Physiological Properties of Late Inspiratory Neurons and Their Possible Involvement in Inspiratory Off-Switching in Cats

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Haji, Akira, Mari Okazaki, Hiromi Yamazaki, and Ryuji Takeda. Physiological properties of late inspiratory neurons and their possible involvement in inspiratory off-switching in cats. J Neurophysiol 87: 1057–1067, 2002; 10.1152/jn.00470.2001. To assess the functional significance of late inspiratory (late-I) neurons in inspiratory off-switching (IOS), membrane potential and discharge properties were examined in vagotomized, decerebrate cats. During spontaneous IOS, late-I neurons displayed large membrane depolarization and associated discharge of action potentials that started in late inspiration, peaked at the end of inspiration, and ended during postinspiration. Depolarization was decreased by iontophoresis of dizocilpine and eliminated by tetrodotoxin. Stimulation of the vagus nerve or the nucleus parabrachialis medialis (NPBM) also evoked depolarization of late-I neurons and IOS. Waves of spontaneous chloride-dependent inhibitory postsynaptic potentials (IPSPs) preceded membrane depolarization during early inspiration and followed during postinspiration and stage 2 expiration of the respiratory cycle. Iontophoresed bicuculline depressed the IPSPs. Intravenous dicouline caused a greatly prolonged inspiratory discharge of the phrenic nerve (apneusis) and suppressed late-inspiratory depolarization as well as early-inspiratory IPSPs, resulting in a small constant depolarization throughout the apneusis. NPBM or vagal stimulation after dicouline produced small, stimulus-locked excitatory postsynaptic potentials (EPSPs) in late-I neurons. Neurobiotin-labeled late-I neurons revealed immunoreactivity for glutamic acid decarboxylase as well as N-methyl-d-aspartate (NMDA) receptors. These results suggest that late-I neurons are GABAergic inhibitory neurons, while the effects of bicuculline and dizocilpine indicate that they receive periodic waves of GABAergic IPSPs and glutamatergic EPSPs. The data lead to the conclusion that late-I neurons play an important inhibitory role in IOS. NMDA receptors are assumed to augment and/or synchronize late-inspiratory depolarization and discharge of late-I neurons, leading to GABA release and consequently off-switching of bulbar inspiratory neurons and phrenic motoneurons.

INTRODUCTION

The rhythmic activity generated within the bulbar respiratory network and transmitted in the motor outflow to the respiratory pump is essential for effective pulmonary gas exchange. Respiratory rhythm generation has been considered to rely on two neuronal mechanisms, one involving reciprocal inhibitions between neurons discharging out of phase and the other for switching from one respiratory phase to another (Bianchi et al. 1995; Ezure 1990; Richter 1996; von Euler 1986). The termination of inspiratory activity (inspiratory off-switching; IOS) is of special importance because it determines the duration of inspiration and leads to an orderly transition to expiration during the respiratory cycle. Afferent inputs from the pontine pneumotaxic nucleus and from the pulmonary stretch receptors as well as N-methyl-d-aspartate (NMDA) receptor-dependent synaptic coupling within the respiratory network play important roles in IOS. Impairment of these functional components results in apneusis characterized by a marked prolongation of inspiration, leading to disturbances of pulmonary gas exchange, metabolic status and state of consciousness (Wilken et al. 1997). Pathways and mechanisms responsible for IOS based on supportive data have been suggested (Feldman et al. 1992; Foutz et al. 1989; Haji et al. 1996b; Pierrefiche et al. 1992), including the probable involvement of late inspiratory (late-I) neurons of the medullary respiratory network. However, the neuronal mechanisms through which late-I neurons participate in IOS are still unclear.

Several lines of evidence suggest that late-I neurons are involved in IOS. First, the discharge activity of late-I neurons is most intense at the time when augmenting inspiratory (aug-I) neurons cease discharging further (Ballantyne and Richter 1984; Cohen and Feldman 1984; Marino et al. 1981; Pierrefiche et al. 1995; Richter 1982). Second, pulmonary afferents that facilitate or delay IOS influence the firing of late-I neurons (Cohen et al. 1993; Oku et al. 1992). Third, late-I neurons send axonal projections to the rostral ventral respiratory group (VRG) region where many inspiratory neurons, presumed target cells for postsynaptic inhibition, are located (Oku et al. 1992). Furthermore, inhibitory postsynaptic potentials (IPSPs) in aug-I neurons during IOS evoked by vagal stimulation are blocked by iontophoresis of bicuculline (Haji et al. 1999), suggesting a GABAergic presynaptic source for those IPSPs that one might expect to be late-I neurons. So far, no other evidence has been presented to date, confirming directly the inhibitory nature of late-I neurons. In addition, although apneusis follows systemic administration of NMDA receptor blockers (Foutz et al. 1989; Haji et al. 1996b; Pierrefiche et al. 1992), the potential contribution of NMDA receptors on late-I neurons to membrane potential and discharge properties and consequently to IOS needs to be clarified.

To address these issues, membrane potential and discharge...
activity were recorded from the propriobulbar late-I neurons located in the rostral VRG in vagotomized, decerebrate cats. We analyzed the electrophysiological and pharmacological characteristics of spontaneous, periodic waves of PSPs in late-I neurons. We compared the drive potentials that occurred during spontaneous IOS with IOS responses evoked by stimulating either the vagal pulmonary afferents or the nucleus parabrachialis medialis (NPBM). We also evaluated the effects of blocking NMDA receptors on the two types of responses and by immunofluorescent labeling demonstrated the presence of NMDA receptors and glutamic acid decarboxylase (GAD), an enzyme necessary for GABA synthesis, in late-I neurons.

METHODS

This study was conducted in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Surgical procedures

Forty adult cats of either gender (2.7–4.5 kg body wt) were anesthetized with inhalation of halothane (2.5–3.0% in oxygen-enriched air during induction and 1.5–2.0% during surgery). The depth of anesthesia during surgery was confirmed by a total absence of nociceptive reflexes and stable blood pressure and heart rate. The trachea was intubated below the larynx, and polyethylene catheters were inserted into a femoral vein for drug administration, a femoral artery to monitor blood pressure, and the urethra to allow drainage of urine from the bladder. To minimize bleeding during and after decerebration and decerebellation, both external carotid arteries were tied distal from the bladder. To minimize bleeding during and after decerebration, both external carotid arteries were tied distal from the bladder. To minimize bleeding during and after decerebration and decerebellation, both external carotid arteries were tied distal from the bladder. To minimize bleeding during and after decerebration and decerebellation, both external carotid arteries were tied distal from the bladder. To minimize bleeding during and after decerebration and decerebellation, both external carotid arteries were tied distal from the bladder.

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Drug administration

For systemic application, dizocilpine (Research Biochemicals, Natick, MA), a noncompetitive antagonist of NMDA receptors, was dissolved in physiological saline and injected into the femoral vein at a dose of 0.3 mg/kg (Foutz et al. 1989; Haji et al. 1996b). For iontophoretic application, multibarrel pipettes were filled with solutions of dizocilpine (20 mM, pH 8), bicuculline (5 mM, pH 3.5; Sigma Chemicals, St. Louis, MO), and tetrodotoxin (0.5 mM, pH 6.5; Sigma) dissolved in physiological saline (Haji et al. 1990, 1992, 1996a). Two other peripheral barrels were filled with physiological saline. One pipette was used to eject current through a drug-free medium to determine whether current pulse altered membrane potential and discharge properties during iontophoresis. The other barrel served as an iontophoresis current sink. Bicuculline and tetrodotoxin were ejected with positive currents (50–100 nA, 1–2 min) and dizocilpine with negative currents (50–100 nA, 3–5 min) from a programmable multichannel iontophoresis current pump (IP-2; Medical System, Great Neck, NY). All drugs were retained with an opposite current (5 nA) between the test periods by a current source.

Histological procedures

The location of the electrode tip in the NPBM was marked after the completion of experiments by passing a positive DC current (1.0 mA, 10 s). The animals were transectually perfused with 1000 ml of physiological saline containing 10% formaldehyde and the
sites were verified by examining histological slices stained with cresyl violet. Neurobiotin was injected intracellularly by positive current pulses (2–4 nA, 100–300 ms, 2–5 Hz) for 6–15 min (Kita and Armstrong 1991). To allow neurobiotin to disperse within the soma, axon and dendrites, all animals were kept alive for 2–6 h after the injection. For immunofluorescent staining of intracellularly injected neurobiotin, the animals were perfused transcardially with 1000 ml of heparinized (10 U/ml) physiological saline followed by 500 ml of 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 0.05% glutaraldehyde. The brain stem was removed. Serial 50-μm coronal slices were cut with a Vibratome (DTK-1000; DosakaEM, Kyoto, Japan). Stimulation pulses (24 nA, 100 ms) were delivered into the brain stem by a concentric microelectrode filled with 2% cresyl violet.

After the injection, for immunofluorescence staining of intracellularly injected neurobiotin, the animals were perfused transcardially with 1000 ml of heparinized (10 U/ml) physiological saline followed by 500 ml of 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde overnight at 4°C, and placed in 10 mM phosphate-buffered saline (PBS, pH 7.4). Serial coronal slices (50 μM) of the brain stem were cut with the Vibratome. After rinsing in PBS, they were mounted onto silane-coated slides and air-dried for 1 h at room temperature. The slices were incubated for 30 min with 10% goat normal serum (DAKO, Carpinteria, CA) in PBS, then incubated for 3 h with fluorescein (FITC)-conjugated streptavidin (1 mg/ml; DAKO) at a 1:100 dilution and 0.1% Triton X-100 (nacalai tesque, Kyoto, Japan) in a dark place. They were mounted with 50% glycerol and 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) in PBS. The FITC-labeled neurons were detected using a fluorescent microscope (eclipse-E600; Nikon, Tokyo) with a B-2E/C block of filter (excitation filter at 465–495 nm, dichroic beam splitter at 505 nm, barrier filter at 515–555 nm).

Immunofluorescence staining of GAD or of NMDA receptors was performed using rabbit antibodies either against GAD 65/67 (anti-GAD 65/67 at a 1:100 dilution, Sigma) or against NMDA receptors (anti-NMDA R1 at a 1:20 dilution, Sigma), respectively. Slices containing late-I neurons identified by their FITC-fluorescent somata and dendrites were preincubated with 10% goat normal serum in PBS for 30 min, then incubated overnight at 4°C with either antibody and 0.1% Triton X-100 in a dark place. After rinsing in PBS, the slices were incubated for 2 h at room temperature with fluorolink Cy3-labeled anti-rabbit IgG (H + L) developed in goat (1 mg/ml; Amer sham Pharmacia Biotech, Uppsala, Sweden) at a 1:1000 dilution. They were mounted with 50% glycerol and 2.5% DABCO in PBS. The Cy3-immunofluorescence was detected with a G-2/E/C block of filter (excitation filter at 540/25 nm, dichroic beam splitter at 565 nm, emission filter at 605/55 nm).

They were also recorded on magnetic tape with a digital recorder (PC 204; Sony, Tokyo). Membrane potential and input resistance were measured during the early-inspiratory, late-inspiratory, postinspiratory, and stage 2 expiratory phases of the respiratory cycle. e-I, early-inspiratory phase; l-I, late-inspiratory phase; PI, postinspiratory phase; E2, stage 2 expiratory phase. ●, mean ± SD. C: membrane potential changes induced by intracellular injection of currents. Traces were taken without current injection (0 nA) and during injection of a constant depolarizing current (+1 nA) and a constant hyperpolarizing current (−1 nA) through a K+-citrate-filled electrode.

**Data recording and analysis**

Recording of neuronal activity started ≥3 h after withdrawal of halothane anesthesia. All recordings were displayed on a computer using signal processing software at a sampling rate of 4,000 Hz and stored on a computer hard disk (Macintosh-PowerLab/4 s; ADInstruments Pty, Castle Hill, Australia). They were also recorded on magnetic tape with a digital recorder (PC 204; Sony, Tokyo). Membrane potential and input resistance were measured during the early-inspiratory, late-inspiratory, postinspiratory, and stage 2 expiratory phases of the respiratory cycle. Power spectrum analysis of synaptic noise and poststimulus averagings of PSPs were performed using libraries included in PowerLab. For power spectrum analyses, the fast Fourier transform (8,192 points at a sampling rate of 4,000 Hz) was applied to the respiratory cycle. e-I, early-inspiratory phase; l-I, late-inspiratory phase; PI, postinspiratory phase; E2, stage 2 expiratory phase.

**RESULTS**

Thirty late-I neurons were identified among 286 inspiratory neurons recorded from the rostral VRG. All recorded neurons exhibited membrane potentials more negative than −50 mV during their inactive phase and action potentials with overshoot followed by after-hyperpolarizations (Fig. 1). The neurons exhibited membrane properties characteristic of late-inspiratory neurons, i.e., stereotypical action potential discharges and waves of PSPs that occurred with fixed temporal relationships.
to phrenic nerve activity (Bianchi et al. 1995; Haji et al. 2000b; Richter 1996). Consistent with the findings of Richter (1982), they were not antidromically-activated by vagus nerve or cervical spinal cord stimulation.

Membrane potential properties during eupnea

Late-I neurons depolarized to threshold and discharged a burst of action potentials toward the end of inspiration, i.e., as the augmenting discharge of phrenic nerve activity approached its peak. Membrane depolarization and discharge of the neurons continued into postinspiration, when phrenic nerve activity declines (Fig. 1A). On average (*n* = 30), membrane depolarization started 129 ± 41 ms before the end of inspiration, peaked exactly at the end of inspiration, and ended 211 ± 86 ms after the onset of postinspiration. The peak frequency of action potentials was 64 ± 42 Hz. When the membrane was steadily depolarized by intracellular injection of a continuous current (0.5–1.5 nA, 30 s), late-inspiratory depolarization became more rounded and prolonged but of reduced amplitude, and the action potential frequency increased. Vice versa, when the membrane was hyperpolarized, the late-inspiratory depolarization became taller and shorter, and action potentials ceased (Fig. 1C). Thus this depolarization was voltage dependent.

A decrementing wave of IPSPs occurred during early-inspiration (Figs. 1A and 2B). In 17 neurons, the IPSP wave exhibited high-frequency oscillation (HFO) at 77 ± 5 Hz. The HFOs occurred in synchrony with HFOs in phrenic nerve discharges (Fig. 2A). This corresponded to HFO observed in postinspiratory (PI) and augmenting expiratory (E2) neurons during inspiration (Huang et al. 1996; Mitchell and Herbert 1974; Remmers et al. 1985; Takeda and Haji 1992). Membrane depolarization and firing were followed by a wave of hyperpolarization that was clearly divided into two components; an intermediate component during postinspiration and a more hyperpolarized component during stage 2 expiration (Figs. 1A and 2B). The IPSPs comprising the hyperpolarizing waves were chloride dependent because they were reversed by intracellular injection of Cl⁻ (Fig. 2B).

Average input resistance measured in five neurons was 7.9 ± 1.5 MΩ during early-inspiration, 7.6 ± 1.4 MΩ during late-inspiration, 9.6 ± 1.5 MΩ during postinspiration (*P* < 0.05 vs. early and late inspiration), and 8.8 ± 1.6 MΩ during stage 2 expiration (Fig. 4B).

Effects of iontophoretically applied dizocilpine, bicuculline, and tetrodotoxin

Iontophoretic application of dizocilpine (50 nA, 3 min) to five late-I neurons caused a significant decrease in the late-inspiratory depolarization and suppression of the action potential firing (Fig. 3). The round shape of depolarizing wave changed into a steep peak but of reduced amplitude and decreased duration. The amplitude of the wave of depolarization, measured as the difference between end-inspiratory and -expiratory potentials, was decreased from a control value of 7.4 ± 1.4 to 4.3 ± 1.1 mV during dizocilpine application (*P* < 0.05). Membrane potential during all phases of the respiratory cycle was relatively hyperpolarized during NMDA receptor blockade by dizocilpine, by 4.3 ± 1.2 mV (*P* < 0.05) during early-inspiration and by 5.7 ± 2.9 mV (*P* < 0.05) during late-inspiration with respect to control.

Iontophoretic application of bicuculline (100 nA, 1 min) to four late-I neurons consistently depolarized membrane potential, as shown in Fig. 4A. The superimposed traces of membrane potential illustrate clearly that bicuculline antagonized early-inspiratory, postinspiratory, and stage 2 expiratory IPSPs. The membrane potential shifted in the positive direction by 2.8 ± 1.3 mV during early-inspiration and by 3.3 ± 2.1 mV during stage 2 expiration. Bicuculline also increased discharge intensity during late-inspiration and induced action potentials during intervals when the neuron was normally silent.

Tetrodotoxin (50 nA, 2 min) blocked or reduced synaptic transmission in three late-I neurons tested in the present investigation (Fig. 4B) (also see Haji et al. 1992). Iontophoresed tetrodotoxin consistently hyperpolarized the membrane throughout the respiratory cycle and blocked action potential generation. Periodic waves of depolarization as well as hyperpolarization were all suppressed, resulting in a marked decline of respiratory fluctuations of membrane potential. Input resistance was increased to 1.4- to 1.7-fold over control in each phase of the respiratory cycle in all cases (no statistical analysis was done because of small samples). These results provide additional evidence that
powerful excitatory and inhibitory synaptic inputs are responsible for membrane potential fluctuations in late-I neurons (Richter 1982, 1996).

**Effects of intravenous injection of dizocilpine**

It was of interest to determine how a generalized blockade of NMDA receptors with intravenously administered dizocilpine would affect membrane potential of late-I neurons. Changes in membrane potential trajectories after intravenous dizocilpine (0.3 mg/kg) were examined in six late-I neurons. In association with a prolonged, “plateau-like” apneustic discharge of phrenic nerve action potentials, the late-inspiratory depolarization of late-I neurons was greatly depressed by 5.8 ± 0.9 mV (P < 0.05) and early-inspiratory hyperpolarization was converted to depolarization (Fig. 5). These changes resulted in a small but constant membrane potential depolarization throughout the period of apneusis. The depolarizing synaptic drive potentials (membrane potential difference between the early-inspiratory and late-inspiratory potentials) were decreased from 8.2 ± 2.1 mV (before) to 2.6 ± 0.6 mV (after dizocilpine, P < 0.05). The firing of action potentials progressively decreased and finally ceased (Fig. 5A, a and b). In addition, inspiratory phase membrane potential depolarization was followed directly by stage 2 expiratory hyperpolarization. The membrane potential during stage 2 expiration was shifted in the negative direction by 2.3 ± 1.2 mV after dizocilpine.

**FIG. 3.** Effects of iontophoresed dizocilpine on membrane potential in a late-I neuron. A: MPs recorded before and during iontophoresis of dizocilpine (50 nA, 3 min). B: traces taken with a higher sweep speed during the a and b intervals in A. • • •, reference membrane potentials.

**FIG. 4.** Effects of iontophoresed bicuculline (A) or tetrodotoxin (B) on membrane potential, discharge properties, and input resistance in late-I neurons. A: effects of iontophoresed bicuculline (100 nA, 1 min). Right: superimposed high-cut filtered (100 Hz) traces showing changes in the membrane potential trajectory during bicuculline iontophoresis. B: membrane potentials recorded from another late-I neuron before and 1 min after iontophoresis of tetrodotoxin (50 nA, 2 min). Input resistance was measured by injecting negative constant current pulses (−1 nA, 100-ms pulse duration, 2 Hz). • • •, reference membrane potentials (−57 mV in A and −70 mV in B).
Postsynaptic responses evoked by single stimulation of NPBM or vagus nerve

Single pulse (0.5–1.0 mA, 0.1 ms) stimulation of NPBM or the vagus nerve evoked a short-lasting inhibition, i.e., a transient pause in the phrenic nerve discharge followed by recovery when applied during mid-inspiration, and premature termination of phrenic nerve activity (IOS) when applied during late-inspiration. NPBM or vagus nerve stimulation, whether applied in mid- or late-inspiration, also evoked short-latency waves of IPSPs followed by long-lasting EPSP wave in late-I neurons (n = 4). Figure 6 illustrates typical examples of stimulus-evoked postsynaptic responses in a late-I neuron. The average latency of early IPSP waves evoked by NPBM stimulation was 3.7 ± 0.8 and 5.4 ± 0.5 ms for the vagus nerve stimulation. The late EPSP wave evoked by NPBM single shocks began after 23.8 ± 6.6 and 27.0 ± 5.6 ms after the vagal stimulation and for either type of stimulation lasted for over 150 ms.

Membrane potentials during IOS evoked by repetitive stimulation of NPBM or vagus nerve before and after dizocilpine

Premature IOS of phrenic nerve discharges occurred in parallel with membrane potential depolarization and enhanced firing of late-I neurons when repetitive pulses (0.2 mA, 0.1 ms, 20 pulses at 50 Hz) were applied during mid-inspiration (n = 5). As illustrated in the control records of Fig. 7A, phrenic nerve discharges were greatly depressed during NPBM or vagus nerve stimulation. The depression was followed at the end of stimulation by premature cessation of firing. Accompanying the stimulus-evoked depression and subsequent cessation of phrenic nerve activity were stimulus-dependent increases of membrane potential depolarization and augmented discharges in late-I neurons. The fast sweep traces in Fig. 7B (a and b) reveal that NPBM or vagus nerve stimulation first evoked membrane hyperpolarization consisting of three to four waves of IPSPs and then waves of summing EPSPs 50–60 ms after the onset of stimulation. The large depolarizations generated bursts of action potentials which continued more than 500 ms after the end of stimulation. Following intravenous administration of dizocilpine, NPBM or vagus nerve stimulation still evoked IOS of phrenic nerve activity, even though late-I neurons were depressed and failed to discharge (Fig. 7, A and B, bottom). Only small, stimulus-locked EPSPs, which did not sum up throughout a train of stimulation, occurred and were immediately followed by an expiratory hyperpolarization at termination of apneusis.
In all six experiments, stimulating electrodes were correctly placed in the NPBM. As shown in Fig. 8A, circular lesions marking the sites of stimulation were found in the medial part of the nucleus parabrachialis. These results are consistent with previous histological findings and reports of inspiratory phase termination evoked by stimulating this anatomical component of the pneumotaxic center (Baker and Remmers 1982; Cohen 1971).

**Presence of NMDA receptors and GAD in late-I neurons**

Immunohistochemical experiments were performed to determine whether NMDA receptors on late-I neurons could be target sites that are at least partly responsible for the actions of dizocilpine on IOS. These studies also probed for the presence of GAD, an enzyme responsible for GABA production, implying that GABAergic inhibition of bulbar inspiratory neurons by late-I neurons accompanies IOS. Immunohistochemical localization of either GAD or NMDA receptors was undertaken in 10 double-labeled, neurobiotin-injected late-I neurons. The neurons were located near or in the nucleus ambiguus of the rostral VRG area (0.5–3.5 mm rostral to the obex, Fig. 8B) and exhibited FITC-green immunofluorescence in the soma and dendrites. The somata were multipolar. The average diameter of the major soma axis was 36.7 ± 8.7 μm, while the minor axis was 21.8 ± 5.1 μm. Each soma gave off 5–10 dendritic trunks (Fig. 9). The main dendrites branched into fine dendrites.
Timing of late-I neuron discharges is determined by NMDA receptor-dependent excitation and GABA<sub>Α</sub> receptor-mediated inhibitions

Membrane properties of late-I neurons were first analyzed in detail by Richter and colleagues, who showed that waves of IPSPs during early-inspiration effectively shunt tonic excitatory synaptic inputs from sources such as aug-I neurons, peripheral and central chemoreceptors, and neurons of the reticular activating system (Richter 1996). This shunting delays the firing of late-I neurons until at least midway through the inspiratory phase. The neurons depolarize steeply during late inspiration with the cessation of early-inspiratory IPSPs. In the present investigation, late-inspiratory membrane depolarization and the accompanying short burst of action potential discharges were suppressed by iontophoretically or systemically administered dizocilpine and abolished by iontophoresed tetrodotoxin, indicating that NMDA-dependent excitatory postsynaptic events were responsible. Furthermore, systemic dizocilpine depressed the late-inspiratory depolarization during IOS evoked by NPBM or vagal stimulation, and immunoreactivity for NMDA receptors was observed in the neurobiotin-labeled late-I neurons. A similar conclusion that postsynaptic NMDA receptors mediate excitation of late-I neurons was reached by Pierrefiche et al. (1991), who reported that extracellularly recorded action potentials of late-I units were decreased during iontophoresis of AP7, a competitive antagonist of glutamate at NMDA receptors. Therefore dizocilpine presumably blocked the NMDA receptors located in the postsynaptic membrane of late-I neurons, although the agent is not selective on NMDA receptors but has some effects on nicotinic receptors (Arias et al. 2001) and on monoamine release and uptake (Callado et al. 2000).

Additional non-NMDA receptor-activated mechanisms must also contribute to membrane depolarization of late-I neurons because small stimulus-evoked rapidly decaying EPSPs evoked by NPBM or vagal stimulation remained during blockade of NMDA receptors by intravenous dizocilpine. Other studies have demonstrated that non-NMDA mechanisms also contribute to depolarization and discharge activity in various types of medullary respiratory neurons (Haji et al. 1996a; Pierrefiche et al. 1991). It seems likely that in late-I neurons, as in other types of neurons, NMDA receptor-activated EPSPs are dependent on antecedent membrane depolarization via non-NMDA receptors, resulting to large, NMDA receptor-activated Ca<sup>2+</sup> currents (Bianchi et al. 1995; Headly and Grillner 1991; Richter et al. 1986; Takeda and Haji 1993).

Inhibitory inputs generated Cl<sup>-</sup>-dependent IPSPs in late-I neurons during early-inspiration, postsinspiration, and stage 2 expiration. The IPSPs were decreased by iontophoresed bicusculine, suggesting that they were activated by GABA activation of postsynaptic GABA<sub>Α</sub> receptors. This is consistent with previous reports indicating that GABA<sub>Α</sub> receptors mediate spike discharge suppression (Champagnet et al. 1982; Schmid et al. 1996) and hyperpolarization (Haji et al. 1992) in various types of respiratory neurons, particularly during inspiration and stage 2 expiration. In addition, it has been reported that bicusculine-salts block non-GABA receptor-mediated responses, including afterhyperpolarization in the hippocampal, thalamic, and cortical pyramidal neurons, which are mediated by Ca<sup>2+</sup>-activated K<sup>+</sup> conductances (Seutin and Johnson 1999). Such
conductances have been suggested to be involved in the postinspiratory repolarizing phase of late-I neurons (Pierrefiche et al. 1995). However, it seems likely that late-I neurons are subject to GABA-mediated inhibition from early-inspiratory (early-I), PI, and E2 neurons. This is consistent with the previous hypothesis (Ezure et al. 1989; Krolo et al. 2000; Richter 1982), where late-I neurons receive the early-inspiratory inhibition from the early-I type of inhibitory neurons since the pattern of early-inspiratory inhibition resembles the discharge pattern of those neurons. Together, these inhibitions account for the narrow time window during which late-I neurons are active. GABAergic early-I and PI neurons discharge in response to glutamate activation of NMDA receptors (Haji et al. 1996b; Pierrefiche et al. 1991, 1992; Yamazaki et al. 2000). This explains why systemic injection of dizocilpine depressed early- and postinspiratory IPSPs in late-I neurons. On the other hand, IPSPs during stage 2 expiration were not depressed, probably because the activity of E2 neurons is not depressed during apneusis produced by dizocilpine (Feldman et al. 1992; Haji et al. 2000a; Pierrefiche et al. 1992; Richter et al. 1997), moreover E2 neurons are GABAergic (Richter et al. 2000).

Late-I neurons suppress discharges of aug-I neurons through GABA\(_A\) receptors and permit phase transition into postinspiration of the respiratory cycle

Feldman and Speck (1978) and Segers and coworkers (1987) demonstrated the absence of cross-correlation between excitation of late-I neurons and inhibition of aug-I neurons. However, the present study together with our previous results (Haji et al. 1999) presented an indirect evidence that aug-I neurons in the medullary respiratory network receive inhibitory synaptic inputs from late-I neurons. Analysis of Cl\(^{-}\)-reversed IPSPs provides additional support. Prominent waves of Cl\(^{-}\)-dependent IPSPs have been observed to arrive in the soma of aug-I neurons during late inspiration (Richter 1996). Aug-I neurons make synaptic contact with medullary and spinal motoneurons that innervate the upper airways and pump muscles of the chest wall and diaphragm (Long and Duffin 1986), thus late-inspiratory inhibition of propriobulbar and bulbospinal aug-I neurons will be reflected in termination of discharges in motoneurons. Intracellular recordings in the present investigation revealed a coincidence between peak depolarization of late-I neurons and the end of the phrenic nerve inspiratory discharge. Furthermore, depolarization and stimulus-locked firing of late-I neurons coincided with termination of phrenic activity during repetitive stimulation of the vagus nerve or NPBM. Also, dizocilpine eliminated the late-inspiratory depolarization and greatly prolonged phrenic nerve discharges. Taken together, the data are strongly supportive of the proposal (reviewed by Bianchi et al. 1995; Richter 1996) that IOS is accomplished by a sequential excitation of late-I and PI neurons that effectively inhibits the activity of aug-I neurons.

Our previous work demonstrated that bicuculline-sensitive IPSPs occur spontaneously during late-inspiration in aug-I neurons (Haji et al. 1992), and during stimulus-evoked IOS (Haji et al. 1999). Coupled with immunohistochemical evidence in this investigation showing that late-I neurons are GABAergic, we propose that an important component of IOS is GABA\(_A\) receptor-activated postsynaptic inhibition of aug-I neurons. However, late-I neurons are reported to be heterogeneous with some of them being excitatory and bulbospinal (Ballantyne and Richter 1984; Cohen and Feldman 1984; Ezure 1990; Monteau et al. 1985). Because the present study selected the nonantidromically activated late-I neurons, such neurons can be excluded.

**Late-I neurons are involved in spontaneous IOS but are not essential for terminating inspiration mediated by pulmonary afferents and the NPBM**

The correlation between depression of cellular excitability in late-I neurons and the occurrence of apneustic phrenic nerve discharges following intravenous administration of dizocilpine suggests that firing of late-I neurons is responsible for spontaneous IOS. Further indirect support is drawn from studies cited in the preceding text showing inhibitory synaptic coupling between late- and aug-I neurons. On the other hand, the present study has also demonstrated that pathways activated by vagus nerve or NPBM stimulation need not evoke firing of late-I neurons to induce premature IOS (Fig. 7). Vagal afferents originating from pulmonary stretch receptors as well as the pneumotaxic center of the rostral pons are known to play a facilitatory role in IOS (Cohen 1979; von Euler 1986). There is also evidence that late-I neurons receive excitatory inputs from slowly adapting pulmonary afferents (Baker and Remmers 1980; Cohen and Feldman 1984; Cohen et al. 1993; Richter et al. 1986) and from the pontine pneumotaxic center (Baker and Remmers 1982; Cohen 1979). This is consistent with the present result that repetitive stimulation of the vagus nerve or NPBM evoked a large depolarization together with a burst of action potential discharge in late-I neurons and a premature IOS. The EPSPs evoked by stimulation either pathway were of short but different latencies, suggesting that they follow separate oligosynaptic pathways. As the pathways diverge, they may distribute to other neurons that play a role in terminating inspiratory discharges, in particular, PI neurons. The latter are activated by vagal afferents (Haji et al. 1996a; Remmers et al. 1986) and are still active during apneusis induced by NMDA receptor blockade (Feldman et al. 1992; Pierrefiche et al. 1998). Therefore we suggest that PI neurons are responsible for stimulus-evoked IOS after excitability is abolished in late-I neurons by dizocilpine. Ezure (1990) also discussed the possibility that the decrementing type of expiratory neurons in the Bötzinger complex, which may belong to the PI neuron group, play a role in IOS, because of their firing properties, excitatory responses to lung inflation, and inhibitory natures. Other candidates responsible for IOS are the bIE neurons (Oku et al. 1992) and early-onset decrementing expiratory neurons (Ezure et al. 1993), although there is no evidence that such neurons are still active after dizocilpine.

**Physiological significance of IOS**

Late-I neurons are activated by lung inflation to normal tidal volume or prematurely by hyperinflation (Baker and Remmers 1980; Cohen and Feldman 1984; Cohen et al. 1993). Their activation constitutes a “reversible off-switch” because lung inflation continues after temporary arrest if peak tidal volume has not been reached. According to von Euler (1983), the IOS threshold is adjustable across a range
of tidal volumes during normal breathing, and IOS is an important means of varying rate and depth of breathing. Brisk discharge activity of late-I neurons induces a sharp break point in inspiratory activity and a well-defined transition to the postinspiratory phase.

Finally, the present study demonstrated neurochemical, physiological and pharmacological characteristics of membrane potential trajectories as well as synaptic inputs from peripheral and central origin in late-I neurons. The current results are consistent with that late-I neurons essentially contribute to spontaneous IOS, although all of the data presented do not directly establish this hypothesis. This may provide further understandings of the neuronal mechanisms responsible for rhythm generation in the central neural network.

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