjective night rats. Recordings were made from all regions of the SCN.

Current-clamp recordings. Slices were placed in a recording chamber mounted on the stage of an upright microscope (Nikon Eclipse E600 FN). Single SCN neurons in slices were visualized using a high-immersion objective lens [40×, 0.8 numerical aperture (NA)], differential interference contrast optics, and a 0.9 NA condenser. An infrared-sensitive video camera was used to display the field of view on a video monitor. To measure the AHP, action potential firing rate, and other properties of membrane potential changes in SCN neurons, recordings were made using the current-clamp mode of the Axopatch 200B amplifier. The bathing solution, ACSF, was bubbled continuously with 95% O₂–5% CO₂. Recordings were obtained at room temperature (20–23°C). For Ca²⁺ substitution experiments, a HEPES-based ACSF was used that contained (in mM): 126 NaCl, 3 KCl, 1.5 MgCl₂, 2.4 CaCl₂, 11 glucose, 25.9 NaHCO₃, and 0.5 amphotericin B (1 mg/ml, in dimethylsulfoxide) was diluted into a potassium gluconate-based pipette solution (1:200) that was otherwise identical to that used in the ruptured-patch current-clamp recordings. The patch pipette tip was filled with amphotericin B-free potassium gluconate-based pipette solution and continuously gassed with 95% O₂ –5% CO₂ for the solutions used in current-clamp recording (Neher, 1992); data were filtered at 2 kHz and sampled at 4 kHz. Linear leak and residual capacitance currents were removed on-line by subtracting a stable level of −40 mV.

Voltage-clamp of Ca²⁺ channel currents. For recording whole-cell Ba²⁺ currents, the extracellular solution contained (in mM): 100 NaCl, 3 KCl, 0.15 MgCl₂, 5 BaCl₂, 17 tetraethylammonium chloride (TEA-Cl), 0.5 4-aminopyridine, 5 CsCl, 26 HEPES, 10 glucose, 0.03 bicuculline methiodide, and 0.001 tetrodotoxin, pH 7.4. A reservoir containing this solution was bubbled continuously with 100% O₂, and a gravity-driven system was used to perfuse the oxygenated solution through the recording chamber at 3 °C for at least 45 min before recordings were made. Series resistance compensation and capacitance transient cancellation were performed using the circuitry of the amplifier. Data were filtered at 2 kHz and sampled at 4 kHz. Linear leak and residual capacitance currents were removed on-line by subtracting scaled and inverted current responses to hyperpolarizing voltage steps of one-quarter the amplitude of the depolarizing test pulse. A liquid junction potential of +1.4 mV was measured for the solutions used in voltage-clamp recording (Neher, 1992); the reported data have not been corrected for this junction potential. All experiments were performed at room temperature.

In all SCN whole-cell voltage-clamp recordings, a series resistance of typically 20–60 MΩ was compensated by ∼80%. Whole-cell Ba²⁺ current amplitude ranged up to ∼300 pA, so in the well clamped compart-
bath perfusion at concentrations 133–4300\( \times \) IC\(_{50}\) values and therefore were expected to block at least 99% of current carried by these Ca\(^{2+}\) channel subtypes. Nonclassical L channels based on the Ca\(_{\alpha}1.3\) (Ca\(_{\alpha}1.3\)) subunit may not have been fully blocked by 10 \( \mu M \) nimodipine (Xu and Lipscombe, 2001). Q-type channels are blocked by \( \omega \)-AgA-IVA, but these channels are less sensitive to this antagonist, so that \( \approx 70\% \) of Q-type current was expected to be blocked at the dose used (dose = 200 nM; IC\(_{50}\) = 90 nM) (Randall and Tsien, 1995). However, there was no significant difference between a mixture of blockers containing \( \omega \)-AgA-IVA and mibefradil. We further dissected the R-type current according to subunit may not have been fully blocked by 10 \( \mu M \) nimodipine (Lipscombe, 2001). Q-type channels are blocked by charybdotoxin, and ryanodine from Sigma; and mibefradil was a generous gift from Peptide Institute (Osaka, Japan); nimodipine from Research Biochemicals (Natick, MA); and mibefradil was a generous gift from Dr. Jean-Paul Cloze and Dr. Eric Ertel (E. Hoffmann-La Roche, Basel, Switzerland).

Mean values are reported together with their SEs, and all error bars indicate SEs of the mean. The number of experiments, \( n \), indicates in all cases the number of neurons studied. Student’s \( t \) test was used to determine statistical significance.

**Results**

**Spontaneous neuronal firing rate correlates with AHP duration**

SCN neurons spontaneously fire action potentials over a range of frequencies (0–15 Hz), with average firing rates being higher during the daytime and lower during the nighttime (Inouye and Kawamura, 1979; Gillette, 1991; Jagota et al., 2000). Clock cells are not tightly synchronized with one another, however, and the circadian time at which peak firing occurs can be considerably out of phase among SCN neurons. We therefore used comparisons of action potential waveforms recorded from cells firing spontaneously at different rates, regardless of circadian time, as a strategy to identify components of the action potential that might be regulated in a circadian manner. From these comparisons, we identified an altered AHP waveform as a major factor regulating firing frequency: the AHP became shorter in duration as spike frequency increased (Fig. 1A). In addition, the action potential width was significantly broader in slower firing neurons, but other action potential properties remained unchanged: the depolarizing ramp preceding the action potential upstroke, interspike membrane potential, action potential height, and maximum hyperpolarization after the action potential were not significantly different between fast and slow firing neurons (Table 1).

Under current-clamp conditions, action potential discharge was followed by a monophasic AHP in the majority of neurons (98 of 110) studied (Fig. 1A, B). These kinds of neurons have been classified previously as cluster I neurons (Pennartz et al., 1998). Cells with a biphasic AHP (cluster II and III neurons) were not included in our analysis. Identification of cluster I neurons was confirmed by testing for the absence of spike frequency adaptation during a train of action potentials evoked by injection of steady positive current (Fig. 1C) (Pennartz et al., 1998).

In this work, we defined AHP as the Ca\(^{2+}\)-dependent component of the hyperpolarization that followed each action potential
Table 1. Membrane properties of slow- and fast-firing cluster I neurons

<table>
<thead>
<tr>
<th></th>
<th>Depolarizing ramp (mV/msec)</th>
<th>Interspike potential (mV)</th>
<th>Spike width (msec)</th>
<th>Spike amplitude (mV)</th>
<th>Maximum hyperpolarization (mV)</th>
<th>AHP t1/2 (msec)</th>
<th>Cd^{2+}-subtracted AHP</th>
<th>IBTX-subtracted AHP</th>
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<tbody>
<tr>
<td>Number of cells (n)</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Slow rate (&lt;3 Hz)</td>
<td>0.23 ± 0.01</td>
<td>−413.2 ± 1.9</td>
<td>4.0 ± 0.1</td>
<td>82.8 ± 1.7</td>
<td>−53.8 ± 1.6</td>
<td>37.3 ± 5</td>
<td>7.1 ± 0.9</td>
<td>11.0 ± 3.8</td>
</tr>
<tr>
<td>Fast rate (&gt;6 Hz)</td>
<td>0.25 ± 0.03</td>
<td>−447.2 ± 2.3</td>
<td>3.0 ± 0.1</td>
<td>77.7 ± 3.6</td>
<td>−58.1 ± 2.2</td>
<td>17.8 ± 0.9</td>
<td>3.1 ± 0.4</td>
<td>26.0 ± 2.9</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>NS</td>
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<td>0.0001</td>
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<td>NS</td>
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Membrane properties were compared between slow (<3 Hz) and fast (>6 Hz) spontaneously firing cluster I SCN neurons. The depolarizing ramp was measured as the rate of voltage change (mV/msec) over the 50 msec preceding action potential threshold. Interspike potential (Vip) was estimated as the plateau region between action potentials. For fast-spiking neurons in which interspike plateaus were mostly brief, Vip was estimated using the longer interspike intervals recorded from these irregularly firing neurons. Spike width was measured at action potential threshold. Spike amplitude was measured from Vip to maximum depolarization. Maximum hyperpolarization reached during the AHP was measured from 0 mV. t1/2 for the AHP and Cd^{2+}-subtracted AHP was estimated as the half-decay time. For measurements of Cd^{2+}-subtracted AHPs, slow-firing cells included those firing at <5 Hz. Statistical significance (p values) for differences between fast- and slow-firing cells is indicated at the bottom of each column, with p < 0.05 considered not significant (NS).

Figure 2. Changes in the Ca^{2+}-dependent AHP over a range of firing frequencies. A. Comparison of two cells, one firing at 1.3 Hz (left panel) and one firing at 11 Hz (right panel). After several minutes of spontaneous firing in control conditions, 30 µM Cd^{2+} was applied to slices. Ensemble-averaged action potential waveforms (4–6 action potentials for each condition) are shown superimposed. Insets show subtraction of waveforms recorded in 30 µM Cd^{2+} from control waveforms (Cd^{2+}-subtracted AHP). Solid line in the inset indicates 0 mV. Calibration: 10 mV, 10 msec. B, Half-time for decay of the Cd^{2+}-subtracted AHP as a function of spontaneous firing frequency. For each neuron analyzed, decay half-time (t1/2) was measured as the time from maximum hyperpolarization to 50% of that value, and the measured t1/2 value was plotted versus spontaneous firing frequency in that particular neuron. The t1/2 versus frequency data were fit with a linear regression, using a maximum likelihood estimate. Correlation coefficient was r = −0.67 C. Amplitude of the Cd^{2+}-subtracted AHP as a function of spontaneous firing frequency. Amplitude of the subtracted AHP was measured from maximum hyperpolarization to 0 mV. Correlation coefficient for the regression fit was r = 0.66.

Ca^{2+}-sensitive K^+ channels underlying the AHP of cluster I neurons

Subtype-specific antagonists were used to identify the kinds of K_{Ca} channels that produce the AHP in cluster I SCN neurons. In this procedure, blockers of various kinds of K_{Ca} channels were applied to spontaneously firing SCN neurons, and after establishment of a stable reduction in AHP amplitude, the AHP was completely blocked by applying 30 µM Cd^{2+}. Percentage reduction in AHP amplitude was calculated according to the equation: percentage reduction = [(AHP_{control} − AHP_{K_{Ca}-antagonist})/AHP_{control}]×100.
diminution in the AHP by the Ca^{2+} channel blocker mibefradil was added at the end of each experiment to fully block the Ca^{2+}-dependent AHP, thus establishing a baseline from which to estimate the contribution of the AHP to the Ca^{2+} channel currents. The Ca^{2+} channel blockers were recorded under control conditions and in the presence of iberiotoxin (100 nM), apamin (100 nM), or both of these channel blockers. Cd^{2+} (100 nM) was added at the end of each experiment to fully block the Ca^{2+}-dependent AHP, thus establishing a baseline from which to estimate the contribution to the AHP of KCa channels that were sensitive to apamin, sensitive to iberiotoxin, or insensitive to both blockers. Superimposed records were always obtained from the same neuron, but each panel of records was recorded from a different neuron. Iberiotoxin reduced AHP amplitude by 48 ± 5%, with a range of 18–55% (n = 6); apamin reduced AHP amplitude by 18 ± 5%, with a range of 8–37% (n = 6); and the combination of apamin and iberiotoxin reduced AHP amplitude by 54 ± 10%, with a range of 32–91% (n = 5). Further diminution in the AHP by the Ca^{2+} channel blocker cocktail after application of apamin and iberiotoxin. The Ca^{2+} channel mixture contained 10 μM nimodipine, 3 μM ω-CTx-GVIA, 200 nM ω-Aga-IVA, 30 μM Ni^{2+}, and 500 nM mibefradil, which will block most of the Ca^{2+} entry into SCN neurons (see also Fig. 5). Example action potentials in the various conditions are shown on the left, and a bar chart showing the percentage reduction in AHP amplitude is shown on the right. The reductions in AHP amplitude produced by the two sets of blockers (apamin + iberiotoxin, Ca^{2+} channel cocktail) were statistically different from one another at the p < 0.05 level.

(AHP_{control} - AHP_{cd}) \times 100\%$. As shown in Figure 3A, iberiotoxin (100 nM), a blocker of certain large conductance KCa channels (BK_{Ca} channels), reduced AHP amplitude by ~40%. A peptide agonist similar to iberiotoxin in action, charybdo-toxins (100 nM), reduced AHP amplitude by a roughly similar amount (28 ± 12%; range, 0–58%; n = 6; data not shown). Also illustrated in Figure 3A is the effect on AHP amplitude of apamin (100 nM), a blocker of certain small conductance KCa channels (SK_{Ca} channels): apamin reduced AHP amplitude by ~20%. Co-application of apamin and iberiotoxin reduced AHP amplitude by only ~55%, suggesting that SCN neurons possess some KCa channels that are insensitive to both apamin and iberiotoxin. Because 30 μM Cd^{2+} may also have actions on channels other than voltage-gated Ca^{2+} channels (Bekkers, 2000), however, we tested the prediction that block of the AHP by apamin and iberiotoxin would be smaller than block by a mixture designed to block Ca^{2+} channels, including L-, N-, P/Q-, R-, and T-type Ca^{2+} channels. Figure 3B shows that the Ca^{2+} channel mixture further reduced AHP amplitude after application of the combination of apamin and iberiotoxin, consistent with the idea that the AHP of SCN neurons is supported by at least three subtypes of KCa channels: apamin-sensitive channels, iberiotoxin-sensitive channels, and channels insensitive to both antagonists. In addition, the wide range in percentage block by iberiotoxin or apamin suggests that there is considerable cell-to-cell variability in the expression of KCa channels (Fig. 3, legend).

We examined whether the iberiotoxin-sensitive portion of the AHP changed with firing frequency, as observed for the Cd^{2+}-subtracted AHP (Fig. 2). When we applied 100 nM iberiotoxin to SCN neurons firing spontaneously between 0.2 and 8.2 Hz (n = 16) and measured the amplitude and decay kinetics of the iberiotoxin-subtracted potential, we found no correlation between firing rate and either decay half-time (correlation coefficient r = 0.08; Table 1) or amplitude (r = −0.25).

**Ca^{2+} channel subtypes present in neurons of the SCN**

Subtypes of Ca^{2+} channels present in SCN neurons were identified using antagonists effective against particular Ca^{2+} channel subtypes. In these voltage-clamp experiments, Ba^{2+} was substituted for Ca^{2+} as the permeant ion to suppress Ca^{2+}-sensitive currents. Figure 4A–D illustrates the action of subtype-selective Ca^{2+} channel antagonists on whole-cell Ba^{2+} current recorded from voltage-clamped SCN neurons in hypothalamic slices. As shown in Figure 4A, the L channel antagonist nimodipine partially blocked Ba^{2+} current, indicating that L-type Ca^{2+} channels carried a fraction of whole-cell Ca^{2+} current channel. For a series of control neurons, total Ba^{2+} current remained stable over the typical duration of experiments (5–15 min), as shown by the data points and dashed line in Figure 4A. N-type and P/Q-type Ca^{2+} channels were also identified in SCN neurons, on the basis of partial block of whole-cell Ba^{2+} current by high concentrations of ω-CTx-GVIA and ω-Aga-IVA (Fig. 4B,C).

Figure 4D shows that application of a combination of antagonists designed to block L-, N-, P/Q-, and T-type channels (10 μM nimodipine, 3 μM ω-CTx-GVIA, 200 nM ω-Aga-IVA, and 500 nM mibefradil) blocked only half of the total Ba^{2+} current in an SCN neuron. At the antagonist concentrations used, classical L-, N-, and P-type channels were expected to be fully blocked. Approximately 70% of any Q-type current present was expected to have been blocked by 200 nM ω-Aga-IVA. We tested whether the current remaining in the presence of L-, N-, P-, and T-type channel blockers was carried by Q-type channels by substituting ω-CTx-MVIIC (3 μM) for ω-Aga-IVA in the mixture. This concentration of ω-CTx-MVIIC would be expected to fully block both P- and Q-type Ca^{2+} channels over the time that it was applied (>10 min) (Randall and Tsien, 1995). We found that the mixture containing ω-CTx-MVIIC did not block significantly more Ba^{2+} current (52 ± 6%; n = 4) than the mixture containing ω-Aga-IVA (51 ± 4%; n = 12; p > 0.1). Thus, most of the current resistant to block by the combination of nimodipine, ω-CTx-GVIA, ω-Aga-IVA (or ω-CTx-MVIIC), and mibefradil was, by definition, R type (Zhang et al., 1993; Randall and Tsien, 1995).

In some neuronal cell types, R-type current is carried, at least...
in part, by α11-based Ca\(^{2+}\) channels (Wang et al., 1999; Foehring et al., 2000; Tottene et al., 2000; Lee et al., 2002). We therefore tested the effect of SNX-482, a toxin selective for α11-based Ca\(^{2+}\) channels, on whole-cell Ba\(^{2+}\) current. Figure 5A illustrates a cell in which a large portion of Ba\(^{2+}\) current (77%) was blocked by a combination of nimodipine, ω-CTx-GVIA, ω-Aga-IVA, and mibefradil. The remaining current was not affected by application of SNX-482 (200 nM) but could be blocked by addition of Cd\(^{2+}\) (30 μM), a nonselective blocker of all voltage-gated Ca\(^{2+}\) channel subtypes. In seven cells tested, SNX-482 blocked <10% of current remaining in the presence of L-, N-, P/Q-, and T-type channel blockers, suggesting that α11-based Ca\(^{2+}\) channels are not significantly expressed in SCN neurons.

R-type current can also be blocked with some selectivity (see Materials and Methods) by the divalent cation Ni\(^{2+}\) (Zamponi et al., 1996; N’Gouemo and Rittenhouse, 2000; Tottene et al., 2000), and so we tested Ni\(^{2+}\) on the current resistant to block by the combination of nimodipine, ω-CTx-GVIA, ω-Aga-IVA, and mibefradil. As illustrated in Figure 5B, Ni\(^{2+}\) (30 μM) blocked ~75% of the resistant Ba\(^{2+}\) current, confirming the identification of R-type current in these neurons. In the presence of nimodipine, ω-CTx-GVIA, ω-Aga-IVA, and mibefradil, Ni\(^{2+}\) blocked 40 ± 6% (n = 7) of the original total Ba\(^{2+}\) current, a percentage not significantly different from Ni\(^{2+}\) block in the absence of the other four Ca\(^{2+}\) channel antagonists (45 ± 6%; n = 6; p > 0.1). This observation suggests that 30 μM Ni\(^{2+}\) does not block Ca\(^{2+}\) currents other than R type in SCN neurons. A summary of the Ca\(^{2+}\) channel antagonist data is illustrated in Figure 5C.

**Effect of Ca\(^{2+}\) channel blockers on the AHP**

In some neurons, tight coupling exists between specific classes of Ca\(^{2+}\) channels and K\(_{\text{Ca}}\) channels (Davies et al., 1996; Marrion and Tavallini, 1998; Pineda et al., 1998). We therefore tested the effect of individual Ca\(^{2+}\) channel blockers on the AHP amplitude in spontaneously firing SCN neurons (Fig. 6). The N-, P/Q-, and T-type Ca\(^{2+}\) channel blockers ω-CTx-GVIA (3 μM), ω-Aga-IVA (200 nM), and mibefradil (500 nM) had few or negligible effects on AHP amplitude (~10%; n = 6 for each), suggesting minimal participation of these kinds of channels in activating the AHP. In contrast, the L-type Ca\(^{2+}\) channel antagonist nimodipine (10 μM) had a significant effect on peak AHP, reducing the amplitude by ~30%.

We also tested whether R-type current was involved in triggering the AHP (Fig. 7A,B). A combination of antagonists that block non-R-type Ca\(^{2+}\) current (nimodipine, ω-CTx-GVIA, ω-Aga-IVA, and mibefradil) only partially suppressed the AHP (46 ± 5%; n = 5), suggesting that R-type current activated K\(_{\text{Ca}}\) channels responsible for the remainder of the AHP. Indeed, block of R current by Ni\(^{2+}\) (30 μM) partially suppressed the AHP (~20%). Coapplication of the L- and R-type blockers nimodipine and Ni\(^{2+}\) had effects that were additive, reducing the AHP by ~50%. This block was not significantly different from that produced by application of a mixture containing all Ca\(^{2+}\) channel blockers (nimodipine, ω-CTx-GVIA, ω-Aga-IVA, mibefradil, Ni\(^{2+}\); 63% ± 5%; n = 7; p > 0.1).

Because Ca\(^{2+}\) release via ryanodine receptors triggers the AHP in some kinds of neurons, we tested the effect of ryanodine, an inhibitor of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Sutko et al., 1985; Akita and Kuba, 2000), on the AHP of cluster I SCN neurons (Fig. 7C). Measured from spike threshold, maximum hyperpolarization after a spike was diminished by 1.4 ± 0.5 mV (n = 4) after 30 min of exposure to 10 μM ryanodine. This was not statistically different from the reduction in afterhyperpolarization magnitude observed after 30 min in control conditions (1.4 ± 1.0 mV; n = 3). Thus release of Ca\(^{2+}\) from ryanodine-sensitive stores appears to play a minimal or no role in generating the AHP in cluster I SCN neurons, despite the importance of ryanodine receptors in mediating light-induced phase delays in the SCN (Ding et al., 1998).

**Effects on firing rate resulting from pharmacological antagonism of the AHP**

In many kinds of neurons, the magnitude and duration of the AHP are important factors in setting interspike interval and, thereby, neuronal firing rate. For example, reduction in the AHP by the action of neurotransmitters can increase firing rate (Madison and Nicoll, 1982; Pedarzani and Storm, 1995). The relation-

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**Figure 4.** Pharmacological identification of Ca\(^{2+}\) channel currents in SCN neurons. A, Plot of peak inward Ba\(^{2+}\) current versus time for a single SCN neuron recorded in a hypothalamic slice. The L channel antagonist nimodipine (10 μM) was applied during the period indicated by the block bar. Nimodipine blocked 29% of total Ba\(^{2+}\) current in this neuron (filled circles). Asterisks connected by a dotted line mark the mean Ba\(^{2+}\) current recorded from seven neurons to which no antagonist was applied. Inset shows current records obtained at the times indicated by the numbers. The slow time course of the tail currents reflects in part the speed of voltage clamp for SCN neurons in slices. B, Application of the N channel antagonist ω-CTx-GVIA (3 μM) to a neuron in another slice blocked 30% of total Ba\(^{2+}\) current in this example. C, Application of the P/Q channel antagonist ω-Aga-IVA (200 nM) to a neuron in another slice blocked 47% of total Ba\(^{2+}\) current in this example. D, A combination of nimodipine (nimodipine) (10 μM), ω-CTx-GVIA (3 μM), ω-Aga-IVA (200 nM), and mibefradil (mibe) (500 nM) was added to block L-, N-, P/Q-, and T-type Ca\(^{2+}\) channels, revealing a prominent component of antagonist-resistant current (R-type Ca\(^{2+}\) current). In all examples currents were activated every 5 sec by 50 msec voltage steps from −80 to −10 mV (voltage at which peak inward current was obtained).
ship between firing frequency of SCN neurons and duration of the Ca\(^{2+}\)-dependent AHP (Fig. 2B) predicts that reducing AHP duration would increase firing rate. We therefore examined how pharmacological antagonism of the AHP affected spike frequency in spontaneously active cluster I SCN neurons (Fig. 8A). In these experiments, Ca\(^{2+}\) channel blockers and KCa channel blockers were tested. Slices were bathed in bicuculline methiodide (30 μM) or picrotoxin (100 μM) to preclude secondary effects of Ca\(^{2+}\) or KCa channel blockers on firing rate, effects that could otherwise have arisen via alteration of Ca\(^{2+}\)-dependent release of GABA. In control experiments, spontaneous firing rates were found to be stable during the initial 15 min after breakthrough, and we therefore confined our measurements to this time period.

KCa channel antagonists did not alter firing rate. Neither block of SKCa channels by apamin (100 nm) nor block of BKCa channels by ibotenic acid (100 nm) had a statistically significant effect on spike frequency (Fig. 8B), nor did individual subtype-selective blockers of Ca\(^{2+}\) channel subtypes alter firing rate. Applied alone, nimodipine, ω-CTx-GVIA, ω-Aga-IVA, mibefradil, or Ni\(^{2+}\) did not significantly affect firing rate (Fig. 8B). However, application of a combination of nimodipine plus Ni\(^{2+}\) had a significant effect on firing rate (p < 0.01; n = 7). In contrast, complete blockade of Ca\(^{2+}\) entry by Cd\(^{2+}\) (30 μM) increased firing rate ~1.5-fold (see Discussion).

**Effects of channel blockers on spike parameters**

Blocks of Ca\(^{2+}\) channels and KCa channels were examined for their effects on spike parameters other than the AHP: interspike potential (V\(_{\text{i SP}}\)) determined as the plateau region of membrane potential between action potentials; spike amplitude, measured from V\(_{\text{i SP}}\) to peak depolarization; and spike width, measured at half amplitude. None of the blockers tested significantly affected V\(_{\text{i SP}}\), indicating that changes in firing rate could not be attributed to changes in V\(_{\text{i SP}}\) (Table 2). This was confirmed for Cd\(^{2+}\) by the fact that membrane potential was not affected when this blocker was applied, whereas spiking was suppressed by tetrodotoxin (1 μM): V\(_{\text{i SP}}\) was ~40 ± 3.1 mV before Cd\(^{2+}\) application and ~39 ± 2.4 mV during Cd\(^{2+}\) treatment (p > 0.1; n = 6).

Action potential amplitude was not affected by KCa channel blockers nor by Ca\(^{2+}\) channel blockers except for Cd\(^{2+}\), which substantially attenuated spike height (Table 2). A possible explanation for the Cd\(^{2+}\) effect is that block of the AHP, complete for Cd\(^{2+}\) but only partial for the other blockers (even the Ca\(^{2+}\) channel blocker mixture) (Fig. 7), prevented normal recovery from inactivation of voltage-gated Na\(^{+}\) channels. In contrast to V\(_{\text{i SP}}\) and spike amplitude, spike width was more sensitive to blockers (Table 2). Action potentials were broadened by the BKCa channel blocker ibotenic acid, consistent with the known role of BKCa channels in action potential repolarization. Action potentials were also broadened by the N-channel blocker ω-CTx-GVIA, by the five-component Ca\(^{2+}\) channel blocker mixture (nimodipine, ω-CTx-GVIA, ω-Aga-IVA, Ni\(^{2+}\), and mibefradil), and by the nonselective Ca\(^{2+}\) channel blocker, Cd\(^{2+}\), presumably because BKCa channel activity was reduced consequent to Ca\(^{2+}\) channel block.

**Discussion**

Firing frequency of neurons is commonly modulated by spike afterhyperpolarizations (Barrett and Barrett, 1976; Yarom et al.,...
blocks SK2 and SK3 subtypes of SKCa channels, whereas ibotenic acid selectively blocks type I BKCa channels. Coapplication of nearly saturating concentrations of apamin and ibotenic acid reduced the amplitude of the AHP by only ~55%, however, indicating that a large fraction of the channels underlying the AHP are insensitive to these blockers. SK1-based channels, which under some conditions have been found to be insensitive to apamin and ibotenic acid, the essentially uncharacterized channels that might support the apamin- and iberiotoxin-insensitive BKCa channels (Meera et al., 2000), intermediate conductance Ca2+-induced slow afterhyperpolarization in hippocampal pyramidal neurons (IaAHP) (Sah and Faber, 2002).
Four types of high voltage-gated Ca$^{2+}$ channels in SCN neurons

On the basis of sensitivity to Ca$^{2+}$ channel antagonists, our work shows that SCN neurons possess four principal components of high-voltage activated Ca$^{2+}$ channel current: L-, N-, and P/Q-currents, which had been previously identified in these neurons (Huang, 1993; Chen and van den Pol, 1998), and also R-type current. The precision in our measurement of relative current magnitude for the various Ca$^{2+}$ channel subtypes was compromised by the imperfect selectivity of the specific channel antagonists, the incomplete block of individual Ca$^{2+}$ channel subtypes, and the inability to voltage clamp distal processes of SCN neurons. Nonetheless, our pharmacological dissection of components of Ca$^{2+}$ channel current yielded an estimate for the size of the L-type component that is similar to that reported for dissociated SCN neurons (Huang, 1993).

Our analysis revealed that R-type current was the largest current component in SCN neurons from slices. The majority of R current was blocked by low concentrations of Ni$^{2+}$, although a proportion (~15% of total Ca$^{2+}$ channel current) remained that was insensitive to this concentration of Ni$^{2+}$. The nonblocked fraction might represent Ni$^{2+}$-insensitive R current (Schramm et al., 1999) or other Ca$^{2+}$ channel components incompletely blocked at the antagonist doses used. Because SNX-482 did not block R current, α1E-based Ca$^{2+}$ channels are not likely to support the R current of SCN neurons. R current identified here may correspond to the large component of Ca$^{2+}$ current that was found in previous studies to be insensitive to the combination of nimodipine and ω-CTx-GVIA (Huang, 1993).

Coupling of Ca$^{2+}$ channels to $K_{Ca}$ channels in SCN neurons

In some neurons, tight coupling exists between specific Ca$^{2+}$ channel subtypes and $K_{Ca}$ channel subtypes (Davies et al., 1996; Marrion and Tavalin, 1998; Borde et al., 2000; Martinez-Pinna et al., 2000), whereas in other neurons, multiple Ca$^{2+}$ channel subtypes contribute to AHP activation (Williams et al., 1997; Pineda et al., 1998). In cluster I neurons of the SCN, both L- and R-type Ca$^{2+}$ currents are involved in AHP activation. N and T channels make small contributions as well, and P/Q channels appear not to contribute. That large-amplitude N and P/Q currents contributed little or nothing to AHP activation suggests that Ca$^{2+}$ channel subtypes are differentially distributed on SCN neurons, with L- and R-type channels located in closest proximity to $K_{Ca}$ channels.

Physiological significance

Neurons studied here were in the daytime phase of the circadian cycle, but we suggest that the relationship between firing frequency and AHP waveform may contribute to circadian control of firing frequency in SCN neurons. This proposed mechanism for circadian regulation of $K_{Ca}$ channel activity and the AHP might arise from circadian changes in the expression, splicing, post-translational modification, or regulation of specific $K_{Ca}$ channels.

In faster firing SCN neurons, a shortened interspike interval appears to rely on speeded decay of the Cd$^{2+}$-sensitive component of the AHP, yet the Cd$^{2+}$-sensitive component of the AHP was larger and action potentials were narrower in faster firing neurons, suggesting that peak $K_{Ca}$ channel activity is augmented in faster firing neurons. Thus in faster firing neurons the number of $K_{Ca}$ channels open at the peak of the AHP is increased, but the AHP terminates more rapidly. These changes in the AHP might be accomplished in various ways. For example, the kinetics of $K_{Ca}$ channel gating might be speeded globally such that the maximal number of channels open simultaneously is increased (greater AHP magnitude) and yet the duration of the AHP is shortened. Alternatively, the increased magnitude of the AHP in faster firing neurons might be attributable to an increase in the number of $K_{Ca}$ channels available to open, and, in addition, SCN channels active in the range –50 to –60 mV and with a reversal potential positive to that of the AHP might exhibit increased activity (e.g., hyperpolarization-activated cation chan-
nels (Akasu et al., 1993) or Na⁺ channels (Pennartz et al., 1997).
Increased activity of these latter kinds of channels might mask the full duration of KCa channel activity and terminate the AHP more rapidly. The fact that faster firing neurons did not hyperpolarize more than slower firing neurons, although the net size of the Cd²⁺-subtracted AHP was greater in faster neurons, is consistent with the idea that these channels might compensate for the hyperpolarizing influence of KCa channels. In this case, circadian dependence on the activity of these channels, as well as of KCa channels, may be involved in determining the duration of the AHP and the frequency of firing.

In spontaneously firing SCN neurons, full suppression of the Ca²⁺-dependent AHP by application of Cd²⁺ increased firing rate (Fig. 8B), whereas partial suppression of the AHP by mixtures of specific Ca²⁺ channel blockers had the opposite effect. This unexpected contrast could arise if voltage-gated Ca²⁺ channels participate both in bringing neurons above firing threshold and in triggering the AHP. In this scenario, the net effect on firing rate of block of Ca²⁺ channels would reflect the balance between a slowed trajectory through firing threshold (decreased firing rate) versus a reduced AHP duration (increased rate). In the case of Cd²⁺ block, we speculate that the effect of full suppression of the AHP dominates over the slowed trajectory through threshold, so that neurons fire faster. With the Ca²⁺ channel blocker mixtures, we speculate that slowed trajectory through threshold dominates over the effect of partial suppression of the AHP, resulting in a net decrease in neuronal firing.

The molecular mechanism of the frequency-dependent change in AHP waveform appears to be based in part on KCa channels that are insensitive to either apamin or iberiotoxin, because application of these specific antagonists did not alter firing frequency. Additionally, there was no correlation between parameters of the iberiotoxin-sensitive component of the AHP and firing rate. We conclude that other KCa channel types, perhaps iberiotoxin-insensitive BK-Ca channels (Meera et al., 2000) or IK-Ca channels, are modulated in a manner that diurnally alters the firing rate of SCN neurons. For these KCa channels, circadian modulation might occur via second messenger-mediated modification of channel function, via changes in the coupling of Ca²⁺ entry (Pennartz et al., 2002) to channel activation, or by altered expression of channels or associated regulatory proteins. A notable recent finding regarding this latter point is that transcription of Slo2, which encodes a Ca²⁺-binding protein that interacts with and regulates the activity of the Slo KCa channel (Schopperle et al., 1998), is under circadian control in Drosophila (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001). More recently, expression of the mammalian ortholog of the BK-type Slo channel, Kenmama, has also been found to be under circadian control (Panda et al., 2002). The convergence on KCa channels of molecular and electrophysiological approaches underscores the likely importance of these channels in the mechanism of circadian firing of SCN neurons.

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