Cellular Mechanisms Underlying Spontaneous Firing in Rat Suprachiasmatic Nucleus: Involvement of a Slowly Inactivating Component of Sodium Current

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Pennartz, C.M.A., M. A. Bierlaagh, and A.M.S. Geurtsen. Cellular mechanisms underlying spontaneous firing in rat suprachiasmatic nucleus: involvement of a slowly inactivating component of sodium current. J. Neurophysiol. 78: 1811–1825, 1997. Neurons constituting the pacemaker of circadian rhythms, located in the suprachiasmatic nucleus, generate spontaneous firing patterns that change across the day-night cycle. Their average spontaneous firing rate is considered an important functional marker of clock activity because it is highest during daytime and low at night. In this study we investigate the ionic mechanisms underlying spontaneous firing in acutely prepared slices and dissociated neurons of the suprachiasmatic nucleus. In current-clamp mode, spontaneous action potentials were consistently preceded by depolarizing ramps. These ramps were Na⁺-dependent, were sensitive to tetrodotoxin (TTX), and disappeared on hyperpolarization. Ramps and associated spikes were not abolished by blockers of the H current (1 mM cesium) or calcium currents (50 μM nickel or 200 μM cadmium). In voltage-clamped neurons in slices or dissociated neurons, TTX-sensitive and Na⁺-dependent inward current was observed to activate well below firing threshold (−60 to −50 mV). The low-threshold component of Na⁺ current inactivated slowly as compared with the fast component that mediates action potentials. However, its inactivation proceeded more rapidly than has been reported for the persistent Na⁺ current in cortical structures. Persistent Na⁺ current was generally absent or small in amplitude. The voltage dependence and kinetics of the slowly inactivating component of Na⁺ current are consistent with the hypothesis that it is partially deinactivated during spike afterhyperpolarizations and contributes significantly to subsequent depolarizing ramps. These observations implicate the slowly inactivating component of Na⁺ current in ionic mechanisms governing spontaneous firing in suprachiasmatic nucleus neurons.

INTRODUCTION

The suprachiasmatic nucleus (SCN) has been shown to contain the biological clock that generates circadian rhythmicity (Inouye and Kawamura 1979; Meijer and Rietveld 1989; Moore and Eichler 1972; Ralph et al. 1990; Stephan and Zucker 1972). By way of its output to hypothalamic and thalamic target structures, the SCN imposes its rhythm on a number of physiological functions, e.g., sleep, corticosteroid and melatonin release, food and water intake, and locomotion (Cassone et al. 1993; Kalsbeek and Buijs 1992; Meijer and Rietveld 1989). A circadian rhythm in spontaneous firing rate, shown to be present in the SCN both in vivo and in vitro (Bos and Mirmiran 1990; Gillette 1991; Groos and Hendriks 1982; Inouye and Kawamura 1979), constitutes an important functional parameter encoding the output of the biological clock. The spontaneous firing rate has been shown to reach a peak during the daytime and a trough during the night. The enhanced firing rate during the daytime correlates well with the increase in release of several peptides synthesized in the SCN, e.g., vasopressin and somatostatin (Earnest and Sladek 1986; Inouye et al. 1993). A central problem in electrophysiological research on the organization of the circadian pacemaker concerns the membrane physiological mechanisms underlying the circadian rhythm in spontaneous firing rate. Although this question remains to be answered for the mammalian brain, it is useful to mention that in the mollusk Bulla gouldiana a K⁺ conductance in basal retinal cells has been implicated in regulating the circadian rhythm in membrane potential (Michel et al. 1993).

Before an efficient approach can be developed to answer the question as to which ionic conductances and/or ionic gradients in SCN neurons are altered across the circadian cycle, it is desirable first to identify the set of ionic currents that are of demonstrable importance in regulating the spontaneous firing rate. Although a number of ionic currents have either been shown or suggested to be present in SCN neurons, little is known about their role in regulating the length of spike intervals. Evidence has been raised for several K⁺ conductances in SCN neurons, including the transient outward K⁺ current (A current) (Bouskila and Dudek 1995; Walsh et al. 1995) and delayed rectifier (Bouskila and Dudek 1995; Walsh et al. 1995). Furthermore, both low- and high-threshold calcium currents have been revealed in at least a portion of SCN neurons (Akasu et al. 1993; Huang 1993; Kim and Dudek 1993). The low-threshold calcium current has been suggested to regulate spontaneous firing (Akasu et al. 1993; Huang 1993; Kim and Dudek 1993). Many SCN neurons also possess an H (or Q) current that may promote spontaneous firing (Akasu et al. 1993; but see De Jeu and Pennartz 1997).

In this study we adopted a straightforward approach to the question of which ionic conductances may be of immediate importance for the generation of spontaneous action potentials. When examining intracellular recordings of spontaneously firing SCN neurons, one is struck by the observation that nearly every spike is preceded by a slow depolarizing ramp (DR) or spike prepotential (cf. Akasu et al. 1993; Thomson and West 1990). The ionic mechanism underlying...
this DR was investigated here with the use of whole cell patch-clamp recording. This technique was employed not only in SCN slices but also in acutely dissociated cells, because the latter preparation allows a more accurate voltage-clamp control of membrane currents (Kay and Wong 1986). Our voltage-clamp recordings indicate that DRs result from the activation of a slowly inactivating component of Na⁺ current. Because this component can be activated at subthreshold membrane potentials and its inactivation is sufficiently slow to contribute to the main portion of the DR directly preceding the spike, this component is proposed to play a central role in the generation of spontaneous firing patterns in SCN neurons.

**METHODS**

**Slice experiments**

Male Wistar rats (180–300 g) were housed in a room with both temperature (22–24°C; humidity 65–75%) and light-dark control for 2–5 wk before use. The rats were anesthetized with Nembutal (60 mg/kg pentobarbital sodium; Sanofi Sante, Maassluis the Netherlands), perfused transcardially with 50 ml of ice-cold artificial cerebrospinal fluid (ACSF), and decapitated with a guillotine. The brain was rapidly removed from the skull and placed in ice-cold, oxygenated ACSF. A block of tissue containing the hypothalamus was cut and glued onto the stage of a vibratome (Vibrasilicer, Campden UK). Transversal slices 250 μm thick were cut while the hypothalamus was cooled by 2°C ACSF and surrounded by agar (4% in physiological saline). The slice storage chamber was continuously gassed with 95% O₂:5% CO₂ and kept at 23–28°C.

After an incubation period of ≥45 min, slices containing the SCN were transferred to a recording chamber, submerged, and held down with a U-shaped frame of flattened platinum wire covered with insulated stainless steel wire (60 μm diam.). The chamber was designed to fit on the fixed stage of an upright microscope (Zeiss Axiopan). A Zeiss water immersion objective (×40, working distance 1.9 mm, numerical aperture 0.75) with Hoffman modulation contrast was used to visualize single SCN neurons. Distinct somata as well as proximal dendrites could be distinguished with the plane of focus descending into the slice by as much as 100 μm. The slice was superfused at a pump speed of 1.5–2.5 ml/min with oxygenated ACSF composed of (in mM) 124 NaCl, 3.5 KCl, 26.2 NaHCO₃, 1.0 NaH₂PO₄, 1.3 MgSO₄, 25 CaCl₂, and 10.0 glucose, pH 7.3, 29–31°C.

Unless otherwise mentioned, pipettes for whole cell recordings were filled with (in mM) 135.0 K⁺ gluconate, 10.0 KCl, 10.0 N₂-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 0.5 ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’’,N’’-tetraacetic acid (EGTA), 5.0 biocytin, and 2.0 Na₂ATP, pH 7.3, osmolality 270–275 mosM. In one series of experiments, K⁺ gluconate was replaced by Cs⁺ gluconate while the other constituents were maintained. Pipette resistance was 4–8 MΩ, corresponding to tip diameters of 1.2–1.7 μm. Pipette tips were brought into close proximity of the somatic membrane of SCN neurons under positive pressure and under visual guidance (Stuart et al. 1993). This method gave access to cells lying at a depth of 30–100 μm beneath the slice surface without the necessity of blowing away overlying tissue beforehand. The seal resistance ranged between 3 and 30 GΩ. After membrane rupture by mouth suction, membrane properties were assessed in voltage- or current-clamp mode while biocytin was allowed to diffuse into the neuron.

**Dissociated cells**

SCN neurons were acutely dissociated by the following procedure, modified after Kay and Wong (1986; Vreugdenhil and Wadman 1995). Slices (thickness 300–400 μm) were prepared as above with the use of rats 25–38 days of age. After a brief storage period in oxygenated ACSF (see above), minislices comprising the SCN and optic chiasm were prepared by carefully removing the surrounding tissue. These minislices were transferred to a dissociation medium containing 120 mM NaCl, 5 mM KCl, 20 mM piperrazine-N,N’-bis-(2-ethanesulfonic acid) (PIPES), 1 mM MgCl₂, 1 mM CaCl₂, 25 mM D-glucose, and 1 mg/ml trypsin type XI (Sigma; pH 7.0, gassed with 100% O₂). This medium was gently stirred while being contained in an incubation chamber held at 32°C as in Kay and Wong (1986). After 35 min, the minislices were washed in dissociation medium without enzyme and triturated with fire-polished Pasteur pipettes with tips of decreasing diameter. A drop of cell suspension was placed onto the glass plate of the recording chamber (see above) and the cells were observed to settle within 5 min, after which superfusion with HEPES buffer was started (composition, in mM: 120 NaCl, 5 KCl, 10 HEPES, 1 MgCl₂, 1 CaCl₂, 24 D-glucose, 3 4-aminopyridine, 20 tetraethylammonium chloride (TEA), 2 CsCl, and 0.2 CaCl₂, pH 7.4, flow rate 1.5–2.5 ml/min). Recordings were made at room temperature (20–23°C). Viable neurons were characterized by their even, non-granular appearance and the possession of at least one dendrite or axon, usually ~30–50 μm in length. The profile of Na⁺ currents described in RESULTS could be obtained within a maximum of 4 h after dissociation.

Whole cell recordings from dissociated cells were obtained with the use of pipettes filled with (in mM) 130 CsF, 10 HEPES, 11 EGTA, 2 MgCl₂, 1 CaCl₂, and 2 Na₂ATP, pH 7.3, osmolality 300–310 mosM, resistance 2.5–5 MΩ. Because of pH adjustment with KOH the pipette solution also contained ~27 mM K⁺. After a cell-attached patch of ≥2 GΩ was established, the membrane was ruptured by mouth suction.

**Data analysis**

Current and voltage traces were recorded by an Axopatch 1D or Axoclamp 2B amplifier (Axon Instruments) and relayed via a Digidata 1200 interface (Axon Instruments) to a computer and stored on hard disk. We used the pClamp 6.02 package and AxoScope 2.0 (both from Axon Instruments) for data acquisition and analysis. Current traces obtained during ramp commands in voltage-clamp mode were filtered at 500 Hz (Bessel filter at 80 dB/decade), whereas current responses to voltage steps were filtered at 5 kHz. Voltage traces obtained in current-clamp mode were not filtered. Sampling rates ranged from 2 kHz (ramp commands) to 10 kHz (step commands) in voltage-clamp experiments and from 5 to 10 kHz in current-clamp mode. In most voltage-clamp experiments, leak subtraction (based on 4 hyperpolarizing pulses before the depolarizing test pulse) was employed to remove linear leakage and capacitance currents. We corrected for the liquid junction potential, which amounted to ~13 mV for K⁺ gluconate-based pipette solution as estimated when a 3 M KCl bath ground electrode (Neher 1992) was used. For CsF- and Cs⁺ gluconate-based pipette solutions, the liquid junction potentials were ~7 and ~9 mV, respectively.

Numerical values are expressed as means ± SE. Data sets were statistically compared with the use of Wilcoxon’s matched-pairs signed rank test.

**Consideration of voltage-clamp errors**

In slice experiments, K⁺ gluconate-filled pipettes configured in whole cell mode usually had series resistances between 10 and 30 MΩ. These values were compensated by 80%. Inspection of the current-voltage curve recorded under voltage-clamp conditions revealed a current range of ±200 pA to be relevant for studying the slowly inactivating component of Na⁺ current. It follows that
maximal errors in voltage command due to series resistance can be estimated at 0.4–1.2 mV with 200 pA of current (Armstrong and Gilly 1992). In estimating the time constant of voltage settling after initiation of a step, the membrane capacitance of SCN cells (usually 5–15 pF) was multiplied with the series resistance. Thus, time constants between 0.04 and 0.09 ms were usually obtained. It can be inferred that 98% of the desired step value will be reached within 0.16–0.36 ms (i.e., 4 times the time constant).

As concerns deviations from isopotentiality throughout voltage-clamped SCN neurons in slices, it was noted that the voltage response to injection of current steps was well fitted by a single exponential, suggesting a favorable electrotonic compactness of SCN neurons (Rall 1977) relative to pyramidal cells of neocortex and hippocampus. However, biocytin staining of neurons recorded in slices often revealed axonal branches originating from thin, varicose dendrites. Because these branch points may well represent the sites of action potential initiation (Haussler et al. 1995), it is hard to exclude the possibility of inadequate space clamp especially in the case of the fast Na⁺ current. Therefore we supplemented our observations with whole cell recordings in dissociated cells, which only retain their proximal dendrites and are characterized by a very high electrotonic compactness.

In dissociated cells, series resistances in whole cell mode ranged between 6 and 13 MΩ and were compensated by 80%. For a current of 200 pA, the estimated voltage error varied from 0.2 to 0.5 mV. In a majority of dissociated cells, it was feasible to voltage clamp the fast Na⁺ current, and its peak value was calculated to result in −2–5 mV of voltage error. We discarded neurons recorded under poor voltage-clamp control, which was evident because of 1) a delayed onset of fast Na⁺ current during voltage steps close to threshold and 2) absence of graded increments in fast Na⁺ current at membrane potentials around firing threshold (Cummmins et al. 1994; Huguenard et al. 1988; Sakmann and Neher 1995).

**Labeling procedures and three-dimensional reconstruction**

For immunocytochemical processing, slices were fixed overnight in 4% paraformaldehyde or 4% formaldehyde solved in 0.1 M phosphate buffer (pH 7.4–7.6 at 4°C) and subsequently stored in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (pH 7.4–7.6; 0.05 M Tris and 0.15 M NaCl) containing 0.05% sodium azide. After the slices were rinsed several times with Tris-buffered saline, they were incubated in a solution containing 0.25% gelatin, 0.5% triton, and 1.2 µg/ml streptavidin-Cy3 (Jackson Immunoresearch). Because whole mount slices gave satisfactory penetration of streptavidin-Cy3, it was not necessary to make subsections of a single slice. After each slice was mounted onto a coated slide, a drop of Vectashield (Vector Labs) was applied to counteract photobleaching. It was feasible to match one to four labeled neurons in one slice to sets of electrophysiological traces, because the positions of individual somata were accurately noted in an overview sketch of each slice during each recording session. Three-dimensional reconstruction of cells was accomplished by computer-aided superimposition of optical sections (1 µm) obtained by a confocal scanning laser microscope (Zeiss-Kontron, Jena, Germany).

**Solutions and drugs**

Drugs used in slice experiments were tetrodotoxin (TTX), choline bicarbonate, choline chloride, TEA, 4-amino pyridine, CdCl₂, CsCl, Cs⁺ methanesulfonate, bicusculine methiodide (all from Sigma), and bicusculine methochloride (Tocris Cookson, Bristol, UK). In sodium-free solutions, sodium chloride and sodium bicarbonate were substituted in equimolar amounts by choline chloride and choline bicarbonate, respectively. Similarly, the sodium chloride concentration was reduced in TEA-containing ACSF. The pH of sodium-free solution was adjusted to 7.4. With respect to ACSF, superfusion of Na⁺-free solution induced a change in offset potential (−4 mV) at the reference electrode, which was corrected for in the measurements described below.

Additional substances used in experiments with dissociated cells were trypsin type XI, PIPES, and CsF (all from Sigma).

**RESULTS**

**Basic membrane properties of SCN neurons in slices**

Whole cell recordings were obtained from a total of 74 neurons. Duration of the recordings varied from 10 min to 4 h. The resting membrane potential and input resistance were −54.9 ± 0.7 (SE) mV and 1.19 ± 0.05 GΩ, respectively. In spontaneously firing neurons, the resting membrane potential refers to the low-pass light-emitting diode readout of the electrode amplifier. The time constant, obtained by fitting an exponential to voltage deflections induced by negative current pulses (−3 to −30 pA), was 25.9 ± 1.5 ms. Whole cell recordings revealed time-dependent inward rectification in voltage responses to hyperpolarizing current in almost every SCN neuron (Fig. 1, A and B). After termination of a negative current pulse, SCN neurons generated one or more rebound spikes riding atop a rebound depolarization of varying magnitude (Fig. 1, A and B). In response to positive current pulses, the cells generated spike trains usually showing no or moderate frequency adaptation and spike train afterhyperpolarizations (AHPs) of modest amplitude (Fig. 1C). The action potential amplitude, measured with respect to resting level, and its width at half-amplitude were 74 ± 2 mV and 1.35 ± 0.05 ms, respectively.

Current-clamp recordings gave a spike threshold estimate of −38 ± 1 mV. Measurements of Na⁺ currents and Na⁺-dependent potentials obtained from slices in the subjective day and night period were pooled because the results were qualitatively similar. Cells producing spikes <60 mV were discarded. Below we first describe our findings obtained in slices and then continue with recordings in dissociated cells.

**DRs in patterns of spontaneous firing**

In attempting to answer the question of which ionic currents may directly regulate the occurrence and frequency of spontaneous firing in SCN neurons, we started by examining membrane potential records without manipulating the cells by current injection. In SCN cells having a relatively high spontaneous firing rate (5–17 Hz), the spike AHP tended to merge into a less steeply rising phase, which, however, could be rapidly followed by an upshoot toward the next action potential (Fig. 2A). In cells with a spontaneous firing rate of only 0.1–4 Hz, the spike AHP was generally well segregated from the portion immediately preceding the spike (Fig. 2B). Almost every time segment preceding a spike was characterized by a slowly rising membrane potential departing from resting level. Henceforth this slowly rising potential is denoted a DR. DRs were observed in both regularly and irregularly firing cells (regularly firing cells were defined as having a coefficient of variation in spike intervals <0.20) (cf. Groos and Hendriks 1979). In some rare cases, spikes were observed without preceding DRs. Conversely, some DRs did not give rise to a spike. The DR could be
FIG. 1. Basic membrane properties of suprachiasmatic nucleus (SCN) neurons recorded in whole cell configuration (current-clamp mode) in slices. A: injection of a current pulse (−90 pA) revealed time-dependent inward rectification (depolarizing sag, indicated by ∗). Following termination of the current pulse, a modest rebound potential carrying 1 or 2 additional spikes was produced. B: example of another neuron producing a large rebound potential and a burst of spikes following a hyperpolarizing current step (−30 pA). Despite the fact that this pulse produced a smaller inward rectification than in A, the rebound potential was much larger. C: depolarizing current pulse (40 pA) evoked a spike train exhibiting modest frequency adaptation. Notice the spike train afterhyperpolarization (AHP) following pulse termination (--- indicates resting level). Resting membrane potentials: −56 mV (A), −63 mV (B), −53 mV (C). Calibration bar: 300 ms (A and B), 200 ms (C).

easily distinguished from spontaneous synaptic inputs by virtue of its slow rising phase.

We next investigated whether it was feasible to evoke a DR in a controlled manner, viz., by way of intracellular injection of positive current pulses (2−20 pA). Figure 2C illustrates that DRs were indeed evoked reproducibly by such current pulses in a cell that was otherwise silent. The DRs became progressively steeper with increasing current intensity. As can be observed in Fig. 2C, the DR was nearly linear in shape across most of its trajectory even when the latency of the first spike became short, i.e., 5−25 ms. The observation that DRs can be reproducibly evoked by current pulses greatly facilitated our analysis because this condition allows well-timed and systematic activation of DRs.

FIG. 2. Spontaneous action potentials are consistently preceded by depolarizing ramps. A: in cells having a high spontaneous firing rate, it was generally difficult to segregate spike AHPs from depolarizing ramps. B: in cells firing at a low rate, spike AHPs ended long before depolarizing ramps were initiated. In this neuron depolarizing ramps invariably triggered spikes. Dotted lines: baseline extensions. C: positive current pulses reliably evoked depolarizing ramps and associated spikes in neurons that did not fire spontaneously. Note the nearly linear shape of the ramp across most of its trajectory and the increase in steepness with higher current intensities. In all current-clamp records given here and below, rectangular blocks below traces represent current pulses. In this example, superimposed current pulses of +5, 10, and 15 pA evoked the bottom, middle, and top responses, respectively. Resting membrane potentials: −52 mV (A), −55 mV (B), −60 mV (C).
Importance of the slowly inactivating sodium current for spontaneous firing

We sought a means to quantitatively assess the importance of the DR and its underlying current in promoting spontaneous firing of SCN neurons. Although no pharmacological agent is known to us that blocks DRs but leaves spikes completely intact, we examined how closely spikes are associated with preceding DRs. If DRs can be defined in terms of shape and rising steepness, it is possible to determine the conditional probability \( P(\text{DR}|\text{spike}) \) that, given the presence of a spike, a DR preceded it. This measure will reveal which portion of all spontaneous action potentials was generated in direct succession to a DR. Conversely, we also determined \( P(\text{spike}|\text{DR}) \), i.e., the probability of spiking given the presence of a preceding DR. This value can be taken as a measure of the capability of DRs to trigger action potentials.

Reasonable boundaries distinguishing DRs were set between a minimal rising speed of 0.03 mV/ms and a maximum of 0.65 mV/ms across a group of 10 cells (the range from minimum to maximum was considerably narrower for an individual cell). In practice, most events satisfying these constraints were nearly linear in their rising slope, whereas they exhibited a sharp peak and a rapid decline toward baseline in the absence of a spike (Fig. 3A). For 10 cells firing at 0.7–3.6 Hz (rates at which DRs and AHPs were well segregated in time), a value of 98.5 \( \pm \) 1.2\% was found for \( P(\text{DR}|\text{spike}) \) and a value of 90.1 \( \pm \) 2.2\% for \( P(\text{spike}|\text{DR}) \). In sporadic cases where spikes were not preceded by a DR, the membrane potential record had a ragged appearance, which can be ascribed at least in part to spontaneous synaptic inputs (cf. Kim and Dudek 1992).

Ionic basis of DRs

Considering the nearly linear rise of DRs up to spike threshold, it can be inferred that active membrane properties of SCN neurons must be involved to cause the voltage to deviate from a passive, monoexponential response. A DR may be caused by the progressive activation of an excitatory current (with \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) as potential charge carriers) or deactivation or inactivation of an inhibitory current (with \( \text{K}^+ \) as a probable charge carrier). The hypothesis that an excitatory current may be activated was tested by examining the effects of a \( \text{Na}^+ \) channel blocker and by removal of \( \text{Na}^+ \) from the external bathing medium.

TTX (0.5–1.0 \( \mu \text{M} \)), a selective blocker of \( \text{Na}^+ \) channels, was observed to abolish spontaneous DRs in addition to action potentials (Fig. 3A; \( n = 8 \)). Likewise, TTX blocked DRs evoked by positive current pulses (Fig. 3B; \( n = 9 \)). Under conditions of \( \text{Na}^+ \) channel block, voltage changes evoked by a current pulse closely resembled a passive membrane response. This was also the case for voltage responses under TTX conditions that reached a steady-state level close to firing threshold (Fig. 3C). In control conditions, the resting membrane potential, input resistance, and membrane resistance increased by 10.2 \( \pm \) 0.3 \( \Omega \) cm\(^2\). Note that the rising phase of this event is much slower than that of a spontaneous synaptic potential. A3: hyperpolarizing the cell to \(-69\, \text{mV} \) by \(-7\, \text{pA} \) (DC) caused disappearance of both depolarizing ramps and spikes. A4: application of 0.5 \( \mu \text{M} \) TTX abolished both depolarizing ramps and spikes even though the cell remained at resting level. All traces in A are from the same neuron. B: TTX suppressed the depolarizing ramp and spike evoked by a positive current pulse (5 pA). Voltage changes followed an exponential time course to a steady-state level under TTX conditions. C: comparison between the control trace (5 pA) shown in B and the voltage response to a current step (15 pA) under TTX conditions. The response under TTX reveals that the membrane also behaves passively when the response reaches a voltage level normally corresponding to firing threshold. Resting membrane potential of the cell in B and C: \(-59\, \text{mV} \). D: dependence of depolarizing ramps on extracellular sodium. In control conditions, a positive current pulse (10 pA) evoked 3 spikes preceded by depolarizing ramps. When \( \text{Na}^+ \) was substituted by choline, the depolarizing ramps and action potentials were abolished. E: recovery of depolarizing ramps and spikes after replacement of choline by \( \text{Na}^+ \). Resting membrane potential of this neuron: \(-54\, \text{mV} \). Action potentials are truncated in B–E.

FIG. 3. Sensitivity of depolarizing ramps to hyperpolarization, tetrodotoxin (TTX), and removal of extracellular \( \text{Na}^+ \). A1 and A2: current-clamp records of spontaneous depolarizing ramps and associated action potentials in the absence of continuous current injection. Asterisk: depolarizing ramp without spike. Note that the rising phase of this event is much slower than that of a spontaneous synaptic potential. A3: hyperpolarizing the cell to \(-69\, \text{mV} \) by \(-7\, \text{pA} \) (DC) caused disappearance of both depolarizing ramps and spikes. A4: application of 0.5 \( \mu \text{M} \) TTX abolished both depolarizing ramps and spikes even though the cell remained at resting level. All traces in A are from the same neuron. B: TTX suppressed the depolarizing ramp and spike evoked by a positive current pulse (5 pA). Voltage changes followed an exponential time course to a steady-state level under TTX conditions. C: comparison between the control trace (5 pA) shown in B and the voltage response to a current step (15 pA) under TTX conditions. The response under TTX reveals that the membrane also behaves passively when the response reaches a voltage level normally corresponding to firing threshold. Resting membrane potential of the cell in B and C: \(-59\, \text{mV} \). D: dependence of depolarizing ramps on extracellular sodium. In control conditions, a positive current pulse (10 pA) evoked 3 spikes preceded by depolarizing ramps. When \( \text{Na}^+ \) was substituted by choline, the depolarizing ramps and action potentials were abolished. E: recovery of depolarizing ramps and spikes after replacement of choline by \( \text{Na}^+ \). Resting membrane potential of this neuron: \(-54\, \text{mV} \). Action potentials are truncated in B–E.
time constant were \(-57 \pm 2\) mV, \(1.1 \pm 0.1\) GΩ, and \(22 \pm 4\) ms, respectively \((n = 10)\). These membrane properties did not change significantly under TTX conditions (resting membrane potential: \(-57 \pm 2\) mV; input resistance: \(1.1 \pm 0.1\) GΩ; time constant: \(24 \pm 3\) ms). Furthermore, spontaneous DRs disappeared or decreased in frequency when the cell was hyperpolarized to a membrane potential below \(-65\) mV (Fig. 3A; \(n = 10\)), indicating that the ionic conductance underlying the DR is voltage dependent.

The involvement of Na\(^+\) current in mediating the DR was further confirmed by experiments substituting choline for Na\(^+\). As in TTX-containing medium, the DR and concomitant action potential were reversibly abolished in Na\(^+\)-free medium \((n = 5\); Fig. 3, D and E). In Na\(^+\)-free medium, voltage responses to positive current pulses followed an exponential time course toward a steady-state level similar to that observed in TTX conditions. Moreover, spontaneous DRs observed in the absence of current injection vanished in Na\(^+\)-free conditions (not shown). It was further noted that the resting membrane potential of cells bathing in Na\(^+\)-free medium was \(4 \pm 1\) mV negative with respect to ACSF. The disappearance of the DR could not be attributed to this modest hyperpolarization, because injection of compensatory current did not result in its reappearance. Although the exact mechanism producing this hyperpolarization is unknown, it may have been caused by manipulation of a tonically active, Ba\(^{2+}\)-sensitive mixed cation conductance (M. T. De Jeu, A.M.S. Geurtsen, and C.M.A. Pennartz, unpublished data). The input resistance and time constant, both estimated from voltage responses to negative current pulses \((-3 \text{ to } -12\) pA) did not change when ACSF was replaced by Na\(^+\)-free medium (ACSF: \(1.6 \pm 0.1\) GΩ, \(45 \pm 10\) ms; Na\(^+\)-free medium: \(1.5 \pm 0.1\) GΩ, \(42 \pm 6\) ms). Time-dependent inward rectification, however, diminished in magnitude. The latter observation can be explained by the loss of the Na\(^+\) component of the H (or Q) current, which underlies this type of rectification (McCormick and Pape 1990).

Although these experiments strongly suggest a prominent role of TTX-sensitive Na\(^+\) current in DRs, it should be noted that the manipulations used above also abolished action potentials and AHPs. By consequence, low-threshold (T-type) Ca\(^{2+}\) potentials, H-current-mediated potentials, and spontaneous synaptic [primarily \(\gamma\)-aminobutyric acid-A (GABA\(_A\))-receptor-mediated] inputs may have been lost or diminished in amplitude. Because these events may contribute to the generation of DRs, we assessed whether DRs were significantly reduced in the presence of Ca\(^{2+}\) current and H current blockers or the GABA\(_A\) receptor antagonist bicuculline. Application of nickel (50 \(\mu\)M), which attenuates T-type Ca\(^{2+}\) current (Fox et al. 1987; Mogul and Fox 1991; Toselli and Taglietti 1992), did not notably alter the shape or rising speed of DRs, although the spontaneous firing rate in nickel decreased slightly (from \(2.2 \pm 0.6\) Hz in control to \(1.7 \pm 0.6\) Hz; \(n = 9\), not shown; resting membrane potential: \(-53.7 \pm 1.7\) mV in control and \(-53.5 \pm 2.3\) mV in nickel). Superfusion with cadmium (200 \(\mu\)M), a more general Ca\(^{2+}\) current blocker at this dose, failed to suppress DRs as well. On average, Cd\(^{2+}\) induced a membrane depolarization (from \(-57.6 \pm 1.6\) mV in control to \(-52.3 \pm 4.4\) mV in Cd\(^{2+}\); \(n = 5\)) and a slight increase in spontaneous firing rate (control: \(3.5 \pm 0.6\) Hz; Cd\(^{2+}\): \(4.4 \pm 1.0\) Hz; \(n = 5\)). Regardless of the precise magnitude of the depolarizing effect, DRs remained largely unchanged during Cd\(^{2+}\) application (Fig. 4, A–C) and this observation also applied when the DC membrane potential was brought back to control level by compensatory injection of constant current. In all cells Cd\(^{2+}\) was observed to diminish spike AHPs, an effect that could be clearly held apart from the lack of marked changes in DRs. Possibly this effect reflects a blocking action on a Ca\(^{2+}\)-dependent K\(^+\) conductance supporting the spike AHP.

Likewise, the H current blocker Cs\(^+\) (1 mM) did not affect DRs (Fig. 4, D–F; \(n = 6\); resting membrane potentials in control vs. Cs\(^+\) condition: \(-57.5 \pm 1.1\) and \(-57.3 \pm 1.4\) mV, respectively; spontaneous firing rates: \(1.9 \pm 0.4\) and \(2.0 \pm 0.3\) Hz). Finally, we confirmed that DRs and spontaneous firing remained intact during block of spontaneous GABA\(_A\) receptor mediated inhibitory postsynaptic potentials by bicuculline (\(n = 3\)). These results indicate an absence of a prominent T-type Ca\(^{2+}\)-current-, H-current-, or GABA\(_A\)-receptor-mediated contribution to spike-triggering DRs in SCN neurons.

Voltage-clamp experiments in slices

All recordings presented thus far indicate that Na\(^+\) current is involved in generating DRs. However, these recordings were obtained in current-clamp mode, which makes it difficult to assess the voltage dependence and kinetics of the underlying current. We therefore examined TTX-sensitive and Na\(^+\)-dependent current in voltage-clamp mode. In a first series of experiments, we used K\(^+\)-gluconate-based pipette solution and applied ramp commands starting from a holding level of \(-100\) mV and running up linearly to a final level of \(-10\) or \(0\) mV (speed: \(69\) or \(77\) mV/s). K\(^+\), Ca\(^{2+}\), and Na\(^+\)/K\(^+\) (H) currents were suppressed by including 20 mM TEA, 0.25 mM NiCl\(_2\) (or 0 mM Ca\(^{2+}\) and 4.0 mM Mg\(^{2+}\)), and 1.0 mM CsCl in the bath medium. In addition, GABA\(_A\)-receptor-mediated currents and A current were usually blocked by 12.5 \(\mu\)M bicuculline and 3.0 mM 4-aminopyridine. In response to the voltage ramp we observed a slow, TTX-sensitive inward current in the range from approximately \(-65\) mV to \(-45\) mV (Fig. 5). The inward current strongly diminished in amplitude when slower ramp commands (rising speed: \(6–8\) mV/s) were used. Furthermore, the inward current disappeared when extracellular Na\(^+\) was replaced by choline (\(n = 4\)). This effect could not be attributed to a partial removal of H current, because this current is deactivated at voltages positive to \(-60\) mV (Akasu and Shoji 1994; De Jeu and Pennartz 1997; McCormick and Pape 1990). The TTX sensitivity of the inward current was confirmed in eight experiments. As can be inferred from the results obtained with step protocols (see below), it should be noted that ramp protocols will not reveal the full-blown magnitude of Na\(^+\) current because it will at least partially inactivate during the time course of the ramp command.

The reduction in TTX-sensitive inward current on use of slow instead of fast ramp commands suggested involvement of an inactivating rather than a persistent Na\(^+\) current (but see Fleidervish and Gutnick 1996). To obtain further evidence for the presence or absence of persistent Na\(^+\) current, we attempted to trigger Na\(^+\) plateau potentials that have
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FIG. 5. Slow, TTX-sensitive inward current as revealed by a fast voltage-clamp ramp command in neurons from SCN slices. Ramp command, starting from a holding potential of $-100 \text{ mV}$ and running up to $-10 \text{ mV}$ (top; speed: $69 \text{ mV/s}$), evoked a current response characterized by a negative slope conductance in control conditions. TTX (1.0 $\mu\text{M}$) abolished the inward current and rendered the current response nearly linear until about $-40 \text{ mV}$, where outward currents became predominant. Medium contained 20 mM tetraethylammonium chloride (TEA), 1 mM CsCl, 0 mM Ca$^{2+}$/4 mM Mg$^{2+}$, and 12.5 $\mu\text{M}$ bicuculline; the main component of the pipette solution was K$^+$/gluconate.

been ascribed to activation of this current in neocortical pyramidal cells (Fleidervish and Gutnick 1996; Stafstrom et al. 1985). K$^+$ currents were blocked by filling pipettes with solution containing Cs$^+$ methanesulfonate (135 mM) as the main compound and Ca$^{2+}$/currents were blocked by 200 $\mu\text{M}$ Cd$^{2+}$/the ACSF. In one of four cells, brief current pulses (30 ms, applied at a baseline voltage of $-60$ to $-70 \text{ mV}$) elicited short-lasting plateau potentials ($\sim 200$–400 ms in duration), whereas they failed to do so in the other three cells. For comparison, Na$^+$ plateau potentials in neocortex were shown to last for $\sim 2$–3 s. Thus our observations suggest the absence of a pronounced and ubiquitous persistent Na$^+$ current in SCN neurons.

In a second series of experiments, we improved the quality of voltage clamp by 1) using Cs$^+$-gluconate- instead of K$^+$-gluconate-based pipette solution and 2) reducing the extracellular Na$^+$ concentration from 151.2 to 27.2 mM. Furthermore, the bath medium contained 20 mM TEA, 3.0 mM 4-aminopyridine, 0 mM Ca$^{2+}$/4.0 mM Mg$^{2+}$, 2.0 mM CsCl, and 12.5 $\mu\text{M}$ bicuculline throughout this series. In six adequately voltage-clamped neurons, we used step commands to examine the

FIG. 4. Ca$^{2+}$/current and H-current blockers fail to suppress depolarizing ramps. A: spontaneous train of action potentials recorded in control artificial cerebrospinal fluid (ACSF). Membrane potential is indicated at right. B: on application of 200 $\mu\text{M}$ Cd$^{2+}$/the firing rate decreased in this cell, whereas the spike AHP decreased in amplitude and depolarizing ramps remained present. No compensatory current was needed to keep the cell at $-49 \text{ mV}$. C: average of 150 spikes from the records containing time segments $A$ and $B$. Overlay of these averages reveals the lack of Cd$^{2+}$/effect on the ramp segment preceding the spike by 140 ms. Solid horizontal line helps to distinguish the ramp. $D$–$F$: same as in $A$–$C$ but for Cs$^+$ (1 mM). Note that Cs$^+$ failed to affect the depolarizing ramp or spike AHP and slightly reduced the firing rate in this cell.
FIG. 6. Demonstration of a slowly inactivating component of Na⁺ current in SCN slices. A: with the use of a holding potential of −90 mV, step commands were applied to levels ranging from −65 to +30 mV with a step size of 5 mV. Extracellular medium contained 20 mM TEA, 3.0 mM 4-amino-pyridine, 2 mM CsCl, 0 mM Ca²⁺/4 mM Mg²⁺, 12.5 μM bicuculline, and 27.2 mM Na⁺. Fast-activating, slowly inactivating inward current was first seen after step to −55 mV. With progressively stronger depolarization, inactivation proceeded more rapidly. At step levels of −45 mV and above, a fast component of Na⁺ current was coactivated.

B: overlay of the current responses obtained with steps to −50 and −45 mV, showing the distinct kinetics of fast and slowly inactivating components of Na⁺ current. Arrow: full inactivation of the fast Na⁺ component, while the slow component remains visible.

C: biexponential fit to current response shown in A, top (step to −40 mV). Time constants of the slowly and fast inactivating component were 5.5 and 0.7 ms, respectively. Dotted line: baseline extension.

D: slowly inactivating inward current evoked by a step command to −50 mV was completely abolished by TTX. E: current-voltage plot of the peak of Na⁺ current. Arrow: isolated slowly inactivating component. Na⁺ reversal potential estimated from this plot: +6/20 ± 3.0 mV. At voltage levels where the fast component of Na⁺ current became dominant in amplitude, it was still possible to discern the slow component by virtue of its longer time constant. Biexponential fitting allowed us to estimate the amplitudes of the fast and slowly inactivating components estimated from biexponential fits to traces such as shown in A. Squares and circles: slow and fast components, respectively. All traces in this figure were recorded from the same neuron.
where \( g \) is the \( Na^+ \) conductance, \( I \) is the peak current, \( V \) is the step voltage, and \( V_{rev} \) is the \( Na^+ \) reversal potential. The resulting conductance-voltage plots were fitted with a Boltzmann function

\[
g = \frac{g_{\text{max}}}{1 + e^{-\frac{V - V_{1/2}}{S}\frac{1}{2}}}
\]

where \( g_{\text{max}} \) represents the maximal conductance, \( V_{1/2} \) the voltage of half-maximal activation, and \( S \) a slope factor. The \( V_{1/2} \) estimate for the slow component (\(-39.8 \pm 3.8 \text{ mV}; n = 6 \)) was more negative than that for the fast component (\(-33.8 \pm 2.4 \text{ mV}; n = 6 \); not significant). The estimates for slope factor \( S \) were \( 3.8 \pm 1.1 \) and \( 2.1 \pm 0.5 \text{ mV per e-fold} \) for the slow and fast component, respectively (not significant).

Sometimes a TTX-sensitive persistent component was observed; this was, however, small in amplitude. At the very highest levels of depolarization tested (above \(-20 \) to \(-10 \text{ mV} \)), the inactivation time constant of the slow component became so brief that it was difficult to segregate from the decay of the fast component.

**Voltage-clamp experiments in dissociated neurons**

Despite the indications for reasonable voltage control of sodium current in the experiments described above, the possibility of space-clamp problems remains an issue for neurons having intact dendrites. Therefore we conducted similar voltage-clamp experiments in dissociated SCN cells (\( n = 16 \)). Activation of \( Na^+ \) current was examined in 11 neurons bathed in HEPES buffer containing 3.0 mM 4-aminopyridine, 20 mM TEA, 2.0 mM CsCl, and 0.2 mM CdCl\(_2\). With the use of a holding potential of \(-90 \text{ mV} \), incremental depolarizing steps were first seen to activate the slowly inactivating component of \( Na^+ \) current at levels between \(-60 \) and \(-50 \text{ mV} \), whereas at more depolarized levels the fast component was superimposed on the slow one (Fig. 7, A and B). Both components were reversibly abolished by TTX (1 \( \mu \text{M} \); Fig. 7D; \( n = 11 \)). In the presence of TTX, there was no sign of residual inward current, confirming the effectiveness of Cd\(^{2+} \) in blocking Ca\(^{2+} \) conductances. As in slices, the activation time constant of the slow component was \(<1.0 \text{ ms} \) and did not exhibit a clear voltage dependence. The decay time constant (\( 18.3 \pm 2.6 \text{ ms at } -50 \text{ mV} \)) was similar to the mean obtained in slices and decreased with progressive depolarization (Fig. 7F). The estimated \( Na^+ \) reversal potential was \(+49.1 \pm 1.3 \text{ mV} \). At levels positive to \(-45 \text{ mV} \), the decay from peak level was fitted well by a biexponential curve (Fig. 7C). Biexponential fits gave peak current estimates that were converted to conductance data (Eqs. 1 and 2). Boltzmann fits to these conductance-voltage curves (Fig. 7G) indicated a \( V_{1/2} \) of \(-43.2 \pm 3.6 \text{ mV} \) and a slope factor \( S \) of \( 3.1 \pm 0.7 \text{ mV per e-fold} \) for the slow component. The fast component was characterized by a significantly more depolarized value of \( V_{1/2} \) (\(-30.2 \pm 3.5 \text{ mV}; n = 6 \); \( P < 0.05 \) according to Student’s \( t \)-test or Wilcoxon’s matched pairs signed rank test) and a slope factor of 5.5 \( \pm 1.1 \text{ mV per e-fold} \) (not significant). Thus fitting procedures applied to data from slices as well as dissociated cells consistently indicated a more negative \( V_{1/2} \) for the slow as compared with the fast component. The difference in slope factors of the fast component measured in slices versus dissociated neurons might be explained by a higher quality of voltage clamp in the latter preparation.

Dissociated cells were also subjected to tests examining the voltage dependence of inactivation of the slow component of \( Na^+ \) current (\( n = 8 \)). Using a conditioning pulse holding the cell at \(-90 \text{ mV} \), we first adjusted the test level to obtain an isolated slowly inactivating component. This test level ranged from \(-60 \) to \(-45 \text{ mV} \). Figure 8A shows the current responses for a single cell with the use of a fixed test level and variable conditioning pulses. It should be noted that a fast component often appeared at conditioning levels negative to \(-100 \text{ mV} \); in such cases the amplitude of the slow component was estimated by biexponential fitting. The peak amplitude of the current reached a plateau value around \(-100 \text{ mV} \) and gradually decreased with more depolarizing conditioning pulses. However, a slowly inactivating component remained present even at conditioning levels 5 or 10 \text{ mV} below test level. Figure 8B illustrates the current-voltage relationship for the inactivation of this component. The plot of fractional current against voltage was fitted with a Boltzmann equation

\[
I_n = \frac{1}{1 + e^{-\frac{V - V_{1/2}}{S}\frac{1}{2}}}
\]

where \( I_n \) represents the current as a fraction of peak size and \( V \) is the conditioning voltage. The values of \( V_{1/2} \) and \( S \) amounted to \(-78.8 \pm 3.6 \text{ mV} \) and \( 8.5 \pm 1.2 \text{ mV per e-fold} \), respectively.

Even though the inactivation curve suggests that hyperpolarizations below \(-50 \) to \(-65 \text{ mV} \) will partially deinactivate the slow component of \( Na^+ \) current, it is important to take into account the temporal aspects of deinactivation as well. We used a protocol consisting of two depolarizing pulses separated by a hyperpolarizing pulse of variable duration to test its speed of recovery (Dodge 1963; Huguenard et al. 1988). Appreciable recovery started to occur at interpulse intervals of 5–10 ms and was nearly complete at 80–135 ms (Fig. 9). The time constant of recovery amounted to \( 25.6 \pm 4.2 \text{ ms} \). It should be added, however, that a second, slower recovery process may have been present (cf. Huang 1993).

**Light microscopic morphology of SCN neurons displaying slowly inactivating sodium current**

In current-clamp mode, spontaneous or evoked DRs were present in all recorded neurons. Furthermore, a slowly inactivating component of \( Na^+ \) current was found in all neurons examined by voltage clamp. Although this suggests that the slowly inactivating component occurs widely throughout the SCN, it is justified to ask whether we selectively recorded from a morphologically distinct type of neuron while other cell types were overlooked. Biocytin labeling of recorded neurons allowed us to assess which morphologically defined cell types (cf. Van den Pol 1980) displayed the slow component. Figure 10 shows three examples of SCN neurons analyzed with a confocal scanning laser microscope and reconstructed by superimposition of successive optical sections. By matching these three-dimensional reconstructions to electrophysiological records, the slowly inactivating component...
Evidence for a slowly inactivating component of sodium current in SCN neurons

Our results confirm the hypothesis that the DR, which directly precedes spontaneous action potentials, is largely...
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A transient inward current was observed at voltage levels clearly below firing threshold. The transient inward current observed at voltage step commands to -60 to -45 mV most likely represents Na⁺ current, because it was abolished by TTX (Figs. 5-7) or washout of extracellular Na⁺. A contribution by a low-threshold Ca²⁺ current can be considered unlikely because, in most slice experiments, the ACSF was devoid of Ca²⁺, whereas for dissociated cells the medium contained 0.2 mM Cd²⁺. Under these conditions all of the inward current was blocked by TTX, confirming the absence of a contribution by a Ca²⁺ current.

**FIG. 8.** Slowly inactivating component of Na⁺ current in dissociated SCN neurons: voltage dependence of inactivation. A: voltage-clamp protocol for studying inactivation of the slowly inactivating component. Conditioning pulses ranged from -120 to -55 mV (only 5 levels are displayed); test level: -50 mV. Following relatively depolarized conditioning steps, an isolated slowly inactivating component was generated. With strongly hyperpolarizing prepulses a mix of fast and slowly inactivating components was evoked. B: current-voltage plot for the slowly inactivating component as a fraction of peak size. Points negative to -80 mV are peak amplitude estimates obtained from biexponential fits to current responses containing a fast component. Plot was best fitted by a Boltzmann distribution with voltage of half-maximal activation (V_{1/2}) = -68 mV and slope factor (S) = 5.3 mV per e-fold.

**FIG. 9.** Time course of recovery from inactivation of the slowly inactivating component of Na⁺ current in dissociated SCN neurons. A: voltage commands, displayed at top, consisted of a rectangular voltage step from -90 mV to -55 mV for 50 ms, followed by an interval of variable duration (indicated at right of each trace) and a 2nd step to -55 mV for ≥130 ms. Double-pulse current responses uncovered a relatively fast recovery from inactivation, taking ~90 ms to complete in full. B: ratio of the 2nd vs. 1st response peak plotted against pulse interval. Points were fitted by a monoexponential function with a time constant of 24 ms.
of a Ca²⁺ current component. Furthermore, application of Ni²⁺ or Cd²⁺ to slices left DRs recorded in current-clamp mode largely intact, indicating lack of a strong Ca²⁺ current contribution. Similarly, DRs were unaffected on blockade of the H current or GABAₐ-receptor-mediated inhibitory postsynaptic potentials. Finally, although our data argue against the presence of a pronounced and ubiquitous persistent Na⁺ current, a small persistent component was occasionally detected in slice experiments. Although this persistent Na⁺ current is unlikely to mediate the main body of the DR just preceding the spike, it may contribute to the initial, slowly rising phase of the DR.

In slices as well as dissociated cells slowly inactivating, TTX-sensitive inward current was activated at more negative voltage levels than was the fast component of Na⁺ current (Figs. 6 and 7). Although this particular combination of voltage dependence and kinetics by no means proves that the fast and slowly inactivating components of Na⁺ current are mediated by distinct molecular species of ionic channels, the kinetic differences between these components might be explained by different gating modes of Na⁺ channels (Alzheimer et al. 1993; Crill 1996; Moorman et al. 1990). In conclusion, the present results support the hypothesis that the Na⁺ current of SCN neurons contains a slowly inactivating component, the characteristics of which are consistent with and can largely explain the generation of DRs observed in current-clamp mode. Furthermore, biocytin staining revealed that DRs and their underlying Na⁺ current were found in morphologically diverse neurons (Fig. 10) sampled throughout different subregions of SCN. These morphological results underscore the general importance of the slowly inactivating component of Na⁺ current for spontaneous firing in SCN cells that may fulfill distinct functions pertaining to circadian timing mechanisms.

**Functional importance of slowly inactivating component of sodium current for spontaneous firing**

The present study raises a number of arguments supporting the viewpoint that activation of the slowly inactivating component of Na⁺ current strongly promotes spontaneous firing in SCN neurons. The finding that in current-clamp records \( P(DR|\text{spike}) \) was 98.5% on average shows that there are only a few spikes generated that are not in direct succession to a clearly manifested DR. These few spikes may have been triggered by spontaneous excitatory postsynaptic potentials (cf. Jiang et al. 1995). Alternatively, a DR may have been present in these cases, but its shape may then have been obscured by spontaneous inhibitory postsynaptic potentials (Kim and Dudek 1992; Thomson and West 1990) or other spontaneous events. Conversely, our finding that \( P(\text{spike}|DR) \) was 90.1% on average indicates that most DRs will give rise to a spike. Although it is not clear why the remaining 9.9% of DRs do not result in spikes, two possibilities are that (1) these DRs were terminated by spontaneous inhibitory postsynaptic potentials (cf. Kim and Dudek 1992) and (2) the slow component inactivated before spike threshold was reached. It should be noted that in a strict sense these correlative data do not prove that DRs directly cause triggering of action potentials. However, the temporal association between DRs and spikes was very tight and the order of events invariably ran from DR to spike. Further arguments supporting the importance of the slowly inactivating component can be derived from the voltage-clamp experiments. Taken together, these results strongly suggest a causal role of this component in spike initiation.

**Voltage-clamp experiments**

Our first voltage-clamp data suggesting the presence of a slowly inactivating component of Na⁺ current came from slice experiments in which neurons were patched with K⁺-gluconate-filled pipettes. When applying voltage ramp commands at high speed (69 or 77 mV/s), we obtained a current-voltage plot with a negative slope, whereas this slope was absent or reduced at low ramp speeds (6–8 mV/s). The negative slope conductance was shown to be Na⁺ dependent and TTX sensitive. Although these results provided a first indication for a slowly inactivating component of Na⁺ current and showed that this current can be discerned in the presence of functional K⁺ conductances, we should note that neocortical neurons express a persistent Na⁺ current.
(Stafstrom et al. 1985) that also declines in amplitude when ramp speed is reduced (Fleidervish and Gutnick 1996). Attempts to evoke Na\(^+\) plateau potentials in our preparation were generally unsuccessful, suggesting the absence of a predominant persistent Na\(^+\) current in the SCN. Further evidence for the predominance of a slowly inactivating component of Na\(^+\) current was derived from experiments in which voltage step commands were used.

In slice experiments in which neurons were recorded with Cs\(^+\)-gluconate-filled pipettes and subjected to voltage step commands, appropriate attention was paid to the pharmacological isolation of Na\(^+\) current. The reduction of extracellular Na\(^+\) concentration, combined with the pharmacological cocktail, prevented the fast Na\(^+\) current to escape from voltage clamp in a majority of cells. This series of experiments indicated the presence of a fast-activating, slowly inactivating component of Na\(^+\) current and allowed us to segregate this component kinetically from the fast component (Fig. 6).

Despite the indications for good voltage-clamp control achieved in slices, it could still be argued that the kinetics and voltage dependence of Na\(^+\) current may have been distorted by space-clamp problems (cf. White et al. 1995). We therefore supplemented the slice experiments with measurements in dissociated cells. These recordings further confirmed the presence of a distinct TTX-sensitive slowly inactivating component at subthreshold voltage levels. The magnitude and voltage dependence of the decay time constant were similar to those found in slice recordings. On account of this set of recordings, we argue that the appearance of a slowly inactivating component of Na\(^+\) current in SCN neurons cannot be attributed to space-clamp problems. These recordings further show that the channels carrying this component are localized, at least partially, in the somata and proximal dendrites of SCN neurons.

Insights into the functional relevance of the slowly inactivating component of Na\(^+\) current for spontaneous firing behavior can be gained by considering its activation, inactivation, and deinactivation properties (Figs. 6–9). The activation curve suggests that this component can be activated at voltages normally traversed by spontaneously firing cells (−60 to −50 mV; resting membrane potential: −54.9 ± 0.7 mV). Once the slow component has been activated, the neuron will be regeneratively depolarized, because a slight increase of Na\(^+\) current leads to self-amplification. Unless the slow component inactivates prematurely, this regenerative depolarization will eventually lead to spike initiation. Spikes are invariably followed by AHPs that bring the membrane potential down to about −65 to −80 mV. The inactivation curve (Fig. 8) suggests that this level of hyperpolarization is sufficient for partial deinactivation, although the tonic level of membrane potential may also help to achieve this. The time constant (25.6 ± 4.2 ms) extracted from the recovery curve of the slow component (Fig. 9) confirms that spike AHPs, which last ∼50–150 ms, allow partial deinactivation at firing rates normally found in SCN neurons (usually <10 Hz.). In summary, our voltage-clamp data confirm the prominent role of the slowly inactivating component of Na\(^+\) current in the generation of spontaneous firing behavior. An unresolved point is how the DR is initiated in cells with low spontaneous firing rates (Fig. 2B). Even if the resting membrane potential lies slightly negative to the range where the slow component is activated, only one or a few stochastic channel openings may suffice in triggering a DR. In the absence of synaptic inputs or action potentials, a current of 5 pA may depolarize a SCN neuron having an input resistance of 1 GΩ by 5 mV maximally, which can be sufficient to activate the slow component. A related question is whether the slow component, with a decay time constant of ∼16 ms at −50 mV, underlies the full time course of DRs (usually lasting 50–150 ms) or only the main portion directly preceding the spike. We cannot exclude that other currents contribute to the slowly rising, initial phase of DRs. In particular, small persistent Na\(^+\) current may be considered to play a role in this phase.

It is interesting to compare the slowly inactivating component of Na\(^+\) current in SCN with Na\(^+\) conductances identified in other brain structures. MacVicar (1985) described Na\(^+\)-dependent spike prepotentials in CA1 pyramidal neurons. Furthermore, TTX has been reported to block a persistent Na\(^+\) current in layer V neurons of sensorimotor cortex (Stafstrom et al. 1985), layer II neurons of medial entorhinal cortex (Alonso and Linías 1989; Klink and Alonso 1993), cerebellar Purkinje cells (Linías and Sugimori 1980), hippocampal cells (French et al. 1990), and some other brain structures (reviewed by Crill 1996). The voltage range showing activation of the slow Na\(^+\) component in SCN roughly matches that reported previously for other CNS structures (Crill 1996; Stafstrom et al. 1985; Uteshev et al. 1995). However, the component of Na\(^+\) current described here differs from this persistent Na\(^+\) current in that it shows pronounced inactivation (Figs. 6 and 7). Previously, slowly inactivating components of Na\(^+\) current were also reported in stellate cells of entorhinal cortex (Alonso and Linías 1989) and neurons of the tuberomammillary nucleus (Uteshev et al. 1995), but inactivation proceeded much more slowly than observed here. In addition to the persistent Na\(^+\) current expressed in neocortical and hippocampal pyramidal cells (Brown et al. 1994; Crill 1996; French et al. 1990; Stafstrom et al. 1985), acutely dissociated pyramidal cells of mature sensorimotor cortex have also been reported (Huguenard et al. 1988) to display a Na\(^+\) current component with similar decay time constants as shown here as well as in a study on dissociated neostriatal neurons (Ogata and Tagebayashi 1990). Thus the slowly inactivating component of Na\(^+\) current in SCN may not be unique in its kinetic characteristics despite the differences with persistent Na\(^+\) current in neocortex and hippocampus. Finally, we note that Huang (1993) recorded a fast component of Na\(^+\) current in acutely dissociated SCN neurons displaying a similar decay time constant as shown here, but did not identify the slowly inactivating component for reasons unclear at present.

**Dynamic interplay of slowly inactivating component of sodium current with other ionic conductances**

As our current knowledge of ionic conductances in SCN is advancing, it is becoming more clear how these conductances interact to produce spontaneous firing patterns. Beginning with the onset of an action potential marked by fast Na\(^+\) current activation, the fast delayed rectifying K\(^+\) current is probably activated (Bouskila and Dudek 1995; Walsh et
al. 1995). The subsequent spike AHP not only renders the membrane refractory but is also likely to cause partial de-inactivation of the slowly inactivating component of Na⁺ current. Following the AHP, the membrane potential may either remain at resting level for an undefined period of time (Fig. 2B) or move directly into the range where the slow component is activated (Fig. 2A). Besides a small persistent Na⁺ current, the low-threshold Ca²⁺ current may play a role particularly in the initiation of DRs (Akasu et al. 1993; Huang 1993; Kim and Dudek 1993), although our results with Ni²⁺ and Cd²⁺ suggest that such a role, if any, would be modest. Among the other known voltage-dependent ionic currents in SCN, the transient K⁺ current (A current) (Bouskila and Dudek 1995; Walsh et al. 1995) may prolong spike intervals (cf. Connor and Stevens 1971) or may delimit spike duration. The H (or Q) current has been suggested to shorten spike intervals by time-dependent inward rectification in SCN neurons (Akasu et al. 1993). However, in our own lab we have not been able to confirm a role for the H current in promoting spontaneous firing in SCN (De Jou and Pennartz 1997).

A particular challenge for future research will be to study ionic channel behavior mediating the fast and slowly inactivating component of Na⁺ current and the electrophysiological events that are able to trigger DRs from rest. Furthermore, it will be of great interest to test whether the slowly inactivating component of Na⁺ current is targeted by neuropeptides or classical neurotransmitters. Finally, examination of daytime modulation of this component and other functionally important conductances will offer insights as to how the putative molecular output of the biological clock (cf. Dunlap 1993; Takahashi 1995, 1996; Welsh et al. 1995) is converted into a circadian rhythm in firing activity.

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