Riluzole-Sensitive Slowly Inactivating Sodium Current in Rat Suprachiasmatic Nucleus Neurons

Nikolai I. Kononenko, Li-Rong Shao, and F. Edward Dudek

Department of Biomedical Sciences, Anatomy and Neurobiology Section, Colorado State University, Fort Collins, Colorado 80523

Submitted 7 August 2003; accepted in final form 9 October 2003.

Kononenko, Nikolai I., Li-Rong Shao, and F. Edward Dudek. Riluzole-sensitive slowly inactivating sodium current in rat suprachiasmatic nucleus neurons. J Neurophysiol 91: 710–718, 2004. First published October 22, 2003; 10.1152/jn.00770.2003. The persistent (i.e., slowly inactivating) fraction of the Na current (I_{Na,s}) regulates excitability of CNS neurons. In isolated rat suprachiasmatic nucleus (SCN) neurons with a ramp-type voltage-clamp protocol, we have studied the properties of a robust current that has the general properties of I_{Na,p} but exhibits a slow inactivation (I_{Na,s}). The time dependence of the development of the inactivation was also studied by clamping of the membrane potential at different levels: time constants ranging from ~50 to ~700 ms, depending on the voltage level, were revealed. The I_{Na,s} (50–150 pA) was present in both spontaneously active and silent neurons. The neurons exhibited I_{Na,s} without visible rundown during ~1-h recordings. I_{Na,s} had a threshold between ~65 and ~60 mV and was maximal at about ~45 mV. Tetrodotoxin (TTX; 1 µM) completely and reversibly blocked I_{Na,s}. Riluzole, an effective blocker of I_{Na,p}, inhibited reversibly I_{Na,s} with an EC_{50} of 1–2 µM. Microapplication of 10 µM riluzole during either extracellular or intracellular recording suppressed spontaneous activity in isolated SCN neurons. In the slice preparation, bath application of 20 µM riluzole resulted in decreased firing rate or complete suppression of spontaneous activity in some neurons (9/14) but had no effect on other neurons (5/14). In riluzole-resistant neurons in cell-attached experiments, low-amplitude current spikes were present in 1 µM TTX. We concluded that I_{Na,s} is ubiquitously expressed by all SCN neurons and that this current is a necessary but not sufficient depolarizing component of the mechanism for spontaneous firing.

INTRODUCTION

The suprachiasmatic nucleus (SCN) of the hypothalamus serves as a circadian master clock in mammals (Gillette and Tischkau 1999). Spontaneous firing of SCN neurons is under circadian modulation, and the ionic mechanisms underlying the spontaneous activity of SCN neurons are, however, not well understood. Circadian oscillations of electrical activity are present at the cell level even for completely isolated SCN cells (Honma et al. 1998; Welsh et al. 1995). The well-known role of the fast Na^+ current is to generate and propagate action potentials. Many CNS neurons have been found to possess slowly inactivating or “persistent” Na^+ current, I_{Na,p} (Alzheimer et al. 1993; Bevan and Wilson 1999; Chao and Alzheimer 1995; Del Negro et al. 2002a,b; Hammarstrom and Gage 1999; Kay et al. 1998; Magistretti et al. 1999; Schwindt and Crill 1977; Taddese and Bean 2002; Urban and Belluzzi 2000; Uteshev et al. 1995; for review, see Crill 1996; Llinas 1988). Activation of I_{Na,p} is in the subthreshold region, and its slowly inactivating or persistent nature suggests that it participates in spontaneous firing of central neurons by contributing to their resting potential and threshold. The question to what extent I_{Na,p} undergoes inactivation is still open, although a biophysical analysis of this issue has been done for, at least, layer-II principal neurons of entorhinal cortex (Magistretti and Alonso 1999). SCN neurons are spontaneously active, and it has been suggested that the slowly inactivating component of Na^+ current contributes to their spontaneous activity (Pennartz et al. 1997).

The goals of the present investigation were to examine to what extent slowly inactivating Na^+ current in SCN neurons (Pennartz et al. 1997) reflects the properties of I_{Na,p} and to estimate its contribution to spontaneous firing of SCN neurons. In isolated rat SCN neurons, we studied the properties of this Na^+ current (termed here I_{Na,s}), which shares the properties of I_{Na,p} except that it has a relatively slow inactivation (~50–250 ms at ~45 mV that corresponds to peak of I_{Na,s}). Using riluzole, an effective blocker of regular I_{Na,p} and I_{Na,s} in SCN neurons, we have estimated the putative contribution of I_{Na,s} to spontaneous activity of both isolated SCN neurons and these cells in the slice preparation.

METHODS

Slice preparation

Male adult Harlan Sprague-Dawley rats, 4–6 wk old, were used for all experiments. All protocols were approved by the Animal Care and Use Committee at Colorado State University. Rats were deeply anesthetized with halothane and killed by decapitation. One coronal hypothalamic slice (350 µm) containing both SCNs was prepared using a Vibratome (Lancer) in ice-cold artificial cerebrospinal fluid (ASCF) containing (in mM) 124 NaCl, 3 KCl, 2.5 CaCl_2, 2 MgSO_4, 1.25 NaH_2PO_4, 26 NaHCO_3, and 10 d-glucose, bubbled with 95% O_2-5% CO_2 to a final pH of 7.4, osmolarity 295–300 mosM. The slice was made during the day at 09:00–10:00 for recordings at 13:00–16:00. After preparation, the slice was placed in an experimental chamber mounted on the stage of an upright microscope (Axioskop 2, Zeiss) and allowed ~2 h to recover. Individual SCN neurons could be clearly distinguished using a water-immersion objective (Olympus ×40) with Nomarski differential interference contrast optics and a cooled charge-coupled device camera (Hamamatsu, Japan). During recording and recording, the slice was superfused at 1.5–2.5 ml/min (34–35°C). Recordings were made from the central region of the SCN. When GABA (100 µM) or TTX (1 µM) were bath applied to evaluate the time required to exchange solutions, complete equilibration of new solutions in the experimental chamber was achieved in 3 min.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked ‘‘advertisement’’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Isolated neurons

For preparation of isolated SCN neurons, the slice was placed in protease K (Sigma, St. Louis MO) in 0.2 mg/ml in PIPES buffer (in mM: 115 NaCl, 5 KCl, 20 PIPES free acid, 1 CaCl₂, 4 MgCl₂, and 25 d-glucose, pH 7.0; aerated with 100% O₂) at 30 °C for 5 min, rinsed in PIPES buffer, and placed in trypsin (Sigma Type XI: 1 mg/ml) in PIPES buffer at 30 °C for about 30 min. The slice was then rinsed four to five times in PIPES buffer and two regions from both SCNs (~500 × 500 μm) were dissected. Neurons were then isolated from each SCN separately by trituration with flame-polished Pasteur pipettes in ice-cold PIPES buffer containing 0.1% DNase. The resulting solution was diluted 1:1 with Neurobasal-A/B-27 (Gibco, Grand Island, NY). Cells were plated on charged plastic culture dishes, incubated at 37 °C (95% O₂ -5% CO₂) for 30 min to allow adherence, rinsed, and covered with Neurobasal A/B-27. Neurons were incubated ≥72 h prior to electrophysiological recordings that were conducted during the subsequent 5 days. The culture dish was mounted on the stage of an inverted microscope (Eclipse TE300, Nikon) and allowed to recover. Individual SCN neurons (9–11 μm) could be clearly visualized using a phase contrast objective (Nikon ×4 and ×40).

Electrophysiological recording

Cell-attached recordings of spontaneous electrical activity from SCN neurons in the slice preparation were obtained at 36 °C with a MultiClamp 700A amplifier (Axon Instruments, cutoff 2 kHz), sampled at 10 kHz, and analyzed with pClamp8 software (Axon Instrument). Micropipettes were fabricated from capillary glass (Garner Glass, Claremont, CA:1.2 mm ID, 1.65 mm OD) and filled with solution containing (in mM) 135 NaCl, 10 EGTA, 1 EDTA, and 10 HEPES, (NaOH to pH 7.4 produced [Na⁺] of ~160 mM). The osmolarity was 290–295 mosM, and the electrodes had a resistance of 4–5 MΩ. The extracellular electrical activity of two SCN neurons was usually recorded simultaneously.

Cell-attached and whole cell recordings from isolated SCN neurons were obtained at room temperature (20–21 °C) with an Axopatch-1D patch-clamp amplifier (cutoff 2 kHz), sampled at 10 kHz, digitally filtered at 0.5 kHz for ramp recording, and analyzed with pClamp8 software. Micropipettes were filled with solution containing (in mM) 120 K⁺-gluconate, 10 HEPES, 1 NaCl, 1 MgCl₂, 1 CaCl₂, 3 KOH (to pH 7.2–7.4), 5 EGTA, and 2 Na₂ATP. Sucrose was added to bring the osmolarity to 300 mosM, and the electrodes had a resistance of 7–8 MΩ. These micropipettes were also used for extracellular cell-attached recording of spontaneous activity of isolated neurons before rupture of the patch membrane. The chamber was superfused with ACSF at 1.5–2.5 ml/min. Offset potential was zeroed just before the electrode contacted the neuronal membrane. All voltage measurements were corrected offline for the liquid junction potential, which amounted to ~15 mV for the K-glutonate-filled micropipette (Neher 1992). The access resistance did not exceed a value of 15 MΩ (typically was ~6–12 MΩ) and was not compensated because maximal whole cell current was <150 pA, and thus the voltage error was <2 mV.

Drugs and puff application

The TTX (Sigma), was used as a 1-mM stock solution in distilled water and then diluted to its final concentration in ACSF before the experiment. Riluzole (Sigma) was prepared at 100 mM in DMSO and then diluted to appropriate concentration in ACSF. For puff application of drugs (TTX- or riluzole-containing ACSF) during ACSF perfusion, a plastic tube (160 μm ID) was located near the SCN neurons (200–250 μm) and gravity pressure was used (1 drop/2 s) to apply the drugs. Tests with 1 μM TTX have shown that complete block of fast Na⁺ current was achieved within 2 s after start of the puff application.

Data analysis

Firing frequency of spontaneously active SCN neurons in the slice preparation, which was averaged for 10 s, was calculated using pClamp version 6.0 (FETCHAN and pSTAT) and plotted with Sigmaplot 2000 version 6.0. Input resistance (Rᵢ) was determined via linear regression applied to the linear portion of the ramp current-voltage (I-V) relationship generated by a slow voltage ramp (100 mV/s; from ~85 to ~65 mV). In some figures, the linear leak current (Iₗ), was used to extract Iₗ₂Naₐ by subtracting Iₗ-off-line from the total current. In some figures, Iₗ₂Naₐ was low-pass-filtered (50 Hz; Clampfit 8.1). Values are given as means ± SE.

RESULTS

Spontaneous activity of isolated neurons

Most isolated SCN neurons (~90% from >100 recorded) exhibited spontaneous activity when recorded after 3 days of incubation. An irregular bursting or regular firing pattern was observed or the SCN neurons were silent (~10%) with episodic action potentials (<1 action potential per min, Fig. 1). By using long-term multielectrode-dish recordings, similar patterns of spontaneous firing have been recorded in isolated and cultured SCN neurons (Honma et al. 1998; Liu and Reppert 2000; Shirakawa et al. 2000; Welsh et al. 1995; see also Walsh et al. 1992). Irregular or bursting activity has been considered a transitional pattern between silence and high-frequency activity during diurnal rhythms (Welsh et al. 1995). In our experiments, the pattern of electrical firing often remained after rupture of the patch membrane (see, for example, Fig. 4, A and B). Voltage-clamp recordings in whole cell mode were obtained from 96 isolated SCN neurons. Recordings with unclamped action potentials were discarded. The neuronal population studied here had a firing rate of 1.2 ± 0.6 Hz (n = 32 neurons; averaging for 5–10 min of recording in cell-attached mode just after seal formation), and input resistance in its

FIG. 1. Extracellularly recorded spontaneous activity of an isolated suprachiasmatic nucleus (SCN) neuron. Recordings were done 3 days after isolation of the SCN neurons at 11:00 to 13:00 in cell-attached mode and obtained within 2 min after gigaseal formation. A: silent neuron; B: neuron with irregular activity; C: burst-firing neuron; D: neuron with regular activity. Current-spike amplitude during voltage-clamp recording ranged between 30 and 80 pA.
linear region between −85 and −65 mV was 3.14 ± 0.34 GΩ (n = 9 neurons).

Slowly inactivating sodium current

To isolate and study the properties of \(I_{Na,S}\), we did not eliminate other ionic currents because we have focused on the contribution of \(I_{Na,S}\) to the electrical properties of SCN neurons during normal electrical activity with standard ionic media. Experiments were first performed on isolated SCN neurons exhibiting spontaneous activity in which the presence of a persistent or slowly inactivating \(Na^+\) current was expected. The protocol used to evoke \(I_{Na,S}\) was to ramp from a holding potential of −85 mV at a rate of 100 mV/s to different values between −35 and −15 mV and then to ramp backward to the original holding potential. The ascending phase of the voltage-clamp command was used to measure the quasi-steady-state \(I-V\) relationship (considered in the following text to be the \(I-V\) relationship). The 100 mV/s rate was chosen as a compromise because a faster rate evoked in most neurons an obvious unclamped action potential-generating \(Na^+\) current (see for example, Del Negro et al. 2002a, Fig. 4), and a lower rate resulted in a decrease of \(I_{Na,S}\) amplitude because of its inactivation, which was studied in detail in the experiments described in the following text (Fig. 3, B and C).

All of the isolated SCN neurons in this study showed a nonmonotonic N-shaped \(I-V\) relationship (e.g., Fig. 2, A and B) with the negative slope region above approximately −65 mV, suggesting the presence of noninactivating or slowly inactivating inward current in these neurons. We observed no inactivation of this inward current after >40 repetitive ramps (1 ramp/2.2 s; \(n = 12\) neurons), and no visible rundown during ~1-h recordings. Figure 2A shows the slow inward current that was completely and reversibly suppressed by the fast \(Na^+\) channel blocker, TTX, \((n = 15)\) neurons. In different neurons, the TTX-sensitive slow inward current had an amplitude of 50–150 pA (95.9 ± 5.5 pA; \(n = 33\) neurons). Interestingly, the slow inward current was always accompanied by a substantial increase in current noise relative to the baseline between −85 and −65 mV (and compare with the current in the presence of TTX). This suggests that the opening of a relatively high-conductance channel generates the slow inward current (Magistretti et al. 1999). Riluzole, a neuroprotective agent with anticonvulsant properties, is an effective blocker of \(I_{Na,P}\) with \(EC_{50}\) 0.5–3 μM in mammalian neurons (Del Negro et al. 2002b; Spadoni et al. 2002; Urbani and Belluzzi 2000), and we tested the effect of 0.1–100 μM riluzole on the slow inward current, \(I_{Na,S}\), in isolated SCN neurons. At a dose of ≥25 μM, riluzole completely and reversibly (Fig. 2B) inhibited the slow inward current and the accompanying current noise (\(n = 17\) neurons). To further characterize the observed inhibition of the slow inward current, a dose-response curve was constructed. The \(EC_{50}\) of riluzole-induced inhibition was between 1 and 2 μM (Fig. 2C). Thus \(I_{Na,S}\) in SCN neurons was directed inward between −65 and −30 mV, blocked by TTX and riluzole at appropriate concentrations, exhibited threshold properties and substantial noise typical for \(I_{Na,P}\), and thus \(I_{Na,S}\) possessed the main features of \(I_{Na,P}\) documented on other neurons.

Properties of \(I_{Na,S}\)

To assess the role of \(I_{Na,S}\) recorded during depolarizing ramps in the properties of spontaneously firing neurons, we studied \(I_{Na,S}\) in voltage-clamp experiments that were conducted just before and after recording of spontaneous activity of the same SCN neuron in current-clamp mode. These two types of data were plotted on the same voltage axis, and one could see that action potential threshold and the development of \(I_{Na,S}\) were in register during the depolarizing ramp between spontaneously occurring action potentials (Fig. 3A; \(n = 6\) neurons).

From experiments on other neurons, the amplitude of \(I_{Na,P}\) depends on the speed of the voltage ramp (Agrawal et al. 2001; Del Negro et al. 2002a). To characterize \(I_{Na,S}\) in SCN neurons and estimate the contribution of this current to the depolarizing ramp under current-clamp conditions, we studied the dependence of \(I_{Na,S}\) on ramp speed. The multispeed voltage-ramp protocol revealed that slower ramp speeds progressively attenuated the amplitude of the slow inward current (Fig. 3B), reflecting the slow inactivation kinetics of \(I_{Na,S}\) (similar to \(I_{Na,P}\) in other neurons) (Agrawal et al. 2001; Del Negro et al. 2002a; Fleidervish and Gutnick 1996). The \(I_{Na,S}\) amplitude, measured at the peak of the \(I-V\) relationship (Fig. 3B) and plotted as a function of the inverse of the ramp slope (Fig. 3C), demonstrated a decay that approximated a single exponential. Magistretti and Alonso (1999) described a biexponential decay, but we observed a monoexponential decay in all experiments with the clear absence of partially

---

**FIG. 2.** Slowly inactivating \(Na^+\) current recorded by whole cell voltage clamp in acutely dissociated SCN neurons. A: effect of 1 μM TTX on \(I-V\) relationship of neuronal membrane determined by applying a 100-mV/s ramp depolarization from −85 to −35 mV. Ramps were applied repetitively (each 2.2 s) and a 3-s TTX puff application on the background of constant ACSF flow was delivered after the 3rd ramp (control). The 6th ramp shows complete suppression of \(I_{Na,S}\) by TTX and the 23rd ramp shows almost complete restoration. B: effect of 25 μM riluzole on \(I_{Na,S}\). Details of experiment are similar to those presented in A. C: dose response of riluzole on \(I_{Na,S}\). Inset: leak-subtracted, low-pass-filtered \(I_{Na,S}\) recorded before and after 5 μM riluzole application. Data points are given as means ± SE, \(n\) within brackets.

*J Neurophysiol.* VOL 91 • FEBRUARY 2004 • www.jn.org
Besides, we observed hysteresis of \( I_{Na,S} \) acutely isolated neurons. Considering the dependence because of the complete absence of dendrites in these neurons at conditions when outward \( K \) current above \(-45 \) mV obviously takes place. Indeed, we observed small outward current during TTX (Fig. 2A) or riluzole (Fig. 2B) applications in this region of membrane potential, with the same voltage scale (abscissa). Dashed lines, 0 current and 0 voltage levels. The landmark of \( I_{Na,S} \) was recorded just before switching off voltage clamp. The plot could be fitted by a single exponential function \( I = I_0 + I_{max} \exp (-s/V) \), where \( s \) is the ramp slope and \( s_0 \) is the slope constant with \( I_0 = 30 \) pA, \( I_{max} = 401 \) pA, and \( s_0 = 144 \) mV/s (smooth line). The relation between the voltage dependence of \( I_{Na,S} \) and the depolarizing ramp that occurs during the interspike interval in spontaneously firing SCN neuron. \( I_{Na,S} \) was recorded just before switching off voltage clamp. The time constant of inactivation of the main component of \( I_{Na,S} \) depended on the holding potential and decreased with depolarization (Fig. 3F, \( n = 5 \) neurons), varying between 50 and 700 ms. The data from the latter experiment probably due to inactivation of \( I_{Na,S} \). Furthermore, comparing the basic slope constant for cortical neurons (~20 mV/s in Magistretti and Alonso 1999) and for SCN neurons (137 mV/s, Fig. 3C), we should expect inactivation of \( I_{Na,S} \) to be there present.

To address this issue directly, membrane currents were recorded when the ascending voltage-ramp command was varied with depolarization and then holding potential. A noninactivating component ranged between 4 and 20% (8.5 \( \pm \) 2.3 pA; \( n = 6 \) neurons) of maximal value of \( I_{Na,S} \), and it possibly represented the actual "persistent" \( Na^+ \) current in SCN neurons. The time course of inactivation of \( I_{Na,S} \) could be fitted by a monoexponential function. The time constant of inactivation of the main component of \( I_{Na,S} \) depended on the holding potential and decreased with depolarization (Fig. 3F, \( n = 5 \) neurons), varying between 50 and 700 ms. The data from the latter experiment suggest that inactivation was partially responsible for the sharp decrease in \( I_{Na,S} \) amplitude after the maximal value was achieved (Figs. 2, A and B, and 3, A, B, and D).
From the experiments described above (Fig. 3A), $I_{Na,S}$ should be an essential component of the mechanism for spontaneous activity of SCN neurons. In light of this, it seemed interesting to determine whether silent neurons exhibit this current or whether it is an attribute of spontaneously active neurons only. Five silent SCN neurons were studied, and representative results are shown in Fig. 4. To determine whether a neuron was actually silent, its extracellular activity in cell-attached mode was first recorded (Fig. 4A). In whole cell mode, these neurons had a resting potential of approximately $-80$ mV, and depolarizing pulses of appropriate amplitude in current clamp evoked action potentials (Fig. 4B). In all silent neurons studied, the regular ramp protocol revealed an $I_{Na,S}$ (Fig. 4C) that exhibited all of the properties of $I_{Na,S}$ in spontaneously active neurons (not shown here), including riluzole sensitivity (Fig. 4D). However, in contrast with spontaneously active cells, the $I-V$ relationship of silent neurons intersected the zero-current axis in the subthreshold region (Fig. 4C), and the potential of intersection corresponded as expected to the resting potential of the neuron recorded in current-clamp mode (Fig. 4B). Thus $I_{Na,S}$ was present in silent neurons, but it alone was not sufficient to produce net inward current on the total $I-V$ relationship of SCN neurons in the subthreshold region (Fig. 4C).

**Effect of riluzole on isolated neurons**

Riluzole has been used previously to estimate the contribution of $I_{Na,P}$ to the generation of the respiratory rhythm in mammals (Del Negro et al. 2002b; Koizumi and Smith 2002). To estimate the contribution of $I_{Na,S}$ to spontaneous activity of isolated SCN neurons, puff applications of $5-100$ µM riluzole-containing artificial cerebrospinal fluid (ACSF) were delivered to the recorded neurons. Because the spontaneous activity of isolated SCN neurons was mostly irregular, an application of $1-3$ s was delivered in a repetitive manner at equal intervals ($2-10$ min) that depended on the recovery from riluzole after the first application. The time required for washout appeared to reflect primarily the location of the neuron in the dish and the variability in the flow of ACSF rather than the individual properties of different SCN neurons, although this issue was not actually studied. Figure 5A shows representative traces that demonstrate suppression of...
neuron firing in cell-attached mode after application of 10 μM riluzole. This concentration of drug produced ~75%-inhibition of $I_{Na,S}$ in SCN neurons (Fig. 2C) and a ~10 mV shift of steady-state inactivation of fast Na⁺ current (Urbani and Bellozzi 2000). The presence of single spikes on the background of suppression of firing suggests that the main target for riluzole is $I_{Na,S}$ and not a steady-state inactivation of fast Na⁺ current. Whole cell experiments ($n = 5$ neurons) showed that the suppressing effect of riluzole on spontaneous firing was due predominantly to hyperpolarization, which obviously was associated with inhibition of $I_{Na,S}$ (Fig. 5B). In all cases ($n = 16$ neurons), application of riluzole resulted in transient suppression of spontaneous activity in both cell-attached and whole cell recordings. In most neurons, application of drug did not exert any effect on the parameters of the action potential (amplitude and duration), but in some cases (5 of 22 applications of riluzole in whole cell recording), riluzole decreased action-potential amplitude (compare, for example, Fig. 5B, top vs. bottom). This effect was considered to be due to the deteriorating state of the neuron after the long-lasting recording (see DISCUSSION for details). We did not find any effect of riluzole application on the linear portion of leakage current in any of the neurons studied or on resting potential of silent SCN neurons in whole cell experiments ($n = 4$ neurons).

**Effect of riluzole on SCN neurons in slice**

To estimate the contribution of $I_{Na,S}$ to spontaneous activity of intact SCN neurons, bath application of 20 μM riluzole on electrical firing of neurons was studied in the slice preparation. Recordings were usually from two neurons simultaneously. From 14 neurons tested, riluzole partially or completely suppressed activity of nine cells and exerted a weak effect or no effect on five cells. Representative effects of riluzole on the firing frequency of two neurons is presented in Fig. 6A. The effect of riluzole was reversible within 30 min after replacement of solution. Analysis of the data suggests that if 4 riluzole-resistant neurons (Fig. 6B, ○) were excluded, the suppressing effect of riluzole was inversely correlated to the average firing rate (Fig. 6B, $P < 0.001$). We hypothesized that riluzole-resistant electrical firing and TTX-resistant membrane-potential oscillations (Kononenko and Dudek 2002; Pennartz et al. 2002; N. I. Kononenko, unpublished results) are manifestations of the same mechanism. Thus SCN neurons, whose activity was completely or partially riluzole-resistant, would thus be expected to exhibit TTX-resistant electrical oscillations. In these neurons, riluzole- and TTX-resistant mechanisms hypothetically operate together with $I_{Na,S}$ to generate spontaneous electrical activity. To test this hypothesis, we studied the effect of 1 μM TTX on the electrical activity of neurons whose activity was not affected or was inhibited by no more than 50% by riluzole. Indeed, five of five tested riluzole-resistant SCN neurons (if activity was recorded simultaneously from 2 cells, riluzole-sensitive cells were excluded) exhibited TTX-resistant electrical oscillations (Fig. 6C).
DISCUSSION

This is the first report showing that a slowly inactivating Na\(^+\) current (I\(_{Na,S}\)) participates in the spontaneous firing of SCN neurons. Furthermore, the anticonvulsant riluzole, which is used in the treatment of epilepsy, produces a dose-dependent inhibition of I\(_{Na,S}\) in isolated SCN neurons and a suppression of spontaneous firing in both isolated neurons and slices.

Comparison with other currents

As described in the present work, I\(_{Na,S}\) shares virtually all of the properties of the well-known I\(_{Na,P}\) in other mammalian neurons. These properties include 1) activation threshold using a ramp protocol (30–120 mV/s) at −65 to −60 mV, and a peak at approximately −45 mV, 2) TTX sensitivity (Agrawal et al. 2001; Alzheimer et al. 1993; Chao and Alzheimer 1995; Del Negro et al. 2002a,b; Fleidervish and Gutnick 1996; Magistretti and Alonso 1999; Taddese and Bean 2002; Urbani and Belluzzi 2000), 3) suppression by riluzole with EC\(_{50}\) 0.5–3 \(\mu\)M (Del Negro et al. 2002b; Spadoni et al. 2002; Urbani and Belluzzi 2000), and 4) a substantial increase in current accompanying the current activation (Agrawal et al. 2001; Chao and Alzheimer 1995; Del Negro et al. 2002b; Rybak et al. 2003; Spadoni et al. 2002). The only apparent difference between the current studied in our present experiments and I\(_{Na,P}\) is its inactivation. This inactivation was most clearly expressed when I\(_{Na,S}\) achieved its maximum with ascending ramp commands and then membrane potential was clamped at a constant level (Fig. 3E). As was expected from comparing slope constants of the current-ramp slope dependences for cortical (Magistretti and Alonso 1999) and SCN neurons (Fig. 3C), direct measurement of time constants of inactivation yielded approximately sevenfold faster value for SCN neurons (50–700 ms) against 3.4–6.8 s for cortical neurons. Similar if not identical to I\(_{Na,S}\), the slowly inactivating component of Na\(^+\) current has been observed in SCN neurons (Pennartz et al. 1997) and cerebellar Purkinje cells (Kay et al. 1998) using a step-command protocol.

Properties of I\(_{Na,S}\)

Our attention focused on the electrical properties that could be directly connected with the spontaneous firing activity of SCN neurons. The threshold for I\(_{Na,S}\) corresponds to the pacemaker potential in spontaneously active SCN neurons. During a current-clamp recording of spontaneous activity, inactivation of K\(^+\) current and the corresponding decrease of the hyperpolarizing afterpotential depolarizes the membrane potential and achieves threshold for I\(_{Na,S}\) (Fig. 3A). Estimation of even the minimal voltage speed during the depolarizing ramp in a spontaneously active neuron (i.e., in the middle of the interspike interval) yields a value of ~60 mV/s, which should produce an I\(_{Na,S}\) that is ~65 pA (Fig. 3C). The voltage speed during the hyperpolarizing afterpotential and the corresponding I\(_{Na,S}\) are much larger. Such inward current is sufficient to evoke an appropriate depolarizing driving force in SCN neurons whose linear input resistance in the slice preparation is \(\approx 1\) G\(\Omega\) (Pennartz et al. 1997). Thus our recordings of I\(_{Na,S}\) under voltage-clamp conditions reveal its characteristics at the relevant physiological conditions during current-clamp recordings.

Like I\(_{Na,P}\) in other mammalian neurons, the amplitude of I\(_{Na,S}\) in SCN cells exhibited dependence on ramp-speed. Agrawal et al. (2001) and Del Negro et al. (2002a) considered the ramp-speed dependence for I\(_{Na,P}\) as an indirect measure of inactivation. In our experiments, ramp-speed dependence of I\(_{Na,S}\) was described perfectly by a single exponential (Fig. 3C), whereas Magistretti and Alonso (1999) reported biexponential decay: they suggested that the existence of a faster exponential component may be due to the presence of Na\(^+\) current components that are kinetically intermediate between fast Na\(^+\) current and the persistent Na\(^+\) current. Our precaution of discarding recordings with unclamped action-potential-generating fast Na\(^+\) current possibly excluded the faster component from our analysis. Alternatively, different neuronal populations may express different forms of slowly inactivating Na currents.

A defining property of I\(_{Na,S}\) was the hysteresis observed at the ascending and descending components of the ramp protocol (Fig. 3D). The simplest interpretation of this phenomenon, consistent with the ramp-speed dependence of I\(_{Na,S}\) (Fig. 3, B and C), is that the hysteresis reflects inactivation of current at membrane depolarization. Indeed, clamping of the membrane potential at the peak of I\(_{Na,S}\) revealed its inactivation (Fig. 3E) with a time constant (\(\tau_i\)) of 50–250 ms at its maximum. Clamping before the maximum current (i.e., at more negative membrane potentials) produced an increase of \(\tau_i\) in all neurons studied (Fig. 3, F and E). It is still uncertain whether the increase of \(\tau_i\) in this case is due to hyperpolarization of the membrane or to the decreased amplitude of I\(_{Na,S}\) itself, and further studies using a voltage-step protocol are needed. Overall, our data are consistent with the interpretation that I\(_{Na,S}\) undergoes inactivation, and its decrease after reaching the maximum (i.e., at approximately −45 mV) is due to inactivation rather than a decrease in electrical driving force. Previously, the time constant of inactivation for slowly inactivating Na\(^+\) current in SCN neurons was reported as ~10 ms (Pennartz et al. 1997), which is much faster than in our experiments (50–250 ms). One explanation could be an additional fast Na\(^+\) current component in SCN neurons, as suggested by Magistretti and Alonso (1999). Alternatively, Pennartz et al. (1997) used a step protocol for the recording of slowly inactivating Na\(^+\) current, and contamination of total current produced by fast Na\(^+\) current could not be excluded. Based on our experiments, we hypothesize a physiological role of inactivation of I\(_{Na,S}\) during sustained electrical firing of SCN neurons: after achievement of threshold for action-potential generation, inactivation avoids large (tens or hundreds of pA) depolarizing current that could prevent complete repolarization of the membrane; this would be sufficient to deinactivate the fast Na\(^+\) current for subsequent action-potential generation.

Previous attempts to find a correlation between the pattern of electrical activity and the properties of I\(_{Na,P}\) have been undertaken in the pre-Bötzinger complex inspiratory neurons (Del Negro et al. 2002a). In light of this, it was interesting to determine whether spontaneously active and silent SCN neurons express different levels of I\(_{Na,P}\). Is expression of I\(_{Na,P}\) sufficient for pacemaker activity? Our experiments did not reveal any significant differences in the properties of I\(_{Na,P}\) between spontaneously active and silent neurons (Fig. 4C). Also, we did not find any significant differences in input resistance between spontaneously active and silent neurons (not shown here). For silent neurons, only an upward parallel
shift of the $I-V$ relationship was observed, with a corresponding intersection of the $I-V$ relationship with the zero-current axis in the subthreshold region. Thus to explain this observation, one can hypothesize that an additional depolarizing force besides $I_{Na,S}$ needs to be expressed in spontaneously active SCN neurons.

**Effect of riluzole on spontaneous firing**

The blockage of $I_{Na,P}$ by riluzole has successfully been used to estimate directly the participation of $I_{Na,P}$ in the generation of the respiratory rhythm in mammals (Del Negro et al. 2002b; Koizumi and Smith 2002). Although riluzole at a concentration that is sufficient to significantly suppress $I_{Na,P}$ (~10 μM) slightly shifts the steady-state inactivation of fast transient sodium current (Urbani and Belluzzi 2000), it did not exert a visible effect on the amplitude of action potentials (Del Negro et al. 2002b). In our experiments, puff application of riluzole (≥5 μM) resulted in hyperpolarization of the membrane and a transient suppression of spontaneous activity in all neurons studied (Fig. 5). Together with this observation, riluzole did visible effect on the amplitude of action potentials (Del Negro et al. 2002b). In our experiments, puff application of riluzole (≥5 μM) resulted in hyperpolarization of the membrane and a transient suppression of spontaneous activity in all neurons studied (Fig. 5). Together with this observation, riluzole did not hyperpolarize the membrane of silent neurons. This is in accordance with the suggestion that the target for riluzole is the $I_{Na,S}$ and the threshold of $I_{Na,P}$ is more positive than the resting potential of silent neurons (Fig. 4, C and D). Thus these results provide additional evidence that $I_{Na,P}$ Participates in spontaneous electrical firing in isolated SCN neurons.

In some cases, we observed a suppressing effect of riluzole on the action-potential amplitude, especially during long-lasting recordings and after a few applications of drug (Fig. 5B, top and bottom). Our present interpretation of this result is that $I_{Na,S}$ contributes to both the apparent neuronal leakage current and the general Na$^+$ current. If a neuron is healthy (i.e., fast $I_{Na}$ is much greater than leakage current), the effect of riluzole on action-potential amplitude is negligible. After prolonged whole cell recording, the leakage current could increase while fast $I_{Na}$ decreased. In these cases, leakage and fast $I_{Na}$ become comparable, and riluzole could then decrease action-potential amplitude.

Although a suppressing effect of riluzole (20 μM) was also generally observed on the electrical firing of SCN neurons in the slice preparation, there were quantitative differences (Fig. 6A). First, some neurons were insensitive to riluzole. Second, the effectiveness of riluzole on the riluzole-sensitive neurons depended on their firing rate: neurons possessing higher firing rate were less sensitive to riluzole. A TTX-resistant mechanism for oscillations in membrane potential has been shown for SCN neurons (Kononenko and Dudek 2002; Pennartz et al. 2002; N. I. Kononenko, unpublished data), and this mechanism alone could permit riluzole-resistant firing. Experiments with TTX application on riluzole-insensitive (completely or partially) neurons in slice preparations suggested that $I_{Na,S}$ alone is not absolutely required for spontaneous firing (Fig. 6C). TTX application suppressed action-potential generation, but low-amplitude oscillations in membrane potential were still present in whole cell recordings (Kononenko and Dudek 2002; Pennartz et al. 2002). At present, there are two different hypotheses regarding TTX-resistant mechanisms of pacemaker oscillations in SCN neurons; further studies are needed to determine whether L-type Ca$^{2+}$-channels (Pennartz et al. 2002) or subthreshold voltage-dependent cation (SVC) channels (Kononenko and Dudek 2002; N. I. Kononenko, unpublished results) underlie pacemaker oscillations.

**Putative clinical effect of riluzole**

At the recommended daily dose, the maximal plasma level of riluzole ranges between 0.9 and 1.6 μM (Urbani and Belluzzi 2000; based on Le Liboux et al. 1997). The EC50 for $I_{Na,S}$ in SCN neurons is 1–2 μM (Fig. 2C), and 10 μM produced complete suppression of spontaneous firing (Fig. 5). Based on the role of SCN neuronal firing in circadian behavior of mammals, riluzole could alter circadian rhythms at clinical levels.

**ACKNOWLEDGMENTS**

The constructive comments of two anonymous referees leading to substantial improvement of the paper are greatly appreciated.

**GRANTS**

This research was supported by National Institute of Mental Health Grant MH-59995.

**REFERENCES**


Magistretti J and Alonso A. Biophysical properties and slow voltage-dependent inactivation of a sustained sodium current in entorhinal cortex layer-II


