Sodium Currents in Medullary Neurons Isolated from the Pre-Bötzinger Complex Region

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The pre-Bötzinger complex (preBötC) in the ventrolateral medulla contains interneurons important for respiratory rhythm generation. Voltage-dependent sodium channels mediate transient current ($I_{NaT}$), underlying action potentials, and persistent current ($I_{NaP}$), contributing to repetitive firing, pacemaker properties, and the amplification of synaptic inputs. Voltage-clamp studies of the biophysical properties of these sodium currents were conducted on acutely dissociated preBötC region neurons. Reverse transcription-PCR demonstrated the presence of mRNA for Nav1.1, Nav1.2, and Nav1.6 α-subunits in individual neurons. A TTX-sensitive $I_{NaP}$ was evoked in all tested neurons by ramp depolarization from −80 to 0 mV. Including a constant in the Boltzmann equation for inactivation allowed prediction of a window current that did not decay to 0 at voltages positive to −20 mV and closely matched the measured $I_{NaP}$. Riluzole (3 µM), a putative $I_{NaP}$ antagonist, reduced both $I_{NaP}$ and $I_{NaT}$ and produced a hyperpolarizing shift in the voltage dependence of steady-state inactivation. The latter decreased the predicted window current by an amount equivalent to the decrease in $I_{NaP}$. Riluzole also decreased the inactivation time constant at potentials in which the peak window/persistent currents are generated. Together, these findings imply that $I_{NaP}$ and $I_{NaT}$ arise from the same channels and that a simple modification of the Hodgkin–Huxley model can satisfactorily account for both currents. In the rostral ventral respiratory group (immediately caudal to preBötC), $I_{NaP}$ was also detected, but peak conductance, current density, and input resistance were smaller than in preBötC region cells.

Key words: sodium channels; persistent current; respiration; pre-Bötzinger; pacemaker; riluzole; central pattern generator

Introduction

Brainstem neurons involved in the control of breathing typically fire bursts of action potentials phase locked to respiratory motor output. Voltage-dependent sodium channels mediate transient current ($I_{NaT}$), underlying action potentials, and subthreshold persistent current ($I_{NaP}$), which contribute to repetitive firing and/or pacemaker properties of neurons (Brumberg et al., 2000; Taddese and Bean, 2002; Do and Bean, 2003). $I_{NaP}$ can amplify dendritic depolarizations and facilitate repetitive firing (Schwindt and Crill, 1995; Stuart and Sakmann, 1995; Parri and Crunelli, 1998). In addition, $I_{NaP}$ can lead to progressive depolarization that brings neurons to their firing threshold (i.e., pacemaker activity) (Alonso and Llinás, 1989; Pape and Driesang, 1998; Bennett et al., 2000; Del Negro et al., 2002a; Taddese and Bean, 2002).

A core role in generating respiratory rhythm has been ascribed by many investigators to a column of neurons in the ventrolateral medulla termed the ventral respiratory column (VRC) (Alheid et al., 2002; Feldman and McCrimmon, 2003; Feldman et al., 2003). The pre-Bötzinger complex (preBötC), a small compartment within the longer VRC, has been implicated as the zone containing the basic circuit or “kernel” for respiratory rhythm generation (Smith et al., 1991; Ramirez et al., 1998; Rekling and Feldman, 1998; Solomon et al., 1999; Gray et al., 2001; Wang et al., 2002; Monnier et al., 2003). $I_{NaP}$ has been demonstrated within preBötC neurons, as has pacemaker activity (Johnson et al., 1994; Koshiya and Smith, 1999; Del Negro et al., 2002a). $I_{NaP}$ and pacemaker activity have been postulated to be essential for respiratory rhythm generation and consequently have been incorporated in recent biophysical models of respiratory neuronal circuits (Butera et al., 1999a; Smith et al., 2000). Nevertheless, this view has been questioned (Del Negro et al., 2002b, 2005; Feldman et al., 2003), and the possibility has been raised that respiratory pacemaker activity may only be essential in pathophysiological states such as gasping (St. John and Paton, 2000, 2002). Regardless of the necessity of pacemaker behavior per se to respiratory rhythm generation, the underlying ionic mechanisms assuredly participate in shaping the discharge pattern of respiratory neurons. Understanding these mechanisms and providing realistic estimates of the biophysical parameters underlying the intrinsic activity is essential for establishing valid neuronal models of the central control of breathing.

Accordingly, we addressed the following questions. First, is...
I_{NaP} density higher in neurons believed to be more intimately associated with rhythm generation (i.e., preBo\textsubscript{C}Tc neurons) than respiratory neurons in adjacent ventral medullary regions [i.e., in the rostral ventral respiratory cell group (rVRG)]. Second, if there are differences in I_{NaP} between neurons from the preBo\textsubscript{C}Tc and the rostral VRG regions, do these differences correlate with differential expression patterns of sodium channel \( \alpha \)-subunits? Third, do the biophysical properties of macroscopic I_{NaP} and I_{NaT} suggest they arise from the same or from different channels? To answer these questions, we examined acutely isolated rat neurons from the preBo\textsubscript{C}Tc and rVRG regions using whole-cell voltage clamp. Preliminary accounts of these data have been presented previously (McCrnimmon et al., 2001; Ptak et al., 2001a,b, 2002).

### Materials and Methods

**Retrograde labeling of cranial motoneurons.** To document the location of the preBo\textsubscript{C}Tc region relative to the nucleus ambiguus and facial nucleus in neonatal and young rats, dianisobenzamide (DAB) immunocytochemistry for the neurokinin-1 (NK1) receptor was used to label the preBo\textsubscript{C}Tc (Gray et al., 1999) in the same animals in which medullary motoneurons had been retrogradely prelabeled with FluoroGold (see Fig. 1A). Subsequently, FluoroGold labeling alone was used to help identify the preBo\textsubscript{C}Tc region in fresh parasagittal sections (300 \( \mu \)m thickness) by its topographical location caudal (~500–1100 \( \mu \)m for 7- to 15-d-old rats) to prelabeled fluorescent motoneurons in the facial nucleus and ventral to fluorescent motoneurons in caudal aspects of the compact and semicom pact divisions of the nucleus ambiguus (see Fig. 1B). The region of the rVRG was similarly identified relative to the fluorescent-labeled motoneurons as the area caudally adjacent to the preBo\textsubscript{C}Tc, lying ventral and caudal to the caudal-most extent of the compact and semicom pact portions of nucleus ambiguus. Just after birth, animals were injected subcutaneously on the back at the level of the shoulders with 50 mg/kg FluoroGold (Fluorochrome, Denver, CO) or its sulfate, hydroxyethylamidine-methanesulfate (Wessendorf, 1991) (Biotium, Hayward, CA) at 1.5% in 0.15 M NaCl to produce global (retrograde) labeling of cranial motoneurons in the ventral medulla (Ambalavanar and Morris, 1989). Fluorescent-labeled motoneurons were observed in the medulary slices at low magnifications with an inverted microscope and epifluorescent UV illumination.

**Preparation of dissociated neurons.** Neurons from the regions of the preBo\textsubscript{C}Tc or rVRG (see below) were obtained from newborn and young (1- to 15-d-old) Sprague Dawley rats (Charles River, Wilmington, MA). Cells were dissociated according to a technique adapted from Raman et al. (2000). Animals were anesthetized with isoflurane and decapitated. Parasagittal vibratome slices of the brainstem (300 \( \mu \)m thick) were collected in Tyrode’s solution containing the following (in mM): 150 NaCl, 4 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 10 HEPES, 10 HEPE, 10 glucose, pH 7.4, 300 mOsm/L. The slices were incubated for 20 min at 33°C in oxygenated Tyrode’s solution containing the following (in mM): 150 NaCl, 3 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 10 EGTA, 10 glucose, pH 7.4, 300 mOsm/L. For measurement of I_{NaP}, the external solution consisted of the following (in mM): 15 NaCl, 110 tetraethylammonium (NME)-Cl, 10 HEPE, 10 CsCl, 1 MgCl\(_2\), 2 BaCl\(_2\), and 0.3 CdCl\(_2\) buffered to pH 7.4 with CsOH (300 \( \pm \) 5 mOsm/L). For measurement of I_{NaT}, the external solution consisted of the following (in mM): 115 NaCl, 45 TEA-Cl, 10 CsCl, 10 HEPE, 1 MgCl\(_2\), 2 BaCl\(_2\), and 0.3 CdCl\(_2\) buffered to pH 7.4 with CsOH (300 \( \pm \) 5 mOsm/L) (Maurice et al., 2001). The quantitative relationship between I_{NaP} and I_{NaT} (see Figs. 4 B, C, 5, 8A–D) was examined using an internal pipette solution containing the following (in mM): 120 TEA-Cl, 15 NaCl, 1.8 MgCl\(_2\), 9 EGTA, 4 Mg-ATP, 14 phosphocreatine, 0.3 mGTP (Tris salt), and 9 HEPE, pH 7.4 with TEA-OH (265 \( \pm \) 5 mOsm/L). The external solution was as follows (in mM): 150 NaCl, 3 KCl, 15 TEA-Cl, 3.9 BaCl\(_2\), 0.1 CdCl\(_2\), and 10 HEPE, adjusted to pH 7.4 with NaOH (300 \( \pm \) 5 mOsm/L). During recording, the external solutions were applied through a gravity-drained capillary perfusion array. Its end was placed ~3 mm from the neuron. Bathing solutions were changed by adjusting the perfusion array using DC solenoid valves (Warner Instruments, Hamden, CT). Solutions were changed in <1 s. All reagents were obtained from Sigma (St. Louis, MO). Membrane potentials (\( V_m \)) are uncorrected for the liquid junction potential, which was small but varied slightly with the external solution (for external [Na\(^+\)] of 15 mM, \(-1.2\) mV; external [Na\(^+\)] of 115 mM, \(-0.6\) mV; external [Na\(^+\)] of 150 mM, \(+1\) mV) measured using a low KCl bridge (Neher, 1992).

Riluzole [2-amino-6-[(trifluoromethoxy)benzothiazole] (Sigma) was dissolved in 10% DMSO to make a 10 mM stock and was diluted with external solutions immediately before use. The concentration of DMSO in the external solution was 0.01% except in experiments shown in Figure 7B, in which the dose dependence of riluzole-mediated inhibition of I_{NaT} was examined. In these experiments, a stock solution of riluzole in 10% DMSO was sequentially diluted, resulting in a DMSO of 0.1% at the highest riluzole concentration (100 \( \mu \)M).

Membrane capacitance (\( C_m \)) was determined from the transient capacitive current evoked by a 20 ms hyperpolarizing step from –70 to –80 mV averaged for 10 trials and calculated according to \( C_m = \pi R_s \), where \( R_s \) is the series resistance. The input resistance (\( R_{in} \)) was calculated by measuring the steady-state passive current (\( I_p \)) evoked with a 20 ms hyperpolarizing step from –70 to –80 mV (\( V_m \)) averaged for 10 trials and calculated according to \( R_{in} = \pi \mu /I_p \).

Several steps were taken to ensure adequate voltage control as described previously (Carr et al., 2002). Briefly, recordings were performed only on cells with relatively short attached primary dendrites to ensure adequate space-clamp control. Data were also disqualified if there was evidence of loss of voltage control or space-clamp errors. These include changes in the activation kinetics of the pulse current evoked by the inactivation protocol, spiking during the ramp experiments, or signs of rundown (>30% response decline relative to that after the initial pre pulse) over the course of the experiments examining kinetics of entry into inactivation.

As in the previous report by Maurice et al. (2001), the activation data curve was fit with a third-order Boltzmann function of the following form: \( g(V) = 1/(1 + \exp[(V - V_{1/2})/k])^3 \), where \( k \) is the slope factor and \( V_{1/2} \) is the half-activation voltage of each of three hypothetical gates. The


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third-order equation provided a better fit of the curve to the raw data in the threshold region of current activation than was the case for the first-order equation. Optimization of the fit in this region of the curve was particularly important for accurate calculation of the window current. The use of a third-order fit is also consistent with the Hodgkin–Huxley kinetic model of Na"+ channels. In the model, activation is described as the opening of three identical and independent m-gates. Accounting for the movement of three hypothetical particles during channel opening is consistent with the better fit provided by the third-order Boltzmann function to the activation data (Maurice et al., 2001). It should be noted that with a third-order function, half-activation of the macroscopic current occurs at more depolarized potentials than those estimated with the first-order Boltzmann function generally applied to the activation data from various CNS neurons studied in previous reports. In contrast, inactivation in the Hodgkin–Huxley model occurs on the closing of one h-gate, so the inactivation-voltage curve was fit with a modified first-order Boltzmann equation of the form \( I_{\infty} = \frac{(1 - I_{\infty})}{(1 + \exp[(V - V_{1/2})/k])} + I_m \), where \( I_{\infty} \) is the relative current, \( V_{1/2} \) is the half-voltage inactivation, \( k \) is the slope factor, and \( I_m \) is the sustained current resulting from activation of a fraction of available channels (open or closed).

The \( I_{\text{act}} \) activation time constant \( (\tau_{\text{act}}) \) was calculated from the time-to-peak \( [I_{\text{act}}(V)] \) data at each voltage step \( V \) by fitting to a first-order exponential in the following form: \( I(t) = I_{\infty} \exp[-t/\tau_{\text{act}}] \). The \( I_{\text{act}} \) inactivation time constant \( (\tau_{\text{inact}}) \) was evaluated from the data on current decay for each voltage step during the activation protocol by fitting to a first-order exponential in the following form: \( I(t) = I_{\infty} \exp[-t/\tau_{\text{inact}}] \). The \( I_{\text{act}} \) is the current value close to \( I_{\infty} \) at the moment \( t \), and \( I_m \) is the steady-state current of the decayed current. The concentration–response relationship for riluzole inhibition of the transient Na"+ current was fit with a Langmuir isotherm of the following form: \( I = I_{\infty} \left[ 1 + [R]/EC_{50} \right] + I_m \), where \( I_{\infty} \) is the fraction of current blocked at saturating riluzole concentrations, \( I_m \) is the fraction of current that is resistant to block and is the concentration of riluzole, and \( EC_{50} \) is the concentration of riluzole producing a block equal to 50% of \( I_{\infty} \) (Carr et al., 2002). The apparent binding for riluzole to block Na"+ channels in the inactivated state \( (K) \) was estimated by the following equation: \( K = R/(1 + [R]/EC_{50})/\exp(\Delta V_{1/2}/k) \) (R), where \( R \) is the riluzole concentration, \( \Delta V_{1/2} \) represents the shift of inactivation curve after application of riluzole, and \( k \) is the slope factor (Bean et al., 1983).

Average values were expressed as mean ± SE. Except where noted otherwise, statistical significance was evaluated by means of the two-tailed Student's \( t \) test for unpaired data. Box plots (Tukey, 1977) are used in Figures 3 and 6. As applied here, the median is the central line and the box extends from the first to third quartiles. Vertical lines extend beyond the box to the most extreme value not more than 1.5 times the interquartile range. Individual values beyond this limit are plotted as circles.

Single-cell and tissue RT-PCR procedures. Procedures were similar to those described by Maurice et al. (2001). Briefly, isolated neurons (unrecorded) were aspirated into sterilized glass electrodes (capillaries treated at 180°C for 8 h before pulling) containing 1–2 μl of a solution consisting of diethyl pyrocarbonate-treated water (DEPC) and Superasin RNase-inhibitor (Ambion, Austin, TX). Sterile gloves were worn during cell collection for RT-PCR to minimize RNase contamination. The glass pipette tip (containing the cell) was broken into a 0.5 ml presisioniolized centrifuge tube containing a 5 μl mixture of bovine serum albumin (143 ng/μl), Superasin (40 U/μl), deoxyNTPs (dNTPs) (10 mM), and random hexamers (50 ng/μl). The centrifuge tube with the sampled neuron was then incubated for an additional 2 hi nP B S containing an avidin-biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). The sections were then incubated for an additional 2 h in PBS containing a avidin-
Transient sodium current in the pre-Bötzinger region

Initial experiments characterized $I_{Na_T}$. For this purpose, the external Na$^+$ concentration was lowered to 15 mM to reduce current amplitude and voltage disparities introduced by series resistance errors. At this concentration, the properties of $I_{Na_T}$ were similar to those reported in studies in which the Na$^+$ concentration gradient between the inside and outside of neurons was reversed, making them nonregenerative (Dargent et al., 1994). Figure 2, A and B, illustrates Na$^+$ currents evoked by standard activation and inactivation protocols, respectively. Steady-state plots were constructed by subtracting the TTX-insensitive leak current (Fig. 2C). In 29 dissociated neurons from the preBötC region, the $V_{1/2}$ activation of $I_{Na_T}$ was $-43.7 \pm 0.6$ mV, with $k = 6.5 \pm 0.2$, based on the fit of the third-order version of the Boltzmann equation. Using 100 ms prepulses, $V_{1/2}$ of steady-state inactivation was $-67.0 \pm 0.3$ mV with $k = 9.8 \pm 0.4$ mV ($n = 29$).

Comparison of the activation properties obtained for $I_{Na_T}$ in preBötC region neurons revealed reasonable agreement with values reported by Maurice et al. (2001) for cortical pyramidal neurons ($V_{1/2} = -38.4$ mV; $k = 5.8$), the only other study applying a third-order Boltzmann function on dissociated neurons. Fitting a first-order equation to the data in the current study yields an activation of $V_{1/2} = -32.4 \pm 0.8$ for preBötC neurons and a slope factor of $4.7 \pm 0.1$. This agrees well with previous studies on $I_{Na_T}$ voltage dependence using acutely dissociated neurons from other CNS regions (median activation, $V_{1/2} = -31.5$ mV) (Huguenard et al., 1988; Sah et al., 1988; Raman and Bean, 1997; Magistretti and Alonso, 1999; Raman et al., 2000; Taddese and Bean, 2002). With respect to inactivation of $I_{Na_T}$, the $V_{1/2}$ ($-67.0$ mV) in the present study was close to the median value for other CNS neurons ($-64.4$ mV) (Huguenard et al., 1988; Sah et al., 1988; Raman and Bean, 1997; Magistretti and Alonso, 1999; Raman et al., 2000; Maurice et al., 2001; Taddese and Bean, 2002). In contrast, the slope factor (9.8) for the preBötC region Na$^+$ channel inactivation was higher than that for neurons from other CNS regions (median inactivation, $k = 5.7$). The higher slope factor is not readily attributed to methodological differences, because first-order Boltzmann functions were used to fit the inactivation data in both instances. To determine whether the higher slope factor may have been attributable to a failure to achieve steady-state inactivation in these neurons, longer prepulses of 200 or 300 ms were tried. However, the longer steps did not significantly shift the voltage-dependent inactivation in the hyperpolarizing direction, nor did the slope factor become steeper. This is also consistent with the relatively short $\tau$ for inactivation, which was $\sim 7$ ms at $-40$ mV and shortened to $\sim 1$ ms at 0 mV (Fig. 3E,F) (see below). In contrast, use of a lowered external sodium concentration (15 mM in the current study) to improve the space clamp may have reduced the slope factor, as has been found for cerebellar neurons (Afshari et al., 2004). Additionally, the shallow slope is consistent with the presence of multiple sodium channels with slight differences in the voltage dependence of inactivation, thereby extending the voltage range over which inactivation occurred.

Results

In the present study, we characterized sodium currents in neurons from the region of the preBötC in the rostral ventrolateral medulla. These were then contrasted with currents recorded from the immediate caudal region of the rVRG. These regions were identified relative to the fluorescent-labeled landmarks (Fig. 1A, B) that were readily observed at low magnifications and that have a fixed relationship to NK1 receptor immunoreactivity in the preBötC region (Gray et al., 1999). This allowed reproducible sampling of neurons from this region and assured sampling from a population of isolated neurons that were enriched in respiratory neurons from the preBötC or, in the contrasting sample, from the rVRG. Despite this, it should be acknowledged that the respiratory nature of individual dissociated neurons was not directly established.
current was blocked with TTX (300 nM). The resulting residual TTX-insensitive current was thus determined and subtracted from the overall Na\(^+\) current to reveal the TTX-sensitive portion of \(I_{\text{NaP}}\). An \(I_{\text{NaP}}\) was evoked in all tested neurons \((n = 29)\), and its properties were similar to those described previously in neurons from other CNS regions (Alzheimer et al., 1993; Kay et al., 1998; Magistretti and Alonso, 1999; Maurice et al., 2001; Carr et al., 2002). This current was activated above ~60 mV, reaching a peak of approximately ~40 mV (Fig. 4A); the current then declined with additional depolarization but remained active.

**Comparison of pre-Bo\¨tzinger region \(I_{\text{NaP}}\) with window current**

Window current is described by the overlap of the nominally steady-state activation and inactivation curves. A distinction between the window current and the persistent current has been presumed (Kay et al., 1998; Magistretti and Alonso, 1999; Maurice et al., 2001), because the predicted inactivation curve of the window current decays to zero, according to the Hodgkin and Huxley (1952) model, whereas the persistent current remains outside a voltage range predicted for the window current. However, Taddese and Bean (2002) recently applied an allosteric gating model of Na\(^+\) channels and postulated that the persistent current originates from incomplete inactivation approaching a steady-state level in the range of 0.5 to 4% of the total Na\(^+\) conductance.

To determine whether the inactivation process in preBo\¨tzinger region neurons is complete, the ratio of \(I_{\text{NaP}}\) to \(I_{\text{NaT}}\) was quantified. To improve the ability to resolve small currents, the current was evoked in 150 mM external Na\(^+\) and measured at the end of a 70 ms depolarizing pulse to ~20 mV. The evoked current was sampled 25 times and then averaged. In this series, the peak of the \(I_{\text{NaT}}\) was ~5.8 ± 0.3 nA, the steady-state current was ~77.7 ± 8.1 pA, and the ratio of \(I_{\text{NaP}}\) to \(I_{\text{NaT}}\) was 1.54 ± 0.25% at ~20 mV (range, 0.7–3.3%; \(n = 14\)) (Fig. 4B). Next, we determined the kinetics of inactivation at this potential (Fig. 4C). This process was estimated by plotting the current evoked by a test pulse to ~10 mV after a variable length conditioning prepulse (5–100 ms) to ~20 mV. The resulting current was fit with the sum of two exponential functions. The fast time constant was ~1.6 ± 0.1 ms, and the slow time constant was ~12.6 ± 0.35 (\(n = 6\)). In these experiments, the peak of \(I_{\text{NaT}}\) was ~7.68 ± 0.7 nA, the steady-state current was ~76 ± 9.4 pA, and the ratio of \(I_{\text{NaP}}\) to \(I_{\text{NaT}}\) was 1 ± 0.1% (\(n = 6\)) (Fig. 4C). These results indicate that in neurons current was lower for rVRG (open bars; \(n = 29\)) compared with preBo\¨tzinger \((n = 29\)) regions.

The features of transient Na\(^+\) current in neurons from the preBo\¨tzinger region. \(A\), Representative current traces (TTX-subtracted) and protocols used to examine the voltage dependence of activation \((\mathbf{A})\) and inactivation \((\mathbf{B}; 100 \text{ ms prepulses})\). \(C\), Plots of the peak conductance \((\mathbf{C})\) versus voltage. Solid lines are fits to the Boltzmann equation for inactivation and activation. Fitting parameters for this neuron were as follows: \(I_{\text{NaP}} = -46.0 \text{ mV}, k = 6.0 \text{ mV} \) (activation; based on the third-order version of the Boltzmann equation); \(I_{\text{NaP}} = -68.3 \text{ mV}, k = 8.5 \text{ mV} \) (inactivation; first-order Boltzmann equation).

To investigate the time constant of activation \((\tau_a)\), the rising currents evoked by the step depolarizing protocol were fitted with an exponential function. The \(\tau_a\) became shorter with increasing depolarization, for instance, from 0.71 ± 0.08 ms at ~40 mV to 0.11 ± 0.01 ms at 5 mV \((n = 29)\) (Fig. 3E). The inactivation time constant \((\tau_i)\), calculated using an exponential fit to the decaying Na\(^+\) current evoked by depolarizing steps, was also shortened from 6.89 ± 0.76 ms at ~40 mV to 0.81 ± 0.06 ms at +5 mV \((n = 29)\) (Fig. 3F).

**Persistent sodium current in the pre-Bo\¨tzinger region**

For characterization of \(I_{\text{NaP}}\), the external Na\(^+\) concentration was elevated to 115 mM to increase the current amplitude, thereby improving the signal-to-noise ratio. Slow ramps from ~80 to ~0 mV (50 mV/s) were used to examine the voltage dependence of the persistent current. This allowed full inactivation of the fast-decaying Na\(^+\) current component but produced minimal slow inactivation (Fig. 4A). At the end of each experiment, Na\(^+\) current was blocked with TTX (300 nM). To improve the ability to resolve small currents, the current was evoked in 150 mM external Na\(^+\) and measured at the end of a 70 ms depolarizing pulse to ~20 mV. The evoked current was sampled 25 times and then averaged. In this series, the peak of the \(I_{\text{NaT}}\) was ~5.8 ± 0.3 nA, the steady-state current was ~77.7 ± 8.1 pA, and the ratio of \(I_{\text{NaP}}\) to \(I_{\text{NaT}}\) was 1.54 ± 0.25% at ~20 mV (range, 0.7–3.3%; \(n = 14\)) (Fig. 4B). Next, we determined the kinetics of inactivation at this potential (Fig. 4C). This process was estimated by plotting the current evoked by a test pulse to ~10 mV after a variable length conditioning prepulse (5–100 ms) to ~20 mV. The resulting current was fit with the sum of two exponential functions. The fast time constant was ~1.6 ± 0.1 ms, and the slow time constant was ~12.6 ± 0.35 (\(n = 6\)). In these experiments, the peak of \(I_{\text{NaT}}\) was ~7.68 ± 0.7 nA, the steady-state current was ~76 ± 9.4 pA, and the ratio of \(I_{\text{NaP}}\) to \(I_{\text{NaT}}\) was 1 ± 0.1% (\(n = 6\)) (Fig. 4C). These results indicate that in neurons
same Na$^+$ transient and the persistent Na$^+$ inactivation induced by the conditioning train affected both the $I_{NaP}$ of ($\text{tent Na}^+$) different scale for time and current), representing 1.2% of the total Na$^+$ current of (20 mV in neurons from the preBo¨tC region. Entry into inactivation was determined using a 500 ms prepulse to $-20$ mV. Inactivation was measured with a 10 ms test pulse to $10$ mV after a 20 ms recovery interval at $-80$ mV (C). The conditioning trains consisted of 15 20 ms pulses separated by 20 ms intervals at $-80$ mV (○). Insets, Transient Na$^+$ current with (●) and without (○) conditioning trains at higher resolution either during beginning of prepulse at $-20$ mV (left inset) or during test pulse at $10$ mV (right inset). The effect of conditioning trains was also studied on persistent Na$^+$ current measured at the end of prepulse (middle inset; note different current scale). Note that the conditioning trains and step pulse reduced the amplitude of $I_{NaP}$ similarly ($\pm$50%). The step pulse similarly reduced $I_{NaP}$ ($\pm$60%).

from the preBo¨tC region, Na$^+$ channels did not completely inactivate at $-20$ mV, at least within several hundred milliseconds.

To examine whether persistent current originates from the same Na$^+$ channels as transient current, we asked whether slow inactivation affected these currents in a similar manner (Taddese and Bean, 2002; Carr et al., 2003; Do and Bean, 2003). To drive the Na$^+$ channels in preBo¨tC neurons into the slow inactivated state, a train of 15 pulses was delivered at 25 Hz (20 ms depolarizations to $-20$ mV separated by 20 ms intervals at $-80$ mV) (Fig. 5). The conditioning pulse train reduced the amplitude of $I_{NaP}$ by $56 \pm 2.8\%$ and the persistent Na$^+$ current by $63.1 \pm 2.7\%$ ($n = 5$) (Fig. 5). This result demonstrates that the process of slow inactivation induced by the conditioning train affected both the transient and the persistent Na$^+$ currents equivalently and is consistent with both currents generated by the same population of Na$^+$ channels.

These data are consistent with the hypothesis that the persistent Na$^+$ current is a window current. Therefore, we directly compared the conductance of the window current with that of the persistent current evoked by a ramp protocol (50 mV/s). Window conductance was computed by taking the product of the Boltzmann equations fit to the steady-state activation (third-order) and inactivation (first-order) plots (see Materials and Methods). The inactivation curve was fit by a Boltzmann equation that had been altered by the addition of a factor that estimated the steady-state fraction of Na$^+$ channels available for inactivation (open or closed; see Materials and Methods). In neurons from the preBo¨tC region, this availability ranged from 0.7 to 3.3% for a prepulse duration of 100 ms. Figure 4C shows a typical experiment with availability of 1.5% at 100 ms. Figure 5A shows an example in which normalized persistent and window conductances are plotted for an individual cell. The shapes and voltage dependence of both conductances are sufficiently coincident to suggest that $I_{NaP}$ and window current may arise from the same type of Na$^+$ channels. This overlap was a typical result. In 8 of 20 neurons, there was no difference between the voltages at which the peak conductance occurred when comparing persistent and window currents, and, for 17 of 20 neurons, the difference was $<2$ mV. When averaged over all preBo¨tC region neurons, the shift was $<1$ mV ($0.9 \pm 0.6$ mV; $n = 20$) with a median of 0 mV. The distribution of these differences is depicted in the box plot shown in Figure 6B. In two neurons from the preBo¨tC region, the
activation voltage dependence of persistent current appeared to be shifted toward a more negative membrane potential than the macroscopic window current and, in one cell, to a more positive value (Fig. 6 B, outliers).

Effect of riluzole on pre-Bötzinger regions $I_{\text{NaP}}$ and $I_{\text{NaT}}$

It has been postulated that persistent current is selectively sensitive to riluzole (Benoit and Escande, 1991; Urbani and Belluzzi, 2000), with the implication that this current arises from different Na$^+$ channels than those producing $I_{\text{NaT}}$. To examine this question, we compared the sensitivity of $I_{\text{NaT}}$ and $I_{\text{NaP}}$ in preBötC region neurons to riluzole at a series of concentrations. Application of riluzole produced a concentration-dependent and reversible reduction in the peak of $I_{\text{NaT}}$ evoked by a step from a holding potential of $-80$ to $-20$ mV (Fig. 7A). The fraction by which the peak amplitude was reduced was plotted as a function of riluzole concentration and fit with the Langmuir isotherm with an EC$_{50}$ value of 2.4 $\mu$M (Fig. 7B). The effects of riluzole on $I_{\text{NaT}}$ were mediated in part by a negative shift in the voltage dependence of rapid inactivation ($V_{1/2}$ control, $-64.7 \pm 1.1$ mV; $V_{1/2}$ riluzole, $-75.1 \pm 1.1$ mV; $p = 0.001; n = 8$) (Fig. 7C), a decrease in peak Na$^+$ conductance ($g_{\text{max}}$ control, 1.29 $\pm 0.10$ nS; $g_{\text{max}}$ riluzole, 0.52 $\pm 0.06$ nS; $p = 0.001; n = 8$), and reduced channel availability at hyperpolarized potentials (9.52 $\pm 1.4%$ reduction at $-110$ mV; $n = 8$) (Fig. 7C). However, riluzole did not change the inactivation slope factor ($k$ control, $7.80 \pm 0.3$ mV; riluzole, $7.86 \pm 0.2$ mV; $n = 8$) (Fig. 7C). In 3 $\mu$M riluzole, there was no significant change in the voltage dependence of activation ($V_{1/2}$ control, $-43.7 \pm 0.95$ mV; $V_{1/2}$ riluzole, $-43.9 \pm 1.1$ mV; $n = 8$) (Fig. 7C). However, a positive shift in the $V_{1/2}$ of activation occurred as the drug concentration was increased to 10 $\mu$M (data not shown). These results suggest that riluzole binds selectively to Na$^+$ channels in their inactivated states.

By shifting the steady-state inactivation curve, riluzole decreased the relative window conductance by $-70%$ and shifted the peak of the window current to a more hyperpolarized potential (approximately $-4$ mV) (Fig. 7D). In addition to its effects on the transient Na$^+$ current, riluzole reduced the $I_{\text{NaP}}$ measured with a slow (50 mV/s) voltage ramp protocol, by $76 \pm 1\%$ ($n = 5$) (Fig. 7E). An equivalent decrease in the amplitude of the $I_{\text{NaP}}$ was also obtained using a step depolarization to $-20$ mV in the presence of riluzole (3 $\mu$M) in all examined neurons (average reduction, $74 \pm 5\%$; $n = 7$) (Fig. 7A). Riluzole shifted the voltage dependence of persistent current activation toward more negative membrane potentials by $-3$ mV ($V_{1/2}$ control, $-53.1 \pm 0.4$ mV; $V_{1/2}$ riluzole, $-56.5 \pm 0.4$ mV; $p = 0.01; n = 5$) (Fig. 7F). The results from these experiments demonstrate that riluzole modulates transient as well as persistent Na$^+$ currents to approximately the same degree, suggesting the presence of a single population of Na$^+$ channels underlying both types of currents.

Mechanism of Na$^+$ channel modulation by riluzole

A variety of investigators (Benoit and Escande, 1991; Hebert et al., 1994; Song et al., 1997; Urbani and Belluzzi, 2000) have interpreted the effects of riluzole on Na$^+$ channels according to a modulated receptor model (Hille, 1977). In this model, the affinity of the drug for Na$^+$ channels depends on the gating state (deinactivated, open, or inactivated). Accordingly, and as observed in previous reports, a riluzole-induced negative shift in the voltage dependence of fast inactivation results from a higher affinity of the drug for Na$^+$ channels in their inactivated state.

To estimate the affinity of riluzole for binding to inactivated Na$^+$ channels in the preBötC region, the apparent dissociation constant ($K_i$) was calculated (see Materials and Methods). The $K_i$ value of $0.3 \pm 0.1$ $\mu$M ($n = 9$) indicated an approximately eightfold greater apparent affinity of riluzole compared with the EC$_{50}$ value, consistent with a highly preferential block of Na$^+$ channels in the inactivated state, as reported previously (Benoit and Escande, 1991; Hebert et al., 1994; Song et al., 1997; Urbani and Belluzzi, 2000). As a result, the effect of riluzole on Na$^+$ current would be expected to depend on the holding potential of a neuron. Removing inactivation by holding a neuron at a very polarized potential (i.e., moving channels into the deinactivated state) would reduce the effect of riluzole. To examine this in preBötC region neurons, the effects of 3 $\mu$M riluzole on both transient and persistent currents were examined with steps to $-20$ mV from different holding potentials in 150 mM external Na$^+$ (Fig. 7C). The evoked current was sampled 25 times and then averaged as described above. When the current was evoked from $-100$ mV, riluzole decreased the peak of $I_{\text{NaT}}$ and $I_{\text{NaP}}$ by $21 \pm 2$ and $62 \pm 2\%$, respectively (Fig. 8A, E) ($n = 11$). The reduction was greater from a holding potential of $-80$ mV (Fig. 8B, E) (peak $I_{\text{NaT}}$, $59 \pm 0.2\%$; $I_{\text{NaP}}, 74 \pm 1\%$; $n = 18$). In comparison, as shown in Figure

![Figure 7](image-url)
dramatically enhanced the reduction of peak currents with steps initiated from depolarized holding potentials (Fig. 8C–E). Stepping from a holding potential of −60 or −55 mV dramatically enhanced the reduction of peak \(I_{\text{NaP}}\) (88 ± 2% from −60 mV; 94 ± 2% from −50 mV; \(n = 11\)) by riluzole. In contrast, there were only modest additional reductions (\(I_{\text{NaP}}\) 88 ± 2% from −60 mV; 80 ± 1% from −55 mV; \(n = 11\)).

These results are consistent with the modulated receptor model (Hille, 1977), with a greater affinity of riluzole for inactivated compared with deinactivated (closed) channels. The relatively greater reduction in \(I_{\text{NaP}}\) versus \(I_{\text{NaT}}\) evoked from holding potentials of −100 or −80 mV suggests that riluzole may preferentially modulate the inactivation of Na⁺ current. With steps initiated from these relatively hyperpolarized potentials, very few currents were evoked in control and with riluzole (3 μM). Sodium currents were determined in a sample of 20 neurons (Fig. 8C–E), one from each of the three regions when determined using conductance–voltage plots (Table 1). The time constants of activation during step depolarizations. To examine the effect of riluzole on the rate of inactivation, the time course was measured at different test potentials in the presence and absence of 3 μM riluzole. Inactivation time constants (calculated from monoexponential fits) were plotted as a function of the test potentials. As shown in Figure 8F, riluzole reduced the inactivation time constant at potentials negative to −30 mV with more pronounced effects at more negative potentials, that is, riluzole accelerated entry into inactivation. Moreover, the predominant effect occurred over the voltage range encompassing the peak in window current, rather than in activation (data not shown).

Together, these are consistent with riluzole having a higher affinity for the inactivated Na⁺ channels and, by decreasing the inactivation time constant at potentials negative to −30 mV, accelerating entry into inactivation.

### Table 1. Biophysical Characteristics of Na⁺ Channels in Neurons from preBötC and rVRG Regions

<table>
<thead>
<tr>
<th></th>
<th>preBötC Region</th>
<th>rVRG Region</th>
<th>(p) (df = 47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak of (I_{\text{NaP}}) (mV)</td>
<td>−21.2 ± 0.6</td>
<td>−16.5 ± 1.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Magnitude of (I_{\text{NaT}}) (nA)</td>
<td>−2.3 ± 0.1</td>
<td>−1.5 ± 0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Activation (V_{1/2}) (mV)</td>
<td>−43.7 ± 0.6</td>
<td>−40.3 ± 0.8</td>
<td>0.02</td>
</tr>
<tr>
<td>(k) (mV)</td>
<td>6.5 ± 0.2</td>
<td>5.6 ± 0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Inactivation (V_{1/2}) (mV)</td>
<td>−67.0 ± 0.6</td>
<td>−66.1 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>(k) (mV)</td>
<td>9.8 ± 0.4</td>
<td>9.2 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Max window conductance (estimated percentile of (I_{\text{NaP}}))</td>
<td>2.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Peak of (I_{\text{NaP}}) (ramp, 50 mV/s)</td>
<td>−42.0 ± 0.5</td>
<td>−39.6 ± 0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Conductance of (I_{\text{NaP}}) (ramp, 50 mV/s)</td>
<td>1.7 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>(I_{\text{NaP}}) (nA/pF)</td>
<td>14.1 ± 1.6</td>
<td>7.6 ± 0.9</td>
<td>0.01</td>
</tr>
<tr>
<td>(I_{\text{NaP}}) (nS)</td>
<td>21.2 ± 2.3</td>
<td>14.1 ± 1.6</td>
<td>0.01</td>
</tr>
<tr>
<td>(I_{\text{NaP}}) (pA/pF)</td>
<td>42.0 ± 3.9</td>
<td>28.7 ± 3.1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Note:** The enhanced effect of riluzole on transient and persistent Na⁺ currents evoked from holding potentials of −100 (A), −80 (B), −60 (C), and −55 (D) mV. Insets, Currents at the end of 50 ms steps at higher resolution. Note the enhanced effect of riluzole on transient and persistent Na⁺ currents with steps initiated from depolarized holding potentials. E, Summary of the reduction of transient and persistent Na⁺ current by riluzole (3 μM). F, Riluzole reduced the inactivation time course at potentials more negative than −30 mV. Currents were evoked by 50 ms step depolarizations from −45 to 0 mV (5 mV increments) from a holding potential of −80 mV. Decaying phases of current traces were fit by a monoexponential function, and the time constant in the absence (C) and presence (H) of riluzole was plotted as a function of the test potentials. RIL, Riluzole; CTRL, control.
and inactivation were almost identical between neurons from the two regions and demonstrated similar voltage dependence, becoming shorter at more depolarized potentials (Fig. 3 E, F). Finally, similar values for \( C_m \) were measured for both the preBo\`tC and the rVRG region (preBo\`tC, 10.9 \pm 0.7 pF; \( n = 10; \) rVRG, 11.3 \pm 0.6, \( n = 5; \) \( p > 0.05 \)), presumably reflecting sampling of similarly sized neurons from either area. In contrast, \( R_{IN} \) was significantly greater for preBo\`tC compared with rVRG neurons (Fig. 3D) (preBo\`tC, 176.8 \pm 12.4 M\( \Omega \); \( n = 13; \) rVRG, 98.5 \pm 7.5 M\( \Omega \); \( n = 13; \) \( p < 0.05 \)).

The differences in steady-state activation and inactivation kinetics resulted in a significantly smaller maximum window conductance in neurons from the rVRG region compared with neurons from the preBo\`tC region (Table 1). Similar to the reduced window current for neurons from the rVRG region, the persistent Na\(^+\) current induced by a slow ramp (50 mV/s) was also smaller ( \( p < 0.001 \)), with a maximum of \(-85.6 \pm 6.5\) pA (Fig. 3B) (\( g_{NaP} \), 0.9 \pm 0.1 nS; \( n = 20 \)) occurring at a potential of \(-39.6 \pm 0.7\) mV (Table 1) compared with the window current for neurons from the preBo\`tC region, in which the maximal persistent current was \(-161.9 \pm 12.7\) pA (Fig. 3B) (\( g_{NaP} \), 1.7 \pm 0.2 nS; \( n = 29 \)) occurring at a potential of \(-42.0 \pm 0.5\) mV (Table 1). In addition, the density of \( I_{NaP} \) was significantly lower in neurons from the rVRG region compared with that measured in neurons from the preBo\`tC region (Fig. 3C, Table 1).

Detection of Na\(^+\) channel \( \alpha \)-subunits with single-cell RT-PCR

In parallel with our electrophysiological experiments, RT-PCR techniques were used to determine the contribution of different Na\(^+\) channel \( \alpha \)-subunits. In CNS neurons, several sodium channel \( \alpha \)-subunit mRNAs are expressed, including Nav1.1, Nav1.2, Nav1.3, Nav1.5, and Nav1.6 (Donahue et al., 2000; Goldin, 2001). Because a null mutation in Nav1.6 reduced the amplitude of persistent Na\(^+\) current in Purkinje neurons and prefrontal cortex pyramidal neurons, it was suggested that this isoform might contribute, importantly, to this current (Raman et al., 1997; Vega-Saenz de Miera et al., 1997; Maurice et al., 2001). Nevertheless, Nav1.6 null mutant mice demonstrated the presence of persistent current that was attributed to Nav1.1/1.2 (Raman et al., 1997; Maurice et al., 2001). In tissue samples from the preBo\`tC and rVRG regions, all four channel mRNAs were detected. In the small neurons sampled from these regions, only Nav1.1, Nav1.2, and Nav1.6 mRNAs were detected. Although Nav1.5 was not detected in single cells, the ability of the primers to detect this subunit in single neurons has been demonstrated for septal neurons (Maurice et al., 2001). Nevertheless, for RT-PCR, the probability of false negatives, although low, is never zero, and the precise number of cells actually expressing the mRNA for each subunit may be somewhat higher than the experimentally determined detection rate.

Detection of these mRNAs is illustrated in Figure 9. The example in Figure 9A shows detection of Nav1.1 and Nav1.6 in isolated single neurons from the preBo\`tC region. Relative detection frequencies for each Na\(^+\) \( \alpha \)-channel subunit mRNA are depicted in Figure 9B. Significant differences in the detection frequency of Nav1.1 and Nav1.6 mRNAs were observed between the preBo\`tC and rVRG regions. Nav1.1 was more prevalent in isolated neurons from the preBo\`tC region (60%; 76 of 127) compared with the rVRG region (24%; 11 of 45; \( \chi^2 = 16.66; df = 1; \) \( p < 0.01 \)). In contrast, Nav1.2 was more frequently observed in neurons from the rVRG region (36%; 16 of 44) than in the preBo\`tC region (16%; 6 of 37; \( \chi^2 = 4.12; df = 1; \) \( p < 0.05 \)). Nav1.6 was detected at similar rates in the preBo\`tC region (50%; 38 of 74 cells) and in the rVRG (46%; 27 of 59 neurons; \( \chi^2 = 0.28; df = 1, \) NS).

Discussion

Our findings suggest that \( I_{NaP} \) is a common property expressed by respiratory neurons. However, a higher \( I_{NaP} \) density and higher \( R_{IN} \) may distinguish the interneurons in the preBo\`tC region from those in the rVRG. The differential distribution of mRNA for the Nav1.1 and Nav1.2 \( \alpha \)-subunits from the preBo\`tC region and rVRG region neurons may account for the different \( I_{NaP} \) magnitudes observed in these distinct respiratory compartments. Taking into account an estimate of Na\(^+\) channel availability for inactivation allows \( I_{NaP} \) to be closely predicted by the window current. Consequently, the preferential effects of riluzole on Na\(^+\) channel inactivation can account for the apparent selectivity of riluzole for \( I_{NaP} \).

Acutely dissociated neurons provide for improved space clamp and more accurate determination of small persistent cur-
rents; however, the loss of synaptic inputs precludes identification of respiratory neurons by their characteristic phasic discharge pattern. Nevertheless, a substantial fraction of the isolated small interneurons was likely to be respiratory, because, in vivo, the vast majority of spontaneously active neurons from the region of the VRC exhibit a respiratory-phasic discharge pattern. Moreover, the small size of respiratory interneurons distinguishes these from larger premotor and motor neurons in the VRC (Alheid et al., 2002). Topographical cues provided by FluoroGold retrograde labeling of nucleus ambiguus motoneurons greatly improved the likelihood that the preBo¨tC and VRG were selectively sampled from the VRC, while additionally facilitating the exclusion of large labeled motoneurons.

Possible basis of $I_{\text{Na}}^{\text{p}}$
Our data indicate that the amplitude and shape of $I_{\text{Na}}^{\text{p}}$ and predicted window currents coincide. Yet, it has been suggested that $I_{\text{Na}}^{\text{p}}$ and window current may derive from different mechanisms (French et al., 1990; Kay et al., 1998; Magistretti and Alonso, 1999; Maurice et al., 2001). Although $I_{\text{Na}}^{\text{p}}$ remains active at a quasi-stable, nonzero value, window current is generally predicted by the Hodgkin–Huxley equations to decline to zero at voltages of at least $-20$ mV. However, in an examination of an allosteric model of Na$^+$ channel gating, strong depolarization failed to fully inactivate Na$^+$ channels (Taddese and Bean, 2002).

In our experiments, the ratio of preBo¨tC steady-state $I_{\text{Na}}^{\text{p}}$ to the peak $I_{\text{Na}}^{\text{p}}$ was $\sim 1.5\%$, consistent with incomplete inactivation in the allosteric model. Altering the Boltzmann equation for sodium channel inactivation by including a constant estimating this steady-state fraction of Na$^+$ channels available for inactivation resulted in a predicted window current that closely approximated the measured $I_{\text{Na}}^{\text{p}}$ (Fig. 6). The observation that a conditioning train of depolarizing pulses was equally effective in driving both $I_{\text{Na}}^{\text{p}}$ and $I_{\text{Na}}^{\text{w}}$ into the inactivated state is also consistent with the same Na$^+$ channel populations underlying both persistent and transient currents and with observations in other brain regions (Fleidervish and Gutnick, 1996; Magistretti and Alonso, 1999; Urban and Belluzzi, 2000; Taddese and Bean, 2002). Nevertheless, the possibility cannot be excluded that, for a subset of neurons, two or more types of Na$^+$ channels differing in their activation voltage dependence may contribute to $I_{\text{Na}}^{\text{p}}$. This type of heterogeneity could account for the shift in the voltage dependence of the peak conductance observed in some neurons (e.g., the outliers in Figure 6B).

Riluzole, $I_{\text{Na}}^{\text{xp}}$, and $I_{\text{Na}}^{\text{NET}}$
We found that $I_{\text{Na}}^{\text{NET}}$ and $I_{\text{Na}}^{\text{xp}}$, evoked from $-80$ mV, exhibited similar sensitivities to riluzole (3 μM), reducing them 59 and 74%, respectively, consistent with a common origin for these two currents. Contributing to this effect was a 10 mV hyperpolarizing shift in the steady-state inactivation curve. A similar displacement was observed in rat dorsal root ganglion neurons (Song et al., 1997), cortical neurons (Urban and Belluzzi, 2000), and Xenopus oocytes in which the Nav1.2 Na$^+$ channel isoform was expressed (Hebert et al., 1994). The shift in the inactivation curve by riluzole can account for its apparent selectivity for $I_{\text{Na}}^{\text{p}}$ because an identity between the window current and $I_{\text{Na}}^{\text{xp}}$ means that a hyperpolarizing shift in the inactivation curve leads to a proportionally greater decrease in the $I_{\text{Na}}^{\text{p}}$ (window current) than is the case for $I_{\text{Na}}^{\text{NET}}$.

As pointed out by Urban and Belluzzi (2000), the effects of riluzole on $I_{\text{Na}}^{\text{xp}}$ and $I_{\text{Na}}^{\text{NET}}$ are consistent with the modulated receptor hypothesis in which both currents are generated by the same channels, but riluzole binds with a higher affinity to channels in the inactivated state (Hille, 1977; Kuo and Bean, 1994). In our experiments, riluzole modulation of Na$^+$ currents depended on the holding potential from which these currents were evoked, implying that riluzole has higher affinity for Na$^+$ channels in the inactivated state. We also found that riluzole decreased the activation time constant at potentials less than $-30$ mV.

Possible contribution of $I_{\text{Na}}^{\text{NET}}$ to breathing rhythm and pattern generation
The finding of substantial persistent current in all neurons tested from the VRC region agrees with previous observations (Del Negro et al., 2002a,b; Rybak et al., 2003). Additionally, peak persistent sodium conductance, current density, and $R_{\text{IN}}$ were greater in interneurons isolated from the preBo¨tC than from the immediately adjacent rVRG region. In brain slices, $I_{\text{Na}}^{\text{NET}}$ density was nominally greater for preBo¨tC pacemaker versus nonpacemaker neurons (Del Negro et al., 2002a,b), suggesting that the higher $I_{\text{Na}}^{\text{NET}}$ density reflects the higher proportion of pacemaker neurons in this region. Pacemaker activity does not depend on the magnitude of the persistent current alone; higher input resistance (as observed for preBo¨tC neurons compared with rVRG cells) also promotes firing from subthreshold activation of persistent currents (Del Negro et al., 2002a; Taddese and Bean, 2002).

Persistent sodium current supports pacemaker activity in various CNS neurons (Taddese and Bean, 2002), including a subset of preBo¨tC respiratory neurons (Thoby-Brisson and Ramirez, 2001; Del Negro et al., 2002a,b, 2005). However, $I_{\text{Na}}^{\text{NET}}$ is also present in nonpacemaker preBo¨tC neurons (Del Negro et al., 2002a, 2005) as well as in rVRG neurons (present study). Accordingly, Del Negro et al. (2002a) argued that pacemaker activity in preBo¨tC neurons depends on the ratio of $I_{\text{Na}}^{\text{NET}}$ to the leak current. In the current study, $I_{\text{Na}}^{\text{NET}}$ density in preBo¨tC neurons averaged 16.7 versus 7.7 pA/pF in rVRG neurons. These are larger than the current densities reported by Del Negro et al. (2002a) for pacemaker or nonpacemaker preBo¨tC neurons (4.3 and 2.0 pA/pF, respectively). The difference probably reflects our use of dissociated neurons with small dendritic arbor compared with relatively intact neurons within their slice preparation. This is also concordant with the smaller $C_{\text{m}}$ in our experiments, approximately one-third of that measured by Del Negro et al. (2002a). Although Del Negro et al. (2002a) reported a difference in $C_{\text{m}}$ between pacemaker and nonpacemaker neurons, our selection of similar-sized small cells for physiological recording from both the preBo¨tC and rVRG is consistent with the similarity in the $C_{\text{m}}$. We obtained for cells from these two regions. The $I_{\text{Na}}^{\text{NET}}$ in our preBo¨tC region neurons was also greater than that reported for many other CNS neurons, even where dissociated cells were used (Raman et al., 1997; Maurice et al., 2001; Carr et al., 2002; Taddese and Bean, 2002).

The mRNAs for α-subunits Nav1.1, Nav1.2, and Nav1.6 were detected in substantial numbers of neurons from both the preBo¨tC and rVRG regions, and this agrees with the RT-PCR performed on tissue samples. The ratio of $I_{\text{Na}}^{\text{NET}}$ to peak sodium current differs between α-subunits when they are expressed in Xenopus oocytes (Nav1.1, ~2% Nav1.2, ~0.5%; Nav1.6, ~2–4%) (Smith et al., 1998). The ratio observed for preBo¨tC neurons (1.5%) is consonant with a key role for Nav1.1 and Nav1.2 α-subunits in determining $I_{\text{Na}}^{\text{NET}}$ for these cells. The greater detection frequency of Nav1.1 in preBo¨tC neurons is consistent with the higher current density found in neurons from this region and, conversely, the higher prevalence of Nav1.2 in the rVRG is
consistent with the lower current density measured in these neurons. Although the presence of pacemaker neurons is characteristic of the preBo\ötzinger complex, it has been argued that they may not be essential for the maintenance of rhythmic activity. Del Negro et al. (2002b, 2005) pharmacologically blocked persistent sodium current with relatively low doses of riluzole (10 μM); this blocked bursting activity in pacemaker neurons in the preBo\ötzinger complex, confirming that $I_{\text{NaP}}$ is required for this activity. However, respiratory rhythm continued (Del Negro et al., 2002b, 2005). Although $I_{\text{NaP}}$-based pacemaker activity may not be essential for generation of respiratory rhythm, $I_{\text{NaP}}$ would be expected to modulate the intrinsic excitability of both pacemaker and nonpacemaker respiratory neurons. Subthreshold persistent sodium current could have an important role in amplifying synaptic currents supporting repetitive firing and leading to rhythm generation (Butera et al., 1999b; Lee and Heckman, 2000; Henze and Buzsáki, 2001).

In summary, $I_{\text{NaP}}$ is evident in all examined interneurons from the preBo\ötzinger and rVRG and may be closely modeled with the window current using the modified Hodgkin–Huxley equations. Higher $I_{\text{NaP}}$ density and input resistance, a combination of parameters favoring pacemaker activity (Tadose and Bean, 2002), are found in the preBo\ötzinger, a region in which VRG pacemaker neurons are more prevalent and that is thought to be crucial for the generation of rhythmic inspiratory activity.

References


