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

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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 post synaptic 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 dizocilpine 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 dizocilpine produced small, stimulus-locked excitatory post synaptic potentials (EPSPs) in late-I neurons. Neurobiotin-labeled late-I neurons revealed immuno-reactivity 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 dizocilline 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 demonstrating 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 to the lingual artery. The head of the animal was fixed on a stereotaxic frame, and decerebellation was performed by aspirating the brain rostral to the midcollicular transsection. A C2–C3 laminectomy and an occipital craniotomy were performed to expose the cervical spinal cord and medulla oblongata, respectively. In six animals, the dorsal surface of the pons caudal to the inferior colliculus was exposed by aspirating the cerebellum. The phrenic nerve and cervical vagus nerve were prepared by a dorsal approach and cut distally in both sides. Bilateral pneumothoraces were performed to reduce movement of the brain stem associated with positive pressure ventilation. The animals were paralyzed with pancuronium bromide (0.3 mg/kg initially and 0.1 mg/kg hourly), and the lungs were artificially ventilated with oxygen-enriched air (FiO2 = 0.3). An expiratory flow resistance of 1–2 cmH2O was applied to prevent collapse of the lungs. Tracheal pressure was kept below 8 cmH2O at maximal lung inflation. The end-tidal concentration of CO2 was continuously monitored (Capnomac; Datex, Helsinki, Finland) and kept at 4.0–4.5% by tuning the rate of ventilation and/or stroke volume. Glucose-lactate Ringer solution was infused at the rate of 3–5 ml·kg⁻¹·h⁻¹. Rectal temperature was maintained with a thermostatically regulated heating pad at 36.5–38°C. Mean arterial blood pressure was kept at or above 100 mmHg. At the end of the experiments, the animals were intravenously given either an anesthetic dose of pentobarbital (30 mg/kg) for histology or doses in excess of 100 mg/kg to produce cardiac arrest and death.

Recording and stimulating procedures

The motor output of the central respiratory network was analyzed by recording nerve discharges from the phrenic nerve through bipolar silver electrodes. Amplified signals (3,000–10,000 times) were filtered (30–3,000 Hz), rectified and integrated with a leaky integrator (time constant, 0.1 s). Membrane potential and discharge properties of late-I neurons in the medulla were recorded either with single micropipettes or with the central micropipette of a six-barrel coaxial array for intracellular recording and extracellular iontophoresis (Haji et al. 1990; Remmers et al. 1997). Recording pipettes were filled with 2 M K-citrate (DC resistance in brain tissue, 30–40 MΩ) or 3 M KCl (10–15 MΩ). The distance between the tip of central recording pipette and tips of the six recessed iontophoresis pipettes was 30–60 μM. For intracellular labeling of late-I neurons, the recording electrodes were filled with 1 M KCH₂SO₄ (20–40 MΩ) containing 4% neurobiotin (Vector Laboratories, Burlingam, CA). Late-I neurons were sought in the rostral VRG area, 3–4 mm lateral to the midline, 0–4 mm rostral to the obex and 2.5–4.5 mm below the dorsal surface of the medulla oblongata (Oka et al. 1992). To determine whether the neurons were vagal or bulboospinal, the occurrence of antidromically conducted action potentials was tested by stimulating the central end of the ipsilateral vagus nerve (0.2- to 0.3-mA intensity, 0.1-ms pulse duration) with bipolar silver electrodes, or the ventrolateral part of the C₁–C₃ spinal cord (0.3–0.5 mA, 0.2 ms) with an array of five concentric stimulating electrodes. To elicit stimulus-evoked IOS responses, a coaxial stimulating electrode was inserted into NPBM (1–2 mm caudal to the margin of the inferior colliculus, 4–5 mm lateral to the midline and 2–3 mm deep from the dorsal surface) (Baker and Remmers 1982; Cohen 1971). The electrode tip was correctly positioned when a premature IOS was induced by stimulation with repetitive pulses (0.1 ms, 10–20 pulses at 50 Hz) with a minimum intensity. The ipsilateral vagus nerve or NPBM was stimulated by trains of pulses with 1.5 times the threshold intensity (0.2–0.4 mA) to induce IOS responses. To analyze excitatory postsynaptic potentials (EPSPs) and IPSPs in late-I neurons during the evoked transient inhibition or a premature IOS, single pulses (0.5–1.0 mA, 0.1 ms) were applied to electrodes in the NPBM or on the vagus nerve according to our previous reports (Haji et al. 1999; Pierrefiche et al. 1998). Stimulation was delivered at a predetermined time during the respiratory cycle using a triggering pulse derived from the integrated phrenic neurogram. For current injection through the recording pipette into a cell, bridge balance or discontinuous current clamp was used (AxoClamp-2B; Axon Instruments, Foster City, CA). Input resistance was measured by injecting a constant negative current (~1 nA, 100 ms, 2 Hz) through the recording pipette filled with a solution of K-citrate.

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 transcardially perfused with 1000 ml of physiological saline containing 10% formaldehyde and the
brain stem was removed. Serial 50-μm coronal slices were cut with a Vibratome (DTK-1000; DosakaEM, Kyoto, Japan). Stimulation 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 dissected, postfixed in the same fixative for 30 min, kept in 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 preincubated 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-diazabicycloc[2,2.2]octane (DABCO; Sigma) in PBS. The FITC-labeled neurons were detected using a fluorescent microscope (eclipse E600; Nikon, Tokyo) with a 20 objectives (numerical aperture, 0.70). In this case, the limit of resolution was 0.45 μm for FITC and 0.49 μm for Cy3. They were saved on computer hard disk. The analysis was performed with a multicolor digital fluorescence imaging system (Argus fish PPC; Hamamatsu Photonics) and computer software (Photoshop; Adobe Systems, Mountain View, CA).

**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 filtered signals (low-pass filter at 250 Hz) occurring during a window of a 2.048-s duration and averaged 15 times. Peak frequency of action potential discharges as well as latency, duration, and amplitude of 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.
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-1 neurons \((n=4)\). Figure 6 illustrates typical examples of stimulus-evoked postsynaptic responses in a late-1 neuron. The average latency of early IPSP waves evoked by NPBM stimulation was \(3.7 \pm 0.8\) and \(5.4 \pm 0.5\) ms for the vagus nerve stimulation. The late EPSP wave evoked by NPBM single shocks began after \(23.8 \pm 6.6\) and \(27.0 \pm 5.6\) 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-1 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-1 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-1 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 bulbary 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 ± 10.0 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.

**FIG. 7.** Effects of intravenous dizocilpine on membrane depolarization in a late-I neuron during IOS induced by repetitive stimulation (0.2 mA, 0.1 ms, 20 pulses at 50 Hz) of NPBM and the vagus nerve (VN). A: traces of MP and PN taken before and after dizocilpine (0.3 mg/kg iv). The stimulus train is indicated by stimulus artifacts. B: traces taken with a faster sweep speed during each stimulation (indicated by a, b, c, and d) in A. ●, reference membrane potentials.

**FIG. 8.** Stimulating sites of NPBM (A) and anatomical localization of late-I neurons (B) identified histologically. A, left: a photomicrograph of a coronal section of the brain stem (P 3.5). A lesion was found in the medial part of NPB. A, right: the drawing superimposes the sites of stimulation (6 dots) obtained from 6 cats. B: reconstruction of location of 10 late-I neurons labeled intracellularly with neurobiotin. ● and ○, the neurons immunoreactive for glutamic acid decarboxylase (GAD) and for N-methyl-D-aspartate (NMDA) receptors, respectively. BC, brachium conjunctivum; BP, brachium pontis; CX, external cuneate nucleus; IO, inferior olive; KF, Kölliker-Fuse nucleus; LRI, lateral reticular nucleus; NA, nucleus ambiguus; NPB, nucleus parabrachialis; S, solitary tract; SOM, medial nucleus of the superior olive; 5 M, motor trigeminal nucleus; 5SP, spinal trigeminal nucleus; SST, spinal trigeminal tract; 12N, hypoglossal nucleus (Berman 1968).
DISCUSSION

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 iontophotically 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\(^{2+}\) currents (Bianchi et al. 1995; Headly and Grillner 1991; Richter et al. 1986; Takeda and Haji 1993).

Inhibitory inputs generated Cl\(^{-}\)-dependent IPSPs in late-I neurons during early-inspiration, postinspiration, and stage 2 expiration. The IPSPs were decreased by iontophoresed bicuculline, suggesting that they were activated by GABA activation of postsynaptic GABA\(_A\) receptors. This is consistent with previous reports indicating that GABA\(_A\) receptors mediate spike discharge suppression (Champagnat 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 bicuculline-salts block non-GABA receptor-mediated responses, including afterhyperpolarization in the hippocampal, thalamic, and cortical pyramidal neurons, which are mediated by Ca\(^{2+}\)-activated K\(^+\) conductances (Seutin and Johnson 1999).

FIG. 9. Immunohistochemical reactivities for GAD and for NMDA receptors in late-I neurons. A: FITC-fluorescent image of a late-I neuron (indicated by a) intracelluarly injected with neurobiotin. B: Cy3-fluorescent image of immunoreactivity for GAD presented in the same section as in A. C: superimposed image of neurobiotin and GAD in the neuron (a). D: FITC-fluorescent image of another late-I neuron (indicated by b). E: Cy3-fluorescent image of immunoreactivity for NMDA receptors in the neuron in the same section as in D. F: superimposed image of neurobiotin and NMDA receptors in the neuron (b). * immunoreactivity for either GAD (B and C) or for NMDA receptors (E and F) in unlabeled neighboring neurons. D, dorsal; M, medial.
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. GABAAergic 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 GABAAergic (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 GABAAergic, 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 nonadendromically 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 stimulating 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 Bötzinguer 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 nature. 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 neuron network.

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REFERENCES


