Norepinephrine Differentially Modulates Different Types of Respiratory Pacemaker and Nonpacemaker Neurons

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INTRODUCTION

Many mammalian neuronal networks generate rhythmic activity. Rhythm-generating networks are found for example in the spinal cord, brain stem, neocortex, thalamus, locus coeruleus, ventral tegmental area, hippocampus, and amygdala. This rhythmicity is associated with major behavioral functions, including the control of breathing, walking, sleep, wakefulness, arousal, movement, motivation, addiction, memory consolidation, cognition, and fear (Ramirez et al. 2004). These networks contain pacemaker neurons, i.e., neurons with the intrinsic ability to generate rhythmic activity in form of bursts of action potentials (Llinas and Sugimori 1980; Ramirez et al. 2004; Schwindt and Crill 1982; Traub and Llinas 1979; Tresch and Kiehn 2000). Because of their intrinsic bursting ability, it has been hypothesized that pacemakers are crucial for understand-

Viemari, Jean-Charles and Jan-Marino Ramirez. Norepinephrine differentially modulates different types of respiratory pacemaker and nonpacemaker neurons. J Neurophysiol 95: 2070–2082, 2006. First published January 4, 2006; doi:10.1152/jn.01308.2005. Pacemakers are found throughout the mammalian CNS. Yet, it remains largely unknown how these neurons contribute to network activity. Here we show that for the respiratory network isolated in transverse slices of mice, different functions can be assigned to different types of pacemakers and nonpacemakers. This difference becomes evident in response to norepinephrine (NE). Although NE depolarized 88% of synaptically isolated inspiratory neurons, this neuromodulator had differential effects on different neuron types. NE increased in cadmium-insensitive pacemakers burst frequency, not burst area and duration, and it increased in cadmium-sensitive pacemakers burst duration and area, but not frequency. NE also differentially modulated nonpacemakers. Two types of nonpacemakers were identified: “silent nonpacemakers” stop spiking, whereas “active nonpacemakers” spontaneously spike when isolated from the network. NE selectively induced cadmium-sensitive pacemaker properties in active, but not silent, nonpacemakers. Flufenamic acid (FFA), a blocker of $I_{\text{CAN}}$, blocked the induction as well as modulation of cadmium-sensitive pacemaker activity, and blocked at the network level the NE-induced increase in burst area and duration of inspiratory network activity; the frequency modulation (FM) was unaffected. We therefore propose that modulation of cadmium-sensitive pacemaker activity contributes at the network level to changes in burst shape, not frequency. Riluzole blocked the FM of isolated cadmium-insensitive pacemakers. In the presence of riluzole, NE caused disorganized network activity, suggesting that cadmium-insensitive pacemakers are critical for rhythm generation. We conclude that different types of nonpacemaker and pacemaker neurons differentially control different aspects of the respiratory rhythm.

METHODS

The transverse slice preparation

Brain stem transverse slice preparation from CD1 mice (P6–P13) were obtained using a technique described in detail previously (Ramirez et al. 1996). The most important steps are summarized here. The experimental procedures were approved by the Institutional Animal Care and Use at the University of Chicago. The mice were deeply anesthetized with ether and decapitated. The isolated brain stem was then placed in 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 MgCl₂, 24 NaHCO₃, 0.5 NaH₂PO₄, and 30 d-glucose (pH 7.4). The brain stem, glued rostral end-up onto an agar block, was mounted into a vibratome (Leica Microsystems, Waukegan, IL) and serially sliced until the rostral boundary of the pre-Bötzinger complex (PBC; Smith et al. 1991) was identified by anatomical landmarks such as disappearance of the facial nucleus and appearance of the inferior olive, the nucleus ambiguous, and the hypoglossal nucleus. A single 650-μm-thick slice was then taken. Because the slices also contained regions caudal to the PBC, we refer to the area encompassed in the slice as the ventral respiratory group (VRG). Slices were transferred into a recording chamber, continuously superfused with oxygenated aCSF, and maintained at a temperature of 30 ± 0.5°C. To initiate and maintain fictive respiratory rhythmic activity the potassium concentration of the perfusate was raised from 3 to 8 mM over 30 min (see also Tryba et al. 2003).

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**Drugs and solutions**

A cocktail of antagonists for N-methyl-d-aspartate (NMDA) receptors [RS-(±)-2-(4-chlorophenoxypyridoinic acid [(RS)±CPP or CPP] 10 μM, Tocris Cookson, Ellisville, MO), non-NMDA receptors [6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) 20 μM, Tocris Cookson], glycine receptors (strychnine 1 μM, Sigma-RBI, St. Louis, MO), and γ-aminobutyric acid type A (GABAₐ) receptors (bicuculline-free base 20 μM, Sigma-RBI) was used to block fast synaptic transmission (Peña et al. 2004). Note that the bicuculline-free base used in the present study has a pharmacology very different from that of the commonly used bicuculline salts (e.g., bicuculline methiodide), and the free base does not block amain-sensitive calcium-activated potassium currents (Debarbieux et al. 1998; Johnson and Seutin 1997; Seutin and Johnson 1999). To block either the persistent sodium or the CAN current we bath-applied riluzole hydrochloride (Tocris Cookson, Ballwin, MO and Sigma-RBI) or flufenamic acid (FFA, Sigma-RBI), which has a different mode of action. We previously demonstrated that the whole cell configuration does not alter the firing pattern of the recorded neuron (Peña et al. 2004). The patch electrodes were pulled from filamented borosilicate glass tubes (G150F-4; Warner Instruments, Hamden, CT) and filled with a solution containing 140 mM K-gluconic acid, 1 mM CaCl₂ 6H₂O, 10 mM EGTA, 2 mM MgCl₂ 6H₂O, 4 mM Na₂ATP, and 10 mM HEPES. The composition of this intracellular solution and the lack of adverse effects on pacemaker activity were previously addressed (Del Negro et al. 2005; Peña et al. 2004). The K-gluconic acid–containing electrode solution resulted in a significant liquid junction potential (LJP, 12 mV), which affected measured membrane potentials. All electrode solution resulted in a significant liquid junction potential (LJP, 12 mV), which affected measured membrane potentials. All electro
de recorded bursts.

**INTRACELLULAR RECORDINGS.** We recorded inspiratory VRG neurons (one neuron per slice) using the blind patch-clamp recording method. Inspiratory neurons were first identified in the cell-attached mode, which revealed their discharge pattern in phase with population activity. Experiments were then performed in whole cell configuration with the neuron recorded in current clamp at the zero current potential. We previously demonstrated that the whole cell configuration does not alter the firing pattern of the recorded neuron (Peña et al. 2004). The patch electrodes were pulled from filamented borosilicate glass tubes (G150F-4; Warner Instruments, Hamden, CT) and filled with a solution containing 140 mM K-gluconic acid, 1 mM CaCl₂ 6H₂O, 10 mM EGTA, 2 mM MgCl₂ 6H₂O, 4 mM Na₂ATP, and 10 mM HEPES. The composition of this intracellular solution and the lack of adverse effects on pacemaker activity were previously addressed (Del Negro et al. 2005; Peña et al. 2004). The K-gluconic acid–containing electrode solution resulted in a significant liquid junction potential (LJP, 12 mV), which affected measured membrane potentials. All membrane potential values were corrected for this LJP as described by Neher (1992).

**Electrophysiology**

**EXTRACELLULAR RECORDINGS.** In the transverse slice preparation population activity recordings were obtained with suction electrodes positioned on the surface of the slice in the area of the nucleus ambiguous (i.e., dorsal to the PBC). The signals were amplified 2,000×, 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 VRG was always in phase with integrated inspiratory activity of the hypoglossal motor nucleus (Telgkamp and Ramirez 1999). Therefore it was used as a marker for inspiratory population activity (Fig. 1A). All recordings were stored on a personal computer using AxoTape (Version 2.0, Axon Instruments, Union City, CA) and analyzed off-line using customized analysis software written with IGOR Pro (Wavemetrics, Lake Oswego, OR). Bursts were automatically detected by the IGOR program. After setting a Y-threshold value in the integrated recording, the program detects voltage deflection above this value. A minimum burst duration was set at 200 ms and a minimum time between bursts was set at 500 ms to minimize the detection of voltage deflections that were not caused by respiratory bursts. Each automatically detected burst was automatically highlighted with a green color. This allowed us to manually scroll through the entire recording to verify that the computer-detected bursts were indeed respiratory bursts and that the computer ignored voltage deflections that were caused for example by artifacts (e.g., static discharges). Once we accepted the computer-detected bursts, for each burst the computer calculated burst duration (width at half-maximal amplitude), amplitude (maximal voltage deflection), rise time, decay time, and burst area (burst area was calculated from the integrated extracellular activity as the area between the baseline and the burst envelope). The computer measured for each burst the voltage beginning 500 ms before and ending 500 ms after crossing the threshold. These values were automatically sorted in tables that were then imported into Excel or other statistics (Sigma plot) and graphics programs (Corel Draw). These values were also used to analyze regularity of the VRG population. In this case the computer calculated the coefficient of variability of the cycle period as described previously (Viemari et al. 2004).

This computerized method was reliable only when used in conjunction with qualitatively good recordings that were characterized by a good signal-to-noise ratio. A good signal-to-noise ratio between the amplitude of the burst and the SD of the recording during the interburst interval was considered as 35.

In case of intracellular recordings this automated program could detect bursts, but the amplitude and burst duration values were not useful because of the presence of action potentials. For this reason we used cursor values to assess changes in the membrane potential (V_m) of automatically detected, intracellularly recorded bursts.

The data were analyzed with SPSS software (SPSS Science Software, Chicago, IL). The burst duration, area, and frequency changes induced by aCSF-containing drugs were assessed by a Student’s paired t-test. In other cases, a one-way ANOVA was used for repeated measures in the same subjects, followed by Tukey’s tests as multiple-comparisons procedure. Statistical significance was assumed to be significant if P < 0.05. Deviations from the mean are given in SE.

**RESULTS**

**Activation of alpha-1 noradrenergic receptors modulates respiratory population activity**

Transverse slice preparations generate two types of inspiratory activity that can be recorded as population discharges from the ventral respiratory group (VRG) under control conditions. We refer to these activities as fictive eupnea and fictive sigh (Lieske et al. 2000). Application of 20 μM norepinephrine (NE) significantly increased the burst duration, area, and frequency of fictive eupneic activity (147 ± 21%, P = 0.033, 218 ± 21%, P = 0.008, 245 ± 45%, P < 0.001, respectively;...
Fig. 1. Alpha-1 noradrenergic receptors activate the respiratory network. A: schematic of the slice preparation (left) illustrating the recording sites of extracellular ventral respiratory group (VRG) activity ($f_{VRG}$) and intracellular whole cell patch-clamp recording site. Right: integrated VRG activity ($f_{VRG}$) (top trace) recorded simultaneously with an inspiratory neuron (bottom trace, membrane potential, $V_m$). B: integrated VRG activity (B1) increases in frequency, duration, and burst area on application of norepinephrine (NE) (B2, 20 μM). Integrated VRG activity (C1) increases in frequency, duration, and burst area on application of phenylephrine (PHE) (C2), an alpha-1 noradrenergic receptor agonist. Integrated VRG activity (D1) is not affected by the alpha-1 noradrenergic receptor antagonist prazosin (D2, 50 μM), but prazosin blocks the effect of norepinephrine (D3, NE). Histograms summarizing the noradrenergic effects on burst duration (E1, ordinate), burst area (E2, ordinate), and burst frequency (E3, ordinate). Significant changes were caused by application of NE and PHE ($^{*}P < 0.05$, Student’s paired $t$-test) compared with control. Results are expressed as mean ± SE.

Table 1. Effects of norepinephrine in the network

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NE</th>
<th>PHE</th>
<th>Control</th>
<th>Prazosin</th>
<th>Prazosin + NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration, s</td>
<td>0.39 ± 0.02</td>
<td>0.56 ± 0.06*</td>
<td>0.41 ± 0.04</td>
<td>0.53 ± 0.03*</td>
<td>0.45 ± 0.04</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>Area, mV</td>
<td>0.11 ± 0.01</td>
<td>0.24 ± 0.04*</td>
<td>0.07 ± 0.02</td>
<td>0.14 ± 0.03*</td>
<td>0.12 ± 0.02</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Frequency, Hz</td>
<td>0.19 ± 0.04</td>
<td>0.37 ± 0.05*</td>
<td>0.2 ± 0.03</td>
<td>0.44 ± 0.05*</td>
<td>0.24 ± 0.02</td>
<td>0.26 ± 0.02</td>
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</table>

NE, norepinephrine; PHE, phenylephrine; *$P < 0.05$, Student’s paired $t$-test.
In the present study we focused on the cellular mechanisms underlying the neuromodulation of fictive eupnea and, more specifically, fictive inspiratory activity. For this reason we obtained intracellular recordings from inspiratory neurons. These neurons are characterized by their rhythmic discharge in phase with VRG population activity (Figs. 1A, 2A, and 3A). We examined the effect of NE in 12 neurons that were embedded in the functional network. NE depolarized all these inspiratory neurons by an average of 3.1 ± 0.3 mV. The duration, area, and frequency of the intracellularly recorded bursts increased by 145 ± 11%, P = 0.016; 205 ± 25%, P = 0.007; and 195 ± 32%, P = 0.007, respectively (Table 2).

To further investigate the cellular effects of NE, we repeated the same experimental procedure in another set of inspiratory neurons (n = 60) that were synaptically isolated before NE application. For this purpose, synaptic transmission was blocked with a cocktail of antagonists containing 10 μM CNQX to block non-NMDA glutamatergic transmission, 10 μM CPP to block NMDA glutamatergic transmission, 20 μM bicuculline to block GABAergic synaptic transmission, and 1 μM strychnine to block glycineric transmission. In 88% of these synaptically isolated inspiratory neurons NE caused a depolarization of 3.7 ± 0.3 mV (n = 53). The depolarization was mimicked by the alpha-1 noradrenergic receptor agonist phenylephrine (20 μM), which depolarized the inspiratory neurons on average by 3.76 ± 0.3 mV (n = 5). Six out of the 60 examined neurons were hyperpolarized on average by 3.48 ± 0.42 mV. This hyperpolarization was blocked by the alpha-2 noradrenergic receptor antagonist yohimbine (50 μM, n = 4), suggesting that NE acted in these neurons on the alpha-2 receptors. Only one out of 60 inspiratory neurons was unaffected by NE. In the remainder of this study, we further investigated the cell type–specific effects of NE. Because the vast majority of neurons were depolarized and because of the overall excitatory effect on the respiratory network, we analyzed only those neurons that were depolarized by NE.

Two types of nonpacemaker neurons

All inspiratory neurons were further classified by characterizing their activity after the blockade of synaptic transmission with the cocktail of antagonists. In the presence of this cocktail rhythmic network activity is lost, as is evident in the integrated traces (Fig. 2B). 31 neurons that were rhythmically active in phase with the inspiratory population activity under control conditions (Fig. 2A) were no longer rhythmic because population activity ceased in the presence of this cocktail (Fig. 2B). These neurons are defined as “nonpacemaker neurons” (Table 3). The population of nonpacemaker neurons was heterogeneous. Of the nonpacemaker neurons (n = 10) 32% became silent (Figs. 2B1 and 3B), whereas 68% (n = 21) of the nonpacemaker neurons continued to generate action potentials after synaptic isolation (Fig. 2B2). We refer to the two types of nonpacemaker neurons as “silent nonpacemaker” and “active nonpacemaker neuron,” respectively (Table 3). Because the cocktail contained CNQX, we hypothesize that the activity in the “active nonpacemaker neurons” was generated by intrinsic membrane properties and was not synthaptically driven by non-NMDA–mediated glutamatergic transmission. The two types of nonpacemaker neurons exhibited no obvious pacemaker properties in response to depolarizing current injections (Fig. 2, C and D). This lack of voltage-dependent bursting was another criterion to define these neurons as nonpacemaker neurons.

NE depolarizes “silent nonpacemaker neurons”

Application of 20 μM NE depolarized all silent nonpacemaker neurons by 3.70 ± 0.45 mV (n = 10, Fig. 3C). In all examined silent nonpacemaker neurons, the NE-induced depolarization was blocked by the alpha-1 noradrenergic receptor antagonist prazosin (n = 2), further confirming the hypothesis that this excitatory effect was mediated by the alpha-1 noradrenergic receptor. To test the effect of NE on the excitability, we injected in six silent nonpacemaker neurons depolarizing current pulses to evoke trains of action potentials in the presence and absence of NE. The application of 20 μM NE increased the mean number of evoked action potentials by 236 ± 32% without inducing pacemaker properties (P = 0.004, n = 6, Fig. 3D).

NE induces bursting properties in “active nonpacemaker neurons”

Application of NE depolarized 20 of the 21 examined active nonpacemaker neurons and transformed their activity pattern into regular bursting activity (pacemaker activity, Fig. 4, A and B, n = 20). Fifteen of these active non-pacemaker neurons were quantitatively analyzed and depolarized by 3.31 ± 0.36 mV in response to 20 μM NE. The five neurons that were not further analyzed had qualitatively similar responses (i.e., were depolarized and showed intrinsic bursting), but could not be quantitatively analyzed because we used either a different concentration of NE or because the recordings were unstable and/or lost shortly after the NE application. One active nonpacemaker was unaffected by NE. Application of 50 μM of the alpha-1 noradrenergic receptor antagonist prazosin blocked the effect of NE in all examined neurons, suggesting that this action resulted from activation of alpha-1 noradrenergic receptors (n = 2, not shown).

The induced pacemaker properties were voltage dependent and could be reset by current injection (Fig. 4B2), indicating that the active nonpacemaker neurons became voltage-dependent pacemaker neurons in the presence of NE. We refer to neurons as “conditional” pacemaker neurons if they do not burst on synaptic isolation, but burst in the presence of a neuromodulator, in this case NE. Thus in our study 20 out of 21 examined “active nonpacemaker neurons” were also conditional pacemaker neurons in the presence of NE.

The properties of these “active nonpacemaker neurons” were further characterized in a scatterplot of interspike intervals (ISIs). In the absence of NE and in the presence of the cocktail, the ISIs were widely distributed (Fig. 4C1). The wide scatter of

<table>
<thead>
<tr>
<th>Duration, s</th>
<th>Control</th>
<th>Norepinephrine</th>
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<tbody>
<tr>
<td></td>
<td>0.38 ± 0.06</td>
<td>0.54 ± 0.05*</td>
</tr>
<tr>
<td>Area, mV</td>
<td>3.9 ± 0.8</td>
<td>8.2 ± 1.8*</td>
</tr>
<tr>
<td>Frequency, Hz</td>
<td>0.19 ± 0.02</td>
<td>0.37 ± 0.05*</td>
</tr>
</tbody>
</table>

*P < 0.05, Student’s paired t-test.
ISIs reflects the irregular discharge pattern of active nonpacemaker neurons. In other words active nonpacemaker neurons were not regular spiking neurons in the absence of synaptic input. Application of NE induced pacemaker activity and resulted in narrow clusters of ISIs (Fig. 4C2). Note that the most prominent ISIs, characteristic for the pacemaker bursts (0.2 s, Fig. 4C1), were very rarely seen in the “nonpacemaker state” (Fig. 4C1). We quantitatively characterized the ISIs of 15 active nonpacemaker neurons by comparing the distribution of 250 ISIs from successive spikes in the presence of the cocktail (Fig. 4D1) and after the subsequent application of NE (Fig. 4D2). Under cocktail conditions, active nonpacemakers were characterized by a mean ISI of 0.38 ± 0.08 s (n = 15, Fig. 4D1) and a high coefficient of variation (0.53 ± 0.07, n = 15). In the presence of NE (Fig. 4D2), the number of ISIs <0.2 s was significantly increased (P < 0.001) compared with the number of ISIs <0.2 s in control conditions, thus confirming the qualitative difference shown in Fig. 4, C1 and C2. The coefficient of variation of the ISI distribution was also decreased in NE (0.26 ± 0.05, n = 15), confirming the qualitative observation that the neurons in the nonpacemaker state are characterized by an irregular discharge pattern, whereas in the bursting mode action potentials are generated in consistently short ISIs.

**NE induces bursting properties by a calcium-dependent mechanism**

As a first step to investigate the cellular mechanisms involved in the induction of pacemaker properties by NE, we performed experiments under low sodium conditions. Active nonpacemaker neurons (Fig. 5A1) became conditional pacemakers in the presence of NE (Fig. 5A2), and the conditional pacemaker activity continued in the presence of low sodium concentrations (Fig. 5A3), but was subsequently blocked by

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**TABLE 3.** Different types of nonpacemaker and pacemaker neurons

<table>
<thead>
<tr>
<th>Neuron Type</th>
<th>Neuron Subtype</th>
<th>Number</th>
<th>NE Effects</th>
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<tbody>
<tr>
<td>Nonpacemaker</td>
<td>Silent</td>
<td>10</td>
<td>Depolarization</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>21</td>
<td>Depolarization and induction of bursts</td>
</tr>
<tr>
<td>Pacemaker</td>
<td>Cadmium-sensitive</td>
<td>14</td>
<td>Depolarization and increased burst area and burst duration</td>
</tr>
<tr>
<td></td>
<td>Cadmium-insensitive</td>
<td>8</td>
<td>Depolarization and increased burst frequency</td>
</tr>
</tbody>
</table>

**FIG. 2.** Two types of nonpacemaker inspiratory neurons. A1, A2: intracellular recording of inspiratory neurons (top traces) discharge rhythmically in phase with the simultaneously recorded VRG population activity (bottom traces). B1: silent nonpacemaker neuron (top trace) loses rhythmic activity after blockade of network activity (bottom trace) with a cocktail containing 10 μM CPP, 20 μM CNQX, 20 μM bicuculline, and 1 μM strychnine. B2: active nonpacemaker neuron loses rhythmic activity (top trace) after blockade of network activity (bottom trace) with the cocktail. Note that the neuron continues to generate spontaneously action potentials. C, D: depolarizing current pulses injected at different strengths (bottom traces), into the synaptically isolated silent nonpacemaker inspiratory neuron (top traces, C1, D1) and into the active nonpacemaker inspiratory neuron (top traces, C2, D2). Note that the 2 types of nonpacemaker neurons exhibit no pacemaker properties in response to the depolarizing current injections.
of pacemaker neurons can be differentiated based on the sensitivity to cadmium under control conditions as previously described (Thoby-Brisson and Ramirez 2001). These pacemaker neurons typically generated tonic burst activity by NE (Fig. 8A), and after isolation from the network, pacemaker properties were lost. Pretreatment with 200 μM cadmium eliminated the rhythmic activity, rendering this nonpacemaker neuron silent. Note that NE significantly increases the number of action potentials (P < 0.05; Student’s paired t-test, n = 6).

200 μM cadmium in all examined neurons (Fig. 5A4, n = 3). Cadmium is a general blocker of calcium channels (Del Negro et al. 2005; Elsen and Ramirez 1998; Peña et al. 2004; Thoby-Brisson and Ramirez 2001).

NE transformed reversibly active nonpacemakers (Fig. 5B1) into conditional pacemakers (Fig. 5B2). After 5 min of washout, pacemaker properties were lost. Pretreatment with 200 μM cadmium (Fig. 5B3) blocked the induction of pacemaker activity by NE (Fig. 5B4, tested in four “active nonpacemaker neurons”), further supporting the conclusion that the induction of pacemaker properties depended on a calcium mechanism. In four active nonpacemaker neurons (Fig. 5C1), bursting was induced by NE (Fig. 5C2), which was subsequently blocked by 500 μM flufenamic acid (FFA), a blocker of the CAN current (Fig. 5, C3 and C4; see also Peña et al. 2004). We therefore propose that NE induces CAN current–dependent bursting properties in active nonpacemaker neurons.

NE alters burst shape, but not frequency, in Cd-sensitive pacemaker neurons

As previously reported, the cocktail of synaptic antagonists identifies not only nonpacemaker neurons but also neurons that continue to burst rhythmically after the blockade of synaptic transmission. These pacemaker neurons typically generated ectopic bursts under control conditions as previously described (Peña et al. 2004; Thoby-Brisson and Ramirez 2001). Two types of pacemaker neurons can be differentiated based on the sensitivity of their bursting properties to cadmium. In the present study 200 μM cadmium was applied at the end of the experiment to classify a given pacemaker neuron as either “cadmium-sensitive” or “cadmium-insensitive” (Tables 3 and 4).

Cadmium-sensitive pacemaker neurons burst in phase with inspiratory population activity (Fig. 6A) and continued to burst after pharmacological isolation from the network (Fig. 6B). As illustrated in Fig. 6C and in Table 4, NE increased burst duration, on average by 157 ± 25% (P = 0.022, n = 14) and burst area by 245 ± 25% (P = 0.02, n = 14) without significantly affecting burst frequency (95 ± 13%, P = 0.131, n = 14).

In the presence of NE we found that the bursting properties of cadmium-sensitive pacemaker neurons were not different from the bursting properties of the conditional pacemaker neurons: Bursting frequency of cadmium-sensitive pacemakers: 0.58 ± 0.08 Hz, n = 14, versus bursting frequency of conditional pacemakers: 0.62 ± 0.09 Hz, n = 15, P = 0.78. Burst duration of cadmium-sensitive pacemakers: 0.85 ± 0.11 s, n = 14 versus burst duration of conditional pacemakers: 1.18 ± 0.16 s (n = 15, P = 0.78). Application of cadmium (200 μM) abolished the bursting in cadmium-sensitive pacemaker neurons, and all examined cadmium-sensitive pacemaker neurons began to discharge tonically (Fig. 6D). In addition, in all four cases in which we applied 500 μM FFA, a blocker of the CAN current (Peña et al. 2004), bursting was abolished (not shown).

NE alters frequency, but not burst shape, in Cd-insensitive pacemaker neurons

As shown in Fig. 7A, “cadmium-insensitive” pacemakers continued to burst after isolation from the network. Note that we refer to cadmium-insensitive pacemakers as those neurons in which pacemaker bursting persists in cadmium. We do not want to imply that calcium-dependent (i.e., cadmium-sensitive) mechanisms play no role in the discharge pattern of these neurons. Application of NE significantly increased in cadmium-insensitive pacemaker neurons the burst frequency on average by 187 ± 34% (Fig. 7B, Table 4, P = 0.016, n = 8), without significantly affecting burst duration and area (118 ± 13%, P = 0.21 and 117 ± 14%, P = 0.39, respectively, n = 8, Table 4). Application of 200 μM cadmium did not abolish bursting (Fig. 7C). However, bursting properties were eliminated in the presence of 20 μM riluzole (Fig. 7D), suggesting that I_{NaP} is essential for the generation of bursting properties in Cd-insensitive neurons (Del Negro et al. 2002, 2005; Peña et al. 2004). Note that the activity shown in Fig. 7D reflects only single action potentials, but not bursts.

Blockade of CAN and persistent sodium currents in the functional neuronal network

In Fig. 8A, we investigated the effect of blocking the CAN current while simultaneously recording from inspiratory neurons. The application of NE (Fig. 8B) increased the intracellularly recorded burst duration by 129 ± 11% (Fig. 8E, P = 0.016, n = 5), burst area by 202 ± 23% (Fig. 8F, P = 0.007,
n = 5), and burst frequency by 148 ± 9% (Fig. 8G, P = 0.009, n = 5). After washout, these values returned to control values. We subsequently applied 500 μM FFA to block the CAN current (Fig. 8C). By 5 min after application of 500 μM FFA alone, there was no significant effect on burst duration (Fig. 8E), area (Fig. 8F), or frequency (Fig. 8G). The subsequent application of NE (Fig. 8D) increased the intracellularly recorded burst frequency by 137 ± 5% (Fig. 8G, P = 0.018, n = 5), but had no significant effect on burst duration (Fig. 8E, 104 ± 3%, P = 0.43, n = 5) or burst area (Fig. 8F, 78 ± 18%,
The involvement of the persistent sodium current. We blocked the presence of 500 nM FFA with riluzole (Fig. 9A; Del Negro et al. 2002, 2005; Peña et al. 2004; Spadoni et al. 2002; Urbani and Belluzzi 2000). After the blockade of Isnap, application of NE was still able to increase the burst frequency (Fig. 9B). This effect was evaluated in a histogram (Fig. 9D). However, the frequency increase was transient, and after 5 min of NE application (Fig. 9, C and D) the rhythm completely disintegrated in all examined slices (n = 7). As part of this disintegration process there was a loss of burst structure (e.g., see Fig. 9C).

**DISCUSSION**

**Recruitment of pacemaker properties**

The data presented in this study indicate that the biogenic amine norepinephrine (NE) alters the duration, area, and frequency of respiratory rhythmic activity through the differential modulation of two populations of pacemaker neurons and two populations of nonpacemaker neurons. By inducing cadmium-sensitive bursting properties in active nonpacemaker neurons NE substantially increases the number of neurons with cadmium-sensitive pacemaker properties in the network. This population of neurons with cadmium-sensitive pacemaker properties consists of those neurons that were cadmium-sensitive pacemakers already before the addition of NE and those neurons that were initially active nonpacemakers and developed cadmium-sensitive pacemaker properties in the presence of NE. In our hands, NE never induced cadmium-insensitive pacemaker properties. We therefore hypothesize that the neuromodulatory milieu determines not only the number but also the types of active pacemaker properties in the respiratory network. We previously demonstrated that cadmium-insensitive pacemaker activity depends on the endogenous activation of the 5-HT2A receptor (Peña and Ramirez 2002). Thus although NE changes the number of neurons with cadmium-sensitive pacemaker properties, changes in endogenous serotonin levels will presumably alter the number of neurons with cadmium-insensitive pacemaker properties. We therefore propose that the number of neurons with cadmium-sensitive pace-
maker properties depends on the level of NE, whereas the number of neurons with cadmium-insensitive pacemaker properties is dependent on the level of serotonin (Peña and Ramirez 2002). This differential dependency may be functionally important because it allows neuromodulators to differentially modulate shape and stability of respiratory activity. The principle described here for the mammalian respiratory system has striking similarities with the situation in invertebrate neuronal networks, in which pacemaker and network activities depend on the presence of, and differential modulation by, amines as well as peptides (Ayali and Harris-Warrick 1999; Destexhe and Marder 2004; Thoby-Brisson and Simmers 1998). To what extent the silent nonpacemaker neurons that were also described in the present study are follower neurons without a particular role in respiratory rhythm generation or show pacemaker properties in the presence of another neuromodulator cannot be determined from our experiments.

**Cellular effects of norepinephrine**

Norepinephrine exerts its primary modulatory effect by acting on two types of noradrenergic receptors. It has excitatory effects through alpha-2 and inhibitory effects through alpha-1 noradrenergic receptors. Although both receptors are present within the respiratory network, the balance between the two receptors is species dependent (Hilaire et al. 2004). It seems that in rats, respiratory effects are primarily mediated through alpha-2 effects (Al-Zubaidy et al. 1996; Hilaire et al. 2004), whereas in mice respiratory effects are primarily excitatory and mediated by the alpha-1 receptor (Viemari et al. 2004). Consistent with results from the "en bloc preparation" in mice (Viemari and Hilaire 2002; Viemari et al. 2004), we find that the noradrenergic effects reported here for slices seem to be mediated by the alpha-1 receptor. Moreover, this excitatory effect of NE was mimicked by phenylephrine and blocked by prazosin, both specific alpha-1 noradrenergic substances.

Norepinephrine caused a general depolarization in the majority of respiratory neurons. However, in addition NE induced cadmium-sensitive properties in active pacemaker neurons. Because of the nonlinear properties associated with bursting, NE exerts not only a quantitative but rather a qualitative change in network activity. Moreover, NE differentially modulated cadmium-sensitive and cadmium-insensitive pacemaker neurons, changing the burst shape in cadmium-sensitive and burst frequency in cadmium-insensitive pacemaker neurons. Again, this modulatory action exerts not only a quantitative but rather qualitative effect on the network as different parameters are differentially modulated. These differential effects were presumably caused by the modulation of different ion channels. Alpha-1 noradrenergic receptor activation has been shown to open nonspecific cationic conductances (Berretta et al. 2000; Carette 1999; Ishibashi et al. 2003), consistent with the hypothesis that NE increased a CAN current in the cadmium-sensitive pacemaker neurons. This modulatory effect
persisted in low sodium concentrations and was blocked by cadmium as well as FFA, a known blocker for the CAN current (Di Prisco et al. 2000; Partridge and Valenzuela 2000; Peña et al. 2004). The modulatory action was different from the effect of NE on cadmium-insensitive pacemaker neurons, which were insensitive to the blockade of calcium channels with cadmium but sensitive to the blockade of the persistent sodium current with riluzole. Thus we propose that the activation of the alpha-1 adrenergic receptor exerts its effect on the isolated respiratory network by modulating different cellular mechanisms.

**Pacemaker neurons have differential roles in regulating inspiratory activity**

The differential modulatory effects of NE on pacemaker activity provided new insights into their functional roles. NE altered in isolated cadmium-sensitive pacemaker neurons burst shape, but not frequency. Blockade of cadmium-sensitive pacemaker properties with 500 μM FFA (Peña et al. 2004) blocked the noradrenergic effect on burst shape, but it did not block the effect on frequency. This result was observed not only at the cellular level but, even more important, also at the network level.

What are the general implications for respiratory rhythm generation? It appears that in the absence of CAN-current bursting mechanisms, the remaining rhythm-generating mechanisms, which include cadmium-insensitive pacemakers, are still capable of generating a norepinephrine-induced frequency increase at the network level, although these mechanisms appear to be unable to generate an increase in burst duration and amplitude. Thus we propose that CAN-current–dependent bursting is critical for modulating the shape of inspiratory network activity in response to NE. However, it is important to emphasize that these lesion experiments do not indicate that CAN-current–dependent bursting is not also important for regulating the timing of the respiratory rhythm. Because FFA did not significantly alter the norepinephrine-evoked frequency response, our data provide only limited insights into the con-

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**FIG. 8.** FFA blocks the NE-evoked increase in burst duration and burst area of inspiratory neurons embedded in the network. A: inspiratory neuron (bottom trace) recorded simultaneously with integrated VRG activity (top trace). B: application of NE 20 μM increases burst duration, burst area, and burst frequency in intracellular recording (bottom trace). C: after washout of NE, the intracellularly recorded inspiratory neuron activity (bottom trace) and the simultaneously recorded VRG activity return to control conditions and are unaffected by FFA, a blocker of the CAN current. D: in the presence of FFA, NE increases bursts frequency but does not increase burst duration and burst area of intracellularly recorded neuron activity (bottom trace). E: histograms summarizing the effects of NE on burst duration (F), burst area (G), and burst frequency (H). Asterisk (*) denotes a significant change compared with control (P < 0.05, ANOVA). This effect was quantified by obtaining for each inspiratory neuron the average burst duration, area, and frequency from 15 successive cycles. Results are expressed as mean ± SE for 5 intracellularly recorded inspiratory neurons.
The difference is not obvious in the integrated extracellular recording (top trace) from the bottom trace is weaker than the third burst. This note the beginning of disintegration in network activity: second burst in the activity (Peña et al. 2004). Similarly, Del Negro et al. (2005) 2020 J.-C. VIEMARI AND J.-M. RAMIREZ maker neurons and reduces at the network level inspiratory rhythm. During the norepinephrine-induced frequency increase potential timing effects could be masked by the modulatory effect on cadmium-insensitive bursting neurons.

Although the cadmium-insensitive pacemaker neurons appear to be unable to contribute to modulation of the burst shape in response to NE, it must be emphasized that pharmacological lesions with riluzole indicate that these neurons play an important role in controlling burst shape under control conditions. Riluzole blocks the majority of cadmium-insensitive pacemaker neurons and reduces at the network level inspiratory burst area, amplitude, and duration (Del Negro et al. 2002, 2005; Peña et al. 2004). Interestingly, riluzole alone had no significant effect on frequency and irregularity of rhythmic activity (Peña et al. 2004). Similarly, Del Negro et al. (2005) observed that riluzole at 20 µM decreases XII amplitude monotonically over a period of 40 min, but does not change the mean period until after the XII amplitude decreased to baseline. The response to NE in the presence of riluzole was qualitatively very different from the response to riluzole alone. We observed that after NE application, there was an initial frequency increase followed by a subsequent cessation of respiratory activity within 5 min. Before the cessation the rhythm was very erratic. Thus the cessation of the rhythm was not characterized by a monotonic amplitude decrease, but rather by a highly irregular respiratory rhythm. In the isolated cadmium-insensitive pacemaker neurons norepinephrine caused a modulation of frequency, but not burst shape. Based on the finding at the single-cell level we hypothesized that the NE-induced frequency increase is eliminated after blockade of the majority of cadmium-insensitive pacemaker neurons with riluzole (Peña et al. 2004); this was not the case, however. In fact the temporary frequency increase was even more pronounced than that in the absence of riluzole.

How can the more pronounced temporary frequency increase be explained? Our previous study showed that riluzole significantly reduced the number of cadmium-insensitive pacemaker neurons, but left intact a small proportion of cadmium-insensitive pacemaker neurons (Peña et al. 2004). The consequences of significantly reducing the number of NaP-dependent pacemaker neurons were previously addressed in a computational model network (Del Negro et al. 2001). In this model network an increase in excitability (in this case caused by extracellular potassium) results in a more pronounced frequency increase, when compared with a model network with more pacemaker neurons (Del Negro et al. 2001). This computational study could thus explain how NE leads to a more dramatic frequency increase.

However, the NE-induced frequency increase was only temporary and rhythm generation ceased on prolonged exposure to norepinephrine (within 5 min). This finding is also consistent with computer modeling studies. When challenged with increased tonic excitation under reduced levels of persistent sodium current the respiratory network reveals a restricted frequency control (Butera et al. 1999). Thus one possible explanation for our finding is that the cessation of the respiratory rhythm that follows the initial frequency increase may be the result of this restricted frequency control in response to the excitatory neuromodulator NE.

Our findings are also interesting for another reason. Riluzole alone did not significantly alter the respiratory frequency and irregularity (Peña et al. 2004), which could suggest that respiratory rhythm generation is unaffected. Yet, the response to norepinephrine reveals major changes in rhythm-generating mechanisms because the rhythm ceases during prolonged exposure. This finding has two important implications: 1) the persistence of regular rhythmic activity after the lesion of a given neuron type does not imply that the lesioned neuron type is not important for rhythm generation. 2) The persistence of regular rhythmic activity, despite the lesioning of the majority of NaP-dependent pacemaker neurons, indicates that the respiratory network is very robust under control conditions, which is consistent with modeling studies (Butera et al. 1999) as well as experimental studies (Peña et al. 2004). Whether the respiratory rhythm remains regular, because of the remaining NaP-dependent mechanisms, because of network mechanisms, or because some Cd-insensitive pacemakers remain unblocked by riluzole (Peña et al. 2004), cannot be solved by the present experiments.

In this context we emphasize that our studies were performed at a certain developmental age window (P6–P13), in a very specific mouse strain (CD1 mice), using a very specific experimental approach (the transverse slice preparation using 650-µm-thick slices), at a temperature of 30°C using a perfus-
ate containing 8 mM potassium. The situation may be very different at a different age window, in a different mouse strain, or different mammalian species, in a different experimental preparation, at a different temperature, or different extracellular potassium concentration, or any other variable that is associated with an experimental procedure. It must also be emphasized that our considerations refer only to the process of rhythm generation itself and that the differential modulation of nonpacemakers and pacemakers by NE is just one step in a complex cellular cascade that will eventually lead to the generation and modulation of the breathing behavior itself. It is well established that respiratory motoneurons, for example, have their own modulatory properties (Parkis et al. 2003) that will greatly influence the respiratory pattern at the behavioral level. Other central structures with a significant contribution to the generation of the breathing patterns include the ventral respiratory group (Janczewski et al. 2002; Mellen et al. 2003; Onimaru and Homma 2003) as well as the pons (Alheid et al. 2004; Hilaire et al. 2004), a structure that contains important noradrenergic nuclei. From the network perspective, however, the isolated respiratory network provides an excellent example illustrating how different functions are distributed among different neuronal elements of the same neuronal network. This network also exemplifies tight cooperation between synaptic and intrinsic membrane properties, a principle that is presumably applicable to most neuronal networks.

Norepinephrine and the control of behavioral functions

The respiratory network provides important insights into another general property of neuronal networks: the modulation of intrinsic and synaptic properties by amines and peptides. Modulators released in a state-dependent manner provide networks with the flexibility required to adjust to changes in environmental and behavioral conditions. When released during arousal (Beane and Marrocco 2004), norepinephrine could alter different properties of inspiratory activity (Horne et al. 2004) by differentially modulating pacemaker neurons. Norepinephrine will also alter membrane properties of respiratory motor neurons (Al-Zubaidy et al. 1996; Funk et al. 1997; Parkis et al. 1995), as well as properties of the peripheral chemoreceptors (Vicario et al. 2000). Norepinephrine not only modulates the respiratory network but is in fact one of the most prominent neuromodulators in the mammalian nervous system. Noradrenergic neurons project from discrete brain stem nuclei (Dahlstrom and Fuxe 1964) into most CNS areas where NE can switch networks into different activity states (McCormick 1992; Steriade 2004). In doing so, this biogenic amine plays critical roles in controlling most behavioral systems, such as the regulation of the sleep–wake cycle, feeding, and cardio-respiration (Aston-Jones et al. 1991; Krantz et al. 2004; Ouyang et al. 2004).

However, the mechanisms underlying these modulatory network effects have been understood in only a few systems, the thalamus being perhaps the best-known example (Bickford et al. 2000). In the lateral geniculate, NE depolarizes relay neurons causing them to switch from a bursting to a tonic mode (McCormick 1992). This switch may play an important role in controlling changes in the arousal state (Le Masson et al. 2002). Although understood in only a few systems, it can already be concluded that norepinephrine is a major behavioral orchestrator, modulating different neuron types in different regions by selectively inducing and altering membrane properties, thereby affecting different aspects of neuronal network function.

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