Identification of Two Types of Inspiratory Pacemaker Neurons in the Isolated Respiratory Neural Network of Mice

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Thoby-Brisson, Muriel and Jan-Marino Ramirez. Identification of two types of inspiratory pacemaker neurons in the isolated respiratory neural network of mice. J Neurophysiol 86: 104–112, 2001. In the respiratory network of mice, we characterized with the whole cell patch-clamp technique pacemaker properties in neurons discharging in phase with inspiration. The respiratory network was isolated in a transverse brain stem slice containing the pre-Bötzing complex (PBC), the presumed site for respiratory rhythm generation. After blockade of respiratory network activity with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 18 of 52 inspiratory neurons exhibited endogenous pacemaker activity, which was voltage dependent, could be reset by brief current injections and could be entrained by repetitive stimuli. In the pacemaker group (n = 18), eight neurons generated brief bursts (0.43 ± 0.03 s) at a relatively high frequency (1.05 ± 0.12 Hz) in CNQX. These bursts resembled the bursts that these neurons generated in the intact network during the interval between two inspiratory bursts. Cadmium (200 μM) altered but did not eliminate this bursting activity, while 0.5 μM tetrodotoxin suppressed bursting activity. Another set of pacemaker neurons (10 of 18) generated in CNQX longer bursts (1.57 ± 0.07 s) at a lower frequency (0.35 ± 0.01 Hz). These bursts resembled the inspiratory bursts generated in the intact network in phase with the population activity. This bursting activity was blocked by 50–100 μM cadmium or 0.5 μM tetrodotoxin. We conclude that the respiratory neural network contains pacemaker neurons with two types of bursting properties. The two types of pacemaker activities might have different functions within the respiratory network.

INTRODUCTION

Neural networks underlying rhythmic activities in vertebrates and invertebrates share similar rhythm-generating mechanisms (Marder and Calabrese 1996). In many of these networks, pacemaker neurons provide a basic rhythmic activity. Phase relationship, discharge pattern, and frequency of the rhythmic output depend critically also on synaptic interactions with other elements of the network and on neuromodulatory inputs from neurons located inside and outside of the neural network (Ayali and Harris-Warrick 1999; Katz 1995, 1998; Thoby-Brisson and Simmers 1998). Thus the resulting rhythmic activity depends on a combination of membrane and synaptic properties.

For the neural network that controls breathing in mammals, the situation seems similar. There is increasing evidence that a subpopulation of respiratory neurons express pacemaker properties (Johnson et al. 1994; Koshiya and Smith 1999; Onimaru et al. 1989; Smith et al. 1991). Pacemaker neurons seem to be primarily located in the ventrolateral medulla in a column called the ventral respiratory group (VRG) (McCrimmon et al. 2000). A relatively high concentration of inspiratory pacemaker neurons has been identified in one particular region of the VRG, the pre-Bötzing complex (Koshiya and Smith 1999; Thoby-Brisson et al. 2000). In brain stem slices containing the pre-Bötzing complex, blockade of synaptic transmission with low calcium concentrations or blockade of non-N-methyl-D-aspartate (NMDA) excitatory transmission (with 6-cyano-7-nitroquinoxaline-2,3-dione: CNQX) eliminated coordinated respiratory network activity (Funk et al. 1993), while respiratory pacemaker neurons remained rhythmically active in an a-synchronized manner (Koshiya and Smith 1999). While excitatory connections are critical for the synchronization of respiratory neuronal activity, inhibitory connections are essential for establishing the different phases of the so-called eupneic respiratory rhythm (inspiration, expiration, and postinspiration) (Lieske et al. 2000; Ramirez et al. 1997; Shao and Feldman 1997) as well as shape of inspiratory activity (Lieske et al. 2000; Ramirez et al. 1997). In the absence of synaptic inhibition, inspiratory activity ceases, but inspiratory neural activity remains rhythmic (Ramirez et al. 1997). Therefore there is increasing interest to understand how inspiratory activity is established. An obvious possibility is that inspiratory activity is generated by inspiratory pacemaker neurons, such as those described by various authors (Johnson et al. 1994; Koshiya and Smith 1999; Thoby-Brisson et al. 2000). The present study aimed at comparing in more detail the intracellularly recorded activity of inspiratory pacemaker neurons in the presence and absence of synchronized population activity (i.e., after blockade of non-NMDA glutamatergic synaptic transmission). Our investigations revealed that inspiratory pacemaker neurons exhibited two significantly different discharge and membrane properties. The identification of heterogeneous pacemaker properties will form an important basis for understanding how these properties may contribute to different aspects of the neural control of respiration.

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METHODS

Preparation of slices

Experiments were performed on brain stem transverse slices from 52 male and female mice (CD1, P6–P13). Only one neuron per slice was examined. The preparation technique, which was previously described in detail (Ramirez et al. 1996) will be summarized here. The animals were decapitated under anesthesia, and the isolated brain stem was placed in an ice-cold artificial cerebrospinal fluid (ACSF) bubbled with carbogen (95% O₂–5% CO₂). The ACSF contained (in mM) 128 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgSO₄, 24 NaHCO₃, 0.5 NaH₂PO₄, and 30 d-glucose, pH of 7.4. The brain stem, glued onto an agar block with the rostral end up, was mounted into a vibratome and serially sliced from rostral to caudal until the rostral boundary of the pre-Bötzinger complex (PBC) became visible. This area was recognized by specific landmarks such as the inferior olive (IO), the nucleus ambiguous (NA), and the hypoglossal nucleus (XII) (Fig. 1A). Five-hundred- to 600-μm-thick slices containing the PBC were transferred into a recording chamber, continuously perfused with oxygenated CSF and maintained at a temperature of 29°C. To obtain and maintain respiratory rhythmic activity the potassium concentration was raised from 3 to 8 mM over 30 min.

Recordings

Population activity recordings were obtained with suction electrodes positioned onto the surface of the PBC. The signals were amplified 2,000 times, filtered (low-pass, 1.5 kHz; high pass, 250 Hz), rectified, and integrated using an electronic filter (time constant of 30–50 ms). Integrated population activity from the PBC was in phase with integrated XII activity (Telgkamp and Ramirez 1999; Thoby-Brisson et al. 2000). Therefore extracellularly recorded PBC activity will be used as a marker for inspiratory population activity (Fig. 1).

Intracellular patch-clamp recordings were obtained from PBC neurons with the blind-patch technique. The patch electrodes were manufactured from filamented borosilicate glass tubes (Clarke GC 150TF), filled with a solution containing (in mM) 140 K-gluconic acid, 1 CaCl₂*6H₂O, 10 EGTA, 2 MgCl₂*6H₂O, 4 Na₂ATP, 10 HEPES. Inspiratory neurons were identified according to their anatomical location and their discharge characteristics. Only inspiratory neurons, active in phase with population activity, were considered in this study. The discharge pattern of each cell type was first identified in the cell-attached mode. Experiments were then performed in the whole cell patch-clamp mode. The firing pattern of the recorded neurons was not altered after establishing the whole cell patch-clamp configuration. The membrane potential values were corrected for liquid junctional potentials (LJP)s as described by Neher (1992). Recordings were stored with a personal computer on Axotape (Version 2.0, Axon Instruments) and analyzed off-line using customized analysis software.

Pharmacology

Drugs were applied at the final concentration of: 20 μM CNQX (Tocris Cookson, Ballwin, MO); 0.5 μM tetrodotoxin (TTX; Sigma, St Louis, MO); 4–200 μM cadmium (Cd; Sigma); 50 μM carbenoxolone (Sigma); and 50 μM 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, C). Depolarizing current injections (top: 0.4 nA; bottom: 1 nA) did not trigger bursting activity in the neuron in CNQX.

RESULTS

Discrimination between pacemaker and nonpacemaker inspiratory neurons

Pacemaker properties were tested for 52 inspiratory neurons (Figs. 1B and 2, Ai and Bi). After blockade of non-NMDA glutamatergic synapses with 20 μM CNQX, 34 neurons became tonically active or inactive (Fig. 1C). Depolarizing current injections triggered no bursting activity in these neurons (Fig. 1C), suggesting that they did not express intrinsic oscillatory properties. Neurons with these properties were considered nonpacemaker inspiratory neurons.

In contrast, 18 neurons remained rhythmically active in CNQX (Fig. 2). These neurons are referred to as pacemaker neurons if the remaining rhythmic activity was also reset by current injection as described in the following text. Three of these neurons were further isolated by adding to the bathing saline 50 μM carbenoxolone to block possible electrical syn-
A rhythmic bursting activity persisted also under such experimental conditions confirming our previous study (Thoby-Brisson et al. 2000). These pharmacological and electrophysiological experiments suggest that the rhythmic activity was generated independent from synaptic excitatory inputs. The pacemaker neurons had two types of discharge properties in CNQX. Eight pacemaker neurons expressed long-duration bursts (0.43 ± 0.03 s) at 1.05 ± 0.12 Hz (Fig. 2Aii). They will be called fast bursters. The 10 other pacemaker neurons expressed bursts with a relatively longer duration (1.57 ± 0.07 s) at a frequency of 0.35 ± 0.01 Hz (Fig. 2Bii). They will be called slow bursters.

The fast burster neurons, which generated short-duration bursts in CNQX, exhibited under control conditions large inspiratory bursts in phase with population activity and brief bursts of action potentials between the inspiratory bursts (Fig. 2Bi). Following the blockade of glutamatergic synapses with 20 μM CNQX (ii), Right: a sample represented at an extended time scale. Ai: voltage response (bottom) to negative current injections (scheme, 0.2-nA steps, 3-s duration) of a fast burster neuron in the presence of CNQX. B: same legend as in A for a slow burster neuron. Action potentials were truncated in Ai and Bii. Note the presence of a hyperpolarization-activated sag depolarization in Bii.

The discharge pattern of the slow burster neurons, which generated the relatively longer bursts, revealed significant differences after isolation from the network. The burst frequency generated in CNQX was significantly higher (0.35 ± 0.01 Hz; n = 8) than the frequency of inspiratory bursts generated under control conditions (0.26 ± 0.01 Hz, n = 8). Similarly, the burst duration in the presence of CNQX was significantly higher (1.57 ± 0.07 s; n = 8) than the duration of inspiratory bursts (1.12 ± 0.04 s) generated under control conditions.

Effect of Cd and TTX on the generation of brief bursts

The possible involvement of calcium currents in the generation of the brief bursts was tested by bath application of Cd, a nonspecific blocker of calcium currents. Cd was applied initially at a low concentration of 4 μM and increased progressively up to 200 μM to block all voltage-dependent calcium currents (Elsen and Ramirez 1998). All the fast burster neurons (Fig. 3A; n = 8), which generated brief bursts in CNQX, continued to burst in 200 μM Cd (Fig. 3B). The bursting frequency decreased significantly from 1.05 ± 0.12 Hz in
CNQX to 0.57 ± 0.02 Hz in CNQX + Cd. The burst duration was not significantly altered (0.43 ± 0.03 s in CNQX and 0.44 ± 0.01 s in CNQX + Cd; *P* = 0.6). However, the application of Cd altered the shape of the burst. Under control conditions and in CNQX, each burst consisted of a short train of action potentials without a significant depolarizing drive potential (Fig. 3A). In the presence of Cd, each burst was characterized by a significant depolarization (Fig. 3B).

The additional application of 0.5 μM TTX suppressed rhythmic bursting activity in all examined fast burster neurons (*n* = 7). TTX abolished initially the depolarizing burst, while action potential generation persisted for approximately 1 min (Fig. 3C, I and 2). All tested neurons became inactive after 3 min in 0.5 μM TTX (Fig. 3C3). These experiments suggest that TTX-sensitive currents are involved in the generation of the brief bursts. The persistence of a bursting activity in the presence of high concentrations of Cd suggests that calcium currents are not essential for the pacemaker properties of the fast burster neurons. The change in the shape of the bursts, however, indicates that calcium currents contribute to the bursting properties.

**Characterization of the brief bursts using current injections**

The voltage dependency, entrainment, and resetting properties of rhythmic brief bursts were examined in the presence of 20 μM CNQX and 200 μM Cd (*n* = 4). Prolonged negative or positive current injections decreased or increased the bursting frequency respectively (Fig. 4, A and B). Hyperpolarizing currents below −1 nA led in all examined neurons to a complete cessation of rhythmic activity (Fig. 4B).

Repetitive injections of brief depolarizing or hyperpolarizing stimuli (−2–−2.5 nA, 50–100 ms) could entrain the generation of the brief bursts (4 neurons tested, Fig. 5, A and B). The entrainment curve in Fig. 5C was obtained by plotting the phase of a current injection within a bursting period against the bursting period of the neuron’s intrinsic activity. Current injections entrained the bursting properties over a limited stimulation range. Entrainment was characterized by a constant phase relationship between current injection and burst generation. Stimulations were not efficient to entrain the neuron’s activity for a stimulation frequency lower than the neuron’s intrinsic bursting frequency irrespective of negative or positive current pulses. For a range of stimulation frequencies (0.87–1 Hz) that were higher than the intrinsic frequency of the neuron, the rhythmic activity of the neuron followed the frequency of the injected current. For stimulation frequencies higher than 1 Hz the bursting activity became unlocked.

The generation of rhythmic brief bursts could also be reset by current injections (50–100 ms, −2–1.5 nA; Fig. 6A; 4 neurons tested). Short hyperpolarizing current injections delivered during an on-going burst resulted in the premature termination of the burst as illustrated on Fig. 6B (see also Fig. 5B). The triggering of all-or-none bursts, the premature termination of an on-going burst, the voltage dependency, the reset, and the entrainment properties are all indicative that the bursting activity was generated intrinsically in the neurons and not synaptically driven by a remaining network.

**Effect of Cd and tetrodotoxin on the generation of long-duration bursts**

Cd effectively blocked the generation of long-duration bursts in all examined slow burster neurons (*n* = 7; Fig. 7A). The pronounced depolarizing potentials underlying each burst were greatly reduced in 50 μM Cd, but low-amplitude bursts consisting of trains of eight to nine action potentials were still generated (Fig. 7Aii). The generation of these low-amplitude bursts ceased in 100 μM Cd and all examined neurons became tonically active (*n* = 7, Fig. 7Aiii). Additional application of 0.5 μM TTX blocked the generation of the remaining action potentials, and the neurons became inactive (Fig. 7Aiv).

To determine whether the bursting mechanism was also TTX sensitive, 0.5 μM TTX was applied in three preparations before the application of Cd. In all cases, action potentials disappeared initially, followed by the elimination of depolarizing bursts as exemplified on Fig. 7B. This suggests that both Cd- and TTX-sensitive currents contribute to the generation of these long-duration bursts.
Characterization of the long-duration bursts using current injections

The voltage dependency, entrainment, and resetting properties of the rhythmic long-duration bursts were examined in the presence of 20 μM CNQX. Hyperpolarizing current injections decreased the frequency of the bursting activity (Fig. 8A). Current injections below 20.5 nA caused in all examined neurons (n = 3) a cessation of bursting activity (Fig. 8B). Depolarizing current injections increased the bursting frequency (Fig. 8A), however, only over a limited current range before bursting activity became erratic (n = 3; Fig. 8B).

Repetitive stimuli (50–100 ms, −2−2 nA) could entrain the generation of long-duration bursts, but only over a limited range (Fig. 9, A and B; n = 3). Stimulation frequencies below the neuron’s intrinsic bursting frequency (less than 0.35 Hz) failed to entrain the neuron’s activity. The neuron followed the stimulation frequency for a stimulation range between 0.35 and 0.5 Hz (Fig. 9C). For higher stimulation frequencies (more than 0.55 Hz), the bursting activity became unlocked. The

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FIG. 4. The frequency of the brief bursts generated by fast burster neurons after isolation from the network with CNQX is voltage dependent. A: intracellular recording of a fast burster neuron (bottom) during current injections (top) in 20 μM CNQX + 200 μM Cd. B: the 4 graphs represent the bursting frequency plotted against the amplitude of the current injected obtained from 4 different fast burster neurons. Note that in the 4 examples illustrated the frequency range and threshold differed for each individual neuron.

FIG. 5. Entrainment property of fast burster neurons in the presence of 20 μM CNQX + 200 μM Cd. A: intracellular recording of a pacemaker neuron (bottom) during repetitive depolarizing current injections (60 ms, 0.5 nA, 1 Hz; top). B: intracellular recording of a fast burster neuron in another slice than in A (bottom) during repetitive hyperpolarizing current injections (60 ms, −1.8 nA, 0.9 Hz; top). *, bursts, which were prematurely terminated by the negative current injection. C: phase diagram illustrating the response of the same neuron shown in B for different frequencies of stimulation (indicated on the graph). The period (Pe) was determined by measuring the duration between the onset of 2 consecutive bursts. The phase (Ph) corresponds to the time from the last burst to the time of the current injection divided by the bursting period. Stimulation artifacts were truncated in A and B.
Entrainment properties were analyzed as described for fast burster neurons. A single brief current injection (50–150 ms, 2–2 nA) could reset the rhythmic long-duration bursts (Fig. 10A, n = 3). These bursts could be terminated prematurely by negative current injection (−0.5 nA, 60 ms). Fast transient stimulation artifacts were truncated in A and B.

Discussion

In the present study we showed the existence of two different types of bursts generated in pacemaker inspiratory neurons within the respiratory neural network. These two types of bursts had distinct physiology and pharmacology (Table 1). One type of bursting activity consisted of brief bursts (0.43 s) occurring at a relatively high frequency (1.05 Hz). The other type of bursts was longer in duration (1.57 s) and occurred at a lower frequency (0.35 Hz). The brief bursts were maintained in high-Cd concentrations but were blocked by TTX. In contrast, the long-duration bursts were blocked by Cd and TTX. The two types of bursts were generated in neurons that exhibited under control conditions different discharge patterns. Fast burster neurons, which generated in CNQX brief rhythmic bursts, expressed under control conditions similar brief bursts between two consecutive inspiratory bursts. Slow burster neurons, which generated in CNQX bursts that resembled the inspiratory bursts, expressed under control conditions only tonic activity between two inspiratory bursts but no brief bursts. Although only one neuron was recorded intracellularly per slice, we believe that the two types of bursters could be found in the same preparation. While searching for pacemaker neurons with the blind-patch technique, we frequently recorded from neurons resembling the two types of bursters in the same slice. However, because these recordings were extracellular these two types of pacemaker were only identified based on their discharge patterns.

The neurons with these two distinct bursts and discharge patterns resemble type 1 and type 2 inspiratory neurons described by Rekling and co-workers (Rekling et al. 1996). According to their classification, type 1 neurons generate bursting activity during the inter-inspiratory interval, generate a hyperpolarization, which slowly repolarizes after an inspiratory burst, and possess no I\textsubscript{h} current. In our study, all the fast burster neurons generating the brief bursts fulfilled these criteria. Type 2 neurons, according to the classification by Rekling and co-workers (Rekling et al. 1996), have a different behavior. The neurons with these two distinct bursts and discharge patterns resemble type 1 and type 2 inspiratory neurons described by Rekling and co-workers (Rekling et al. 1996). According to their classification, type 1 neurons generate bursting activity during the inter-inspiratory interval, generate a hyperpolarization, which slowly repolarizes after an inspiratory burst, and possess no I\textsubscript{h} current. In our study, all the fast burster neurons generating the brief bursts fulfilled these criteria. Type 2 neurons, according to the classification by Rekling and co-workers (Rekling et al. 1996), have a different behavior.

**Fig. 7.** Burst generation in slow burster neurons is blocked by cadmium and tetrodotoxin. A: intracellular recording of a slow burster neuron (bottom) obtained simultaneously with integrated population activity (top) in the presence of 20 μM CNQX (i), CNQX + Cd (50 μM: ii; 100 μM: iii). Aiv: additional perfusion with 0.5 μM TTX abolished action potentials generation. B: intracellular recording from a slow burster neuron (bottom, different neuron than in A) obtained simultaneously with integrated population activity (top) in the presence of 20 μM CNQX (i) and in 20 μM CNQX + 0.5 μM TTX (ii). The last burst generated during TTX application is shown at the beginning of the recording. No further bursts were generated afterward.
ling et al. (1996) exhibit only regular spiking and never bursting activity in the inter-inspiratory interval, present a short-duration hyperpolarization after an inspiratory burst, and possess an \( I_h \) current. All these criteria were fulfilled also in the present study for the slow burster neurons that exhibited the long-duration bursts. However, it must be emphasized that the nomenclature for the two types of inspiratory neurons as used by Rekling et al. (1996) cannot be used for the present study because neurons were characterized in different ACSF solutions. For example, in Rekling’s study the calcium concentration was 50% reduced compared with the concentration of 1.5 mM that we used. Moreover, our slices were perfused with a CSF containing 8 mM \( K^+ \), instead of 5.4 mM, as used by Rekling et al. (1996). In addition, as we described previously.

**Fig. 8.** Voltage-dependent properties of slow burster neurons tested in 20 \( \mu M \) CNQX. **A:** intracellular recording of a slow burster neuron (bottom) during current injections (top). **B:** the 3 graphs represent the bursting frequency plotted against the amplitude of the current injected obtained from 3 different slow burster neurons. Note the individual differences between these examples.

**Fig. 9.** Entrainment property of slow burster neurons in the presence of 20 \( \mu M \) CNQX. **A:** intracellular recording of a slow burster neuron (bottom) during repetitive brief hyperpolarizing current injections (60 ms, \(-2 \) nA, 0.5 Hz; top). **B:** intracellular recording of a slow burster neuron obtained in a different slice than in **A** (bottom) during repetitive depolarizing current injections (60 ms, 1 nA, 0.5 Hz; top). **C:** phase diagram illustrating the response of the same neuron as in **B** for different stimulation frequencies (indicated on the graph). The period (Pe) and the phase (Ph) were determined as explained for fast burster neurons. Stimulation artifacts were truncated in **A** and **B**.
(Thoby-Brisson et al. 2000), the presence or absence of the $I_h$ current was not an unambiguous property to discriminate between the two types of inspiratory neurons. Some neurons, which were tonically active during the interval between two inspiratory bursts and therefore classified as type 2, had no $I_h$ current. Moreover, discrimination between two types of inspiratory neurons based on the discharge pattern is also ambiguous, as some type 2 neurons were inactive between two inspiratory bursts and did not show tonic activity. Therefore although our study clearly indicated that inspiratory pacemaker neurons in the PBC exhibit two significantly different bursting properties, uncertainty remains as to whether these two bursting properties are expressed in two “separate-able” types of inspiratory neurons.

Our results are important since previous models of respiratory rhythm generation have not considered the possibility of two types of bursting properties (Butera et al. 1999a,b; Rekling and Feldman 1998; Smith 1997; Smith et al. 1995, 2000). However, the demonstration of neurons with heterogeneous pacemaker properties in a rhythm-generating network is not new. For example, within the pyloric network of crustaceans, distinct pharmacologies characterize the two types of pacemaker activities generated by two types of neurons: the AB and PD neurons (Bal et al. 1988; Harris-Warrick et al. 1992). These neurons also differ in their neurotransmitter content (Marder and Eisen 1984a), their intrinsic membrane properties (Bal et al. 1988; Marder and Eisen 1984b), and their response to neuromodulators (Flamm and Harris-Warrick 1986; Hooper and Marder 1987). These differences enable the pyloric network to switch its configuration in response to neuromodulatory inputs and state-dependent changes. We expect that the characterization of the two types of pacemaker activities in the respiratory network may also be an important step toward understanding the mechanisms underlying rhythm generation, neuromodulation, or responsiveness to metabolic changes, such as those occurring during hypoxia. Indeed we have previously postulated that there might be different types of pacemaker activities within the respiratory network (Thoby-Brisson and Ramirez 2000). We described neurons generating bursts that were either maintained or inactivated during prolonged anoxic conditions. Neurons that generated brief bursts, as characterized in the present study, are identical with the neurons, which remained rhythmically active in anoxia. Neurons that generated the long-duration bursts were identical with the neurons inactivated during anoxia (Thoby-Brisson and Ramirez 2000).

**Membrane conductances involved in the two types of bursting activity**

The membrane conductances responsible for the rhythmic bursting activity have not been identified experimentally in any respiratory pacemaker neuron. So far, the $I_h$ current is the only conductance characterized in pacemaker neurons (Thoby-Brisson et al. 2000). Although this current plays an important role in modulating the respiratory frequency, it is not essential for burst initiation since bursting activity in inspiratory pacemaker neurons persisted after $I_h$ current blockade (Thoby-Brisson et al. 2000).

In the present study, we have demonstrated that both types of bursts were abolished in TTX, suggesting that a TTX-sensitive sodium current is critical for their generation. In fact, the computational study by Butera et al. (1999a) has previously postulated that one type of sodium current, the persistent sodium current, is important for burst generation. Our results showing that both types of bursting activity by pacemaker neurons were blocked by TTX are consistent with this hypothesis given that most known persistent sodium currents are TTX sensitive (Baker and Bostock 1997; Crill 1996; Elson and Selverston 1997; Ju et al. 1996). However, a persistent sodium current is only one possible mechanism to explain the blockade of bursting properties with TTX. Alternatively, the bursting activity could also involve a transient sodium current or a TTX-sensitive resurgent sodium current (Raman and Bean 1997). Further voltage-clamp recordings will be necessary to determine which TTX-sensitive current is involved in the bursting activity of pacemaker inspiratory neurons.

The application of Cd affected differentially the generation of the two types of bursts. Although the application of Cd allows no specific conclusions on the underlying bursting mechanisms, these experiments demonstrate that the two types of bursts do not share similar calcium-dependent mechanisms for burst initiation or termination. In Cd, the brief bursts exhibited a larger amplitude suggesting that the inward TTX-sensitive current (discussed in the preceding paragraph) is opposed by a Cd-sensitive outward current. A balance between these two currents may lead to the brief burst of action potentials, which were typically characterized by their small amplitude. In contrast, low concentrations of Cd abolished the long-duration bursts, and the neurons exhibited only low-amplitude

**TABLE 1. Overview of the electrophysiological properties of fast and slow pacemaker inspiratory neurons**

<table>
<thead>
<tr>
<th>Pacemaker Neuron</th>
<th>Fast</th>
<th>Slow</th>
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<tbody>
<tr>
<td>Pattern of discharge between two inspiratory burst before isolation</td>
<td></td>
<td></td>
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<tr>
<td>$I_h$ current</td>
<td>Bursting</td>
<td>Tonic</td>
</tr>
<tr>
<td>Burst duration (s) after isolation</td>
<td>0.43 ± 0.03</td>
<td>1.57 ± 0.07</td>
</tr>
<tr>
<td>Burst frequency (Hz) after isolation</td>
<td>1.05 ± 0.12</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Ionic bursting mechanisms</td>
<td>TTX sensitive, Cd insensitive</td>
<td>TTX sensitive, Cd sensitive</td>
</tr>
</tbody>
</table>

Values are means ± SD. Cd, cadmium.
bursts. At higher Cd concentrations, these bursts were abolished and the neurons became tonically active. This suggests that an inward calcium current is involved in the triggering of the long-duration bursts. Further experiments will be necessary to determine which calcium conductance(s) is (are) involved in the two bursting activity of inspiratory pacemaker neurons. However, the present study shows that bursting activity in inspiratory pacemaker neurons may depend on a complex interplay between calcium and sodium currents. Differences in this interplay may explain the distinct properties of the two types of inspiratory pacemaker activities. It is important to note that the exact balance between calcium and sodium currents may also be species-specific. Our study was performed in respiratory neurons of mice, which may have different bursting properties than pacemaker neurons that were recorded in rats (Johnson et al. 1994; Koshiya and Smith 1999; Onimaru et al. 1989; Smith et al. 1991).

Although it is tempting to speculate whether the two types of pacemaker activities as characterized in the present study fulfill different functions in the intact respiratory network, our experiment did not address this issue. However, the description of two pharmacologically distinct burst mechanisms and the observation that these two mechanisms are associated with two different responses during hypoxia (Thoby-Brisson and Ramirez 2000) may provide a first important step for future studies investigating possibly differential roles in respiratory rhythm generation.

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