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Inspiratory-activated and inspiratory-inhibited airway vagal preganglionic neurons in the ventrolateral medulla of neonatal rat are different in intrinsic electrophysiological properties

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ABSTRACT

This study investigates the firing properties of the inspiratory-activated and inspiratory-inhibited airway vagal preganglionic neurons located in the external formation of the nucleus ambiguus. The results showed that inspiratory-activated and inspiratory-inhibited neurons are distributed with different density and site preference in this area. Inspiratory-inhibited neurons exhibit significantly more positive resting membrane potential, more negative voltage threshold and lower minimal current required to evoke an action potential under current clamp. The afterhyperpolarization in inspiratory-activated neurons was blocked by apamin, a blocker of the small-conductance Ca^{2+}-activated K^+ channels; and that in inspiratory-inhibited neurons by charybdotoxin, a blocker of the large-conductance Ca^{2+}-activated K^+ channels. Under voltage clamp, depolarizing voltage steps evoked tetrodotoxin-sensitive rapid inward sodium currents, 4-aminopyridine-sensitive outward potassium transients and lasting outward potassium currents. 4-Aminopyridine partially blocked the lasting outward potassium currents of inspiratory-activated neurons but was ineffective on those of inspiratory-inhibited neurons. These findings suggest that inspiratory-activated and inspiratory-inhibited neurons are differentially organized and express different types of voltage-gated ion channels.

1. Introduction

The airway vagal nerves include the superior and inferior laryngeal nerves and the tracheobronchial branch of the vagus. These nerves dominate the control of airway function via modulating the activities of the tracheobronchial effectors including airway smooth muscles, submucosal secretory glands and the vasculature. Abnormalities in airway parasympathetic pathways participate in some chronic airway diseases such as bronchial asthma and obstructive sleep apnea syndrome (Smith et al., 1998; Lee and Widdicombe, 2001; Lutz and Sulkowski, 2004; Kato, 2007; Loehrl, 2007).

The airway vagal nerves originate from the airway vagal preganglionic neurons (AVPNs), which are the final common path connecting the central nervous system (CNS) with the airway. AVPNs project preganglionic nerves to the intrinsic tracheobronchial ganglia, whose postganglionic nerves innervate airway effectors (Baker et al., 1986; Maize et al., 1998; Dey, 2003). Previous retrograde tracing studies show that AVPNs are primarily located in three sites in the medulla: the compact portion of the nucleus ambiguus (cNA), the external formation of the nucleus ambiguus (eNA) and the dorsal motor nucleus of the vagus (DMV) (Haxhiu and Loewy, 1996; Kc et al., 2004; Atoji et al., 2005; Chen et al., 2007; Mazzone and McGovern, 2010). However, since AVPNs in the DMV are found to predominantly project to tracheobronchial secretory glands and blood vessels (Kalia and Mesulam, 1980; Haselton et al., 1992; Haxhiu et al., 1993; Haxhiu and Loewy, 1996), and stimulation of these neurons has little impact on airway resistance (Haselton et al., 1992; Kc et al., 2004), only AVPNs in the ventrolateral medulla

Abbreviations: ACSF, artificial cerebral spinal fluid; AHP, afterhyperpolarization; AP, action potential; AP_5, D-2-amino-5-phosphonovalerate; 4-AP, 4-aminopyridine; AVPNs, airway vagal preganglionic neurons; CbTx, charybdotoxin; cNA, compact portion of the nucleus ambiguus; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CNS, central nervous system; DMV, dorsal motor nucleus of the vagus; eNA, external formation of the nucleus ambiguus; EPSCs, excitatory postsynaptic currents; EPSPs, excitatory postsynaptic potentials; IPSCs, inhibitory postsynaptic currents; IPSPs, inhibitory postsynaptic potentials; LK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel; NA, nucleus ambiguus; NTS, the nucleus of the tractus; PAG, periaqueductal gray; pre-BötC, the pre-Bötzinger complex; RMP, resting membrane potential; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel; TTX, tetrodotoxin.

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are thought to be related to the control of airway smooth muscles. Furthermore, when tracers were directly applied to the laryngeal nerves or to the tracheobronchial branch of the vagus, retrogradely labeled AVPNs in the ventrolateral medulla were either exclusively within the cNA (Irnaten et al., 2001a,b; Barazzoni et al., 2005; Okano et al., 2006; Chen et al., 2007) or exclusively within the eNA (Kc et al., 2004), respectively. Therefore, the control of tracheobronchial smooth muscles is likely dominated by AVPNs in the eNA.

In previous studies from our laboratory, AVPNs in the eNA were divided into inspiratory-activated and inspiratory-inhibited neurons (Chen et al., 2007; Qiu et al., 2011; Hou et al., in press). During the inspiratory phase, inspiratory-activated AVPNs receive bursting excitatory inputs while inspiratory-inhibited AVPNs receive bursting inhibitory inputs. However, the firing properties of AVPNs in the eNA have not been studied. Peripherally in the paratracheobronchial ganglion of cats, it is shown in vivo that some neurons burst during the inspiratory phase, and these "phasic" neurons primarily project to the tracheobronchial smooth muscles; others fire tonically during inspiratory intervals, and these "tonic" neurons primarily project to the intercartilaginous spaces (Mitchell et al., 1987). Postganglionic neurons with similar "phasic" or "tonic" firing properties are also proved both in humans and in animals in vitro; and they are proved to be different types of neurons with distinct intrinsic membrane properties (Baker, 1986; Lees et al., 1997; Myers, 1998; Myers et al., 1990). It is thus reasonable to conceive that the "phasic" postganglionic neurons are preferentially innervated by inspiratory-activated AVPNs; and the "tonic" postganglionic neurons by inspiratory-inhibited AVPNs. However, it is still unknown whether inspiratory-activated and inspiratory-inhibited AVPNs are intrinsically different or the same, just differentially modulated by inspiratory synaptic inputs. In the present study, AVPNs in the eNA were retrogradely labeled with a fluorescent tracer applied to the extrathoracic trachea and identified in brainstem slices with rhythmically bursting hypoglossal rootlets, and their electrophysiological properties were examined using patch-clamp technique. We aimed to test the hypothesis that inspiratory-activated and inspiratory-inhibited AVPNs are two types of neurons with different intrinsic membrane properties.

2. Materials and methods

Ethical approval: A total of 58 newborn rats were used in this study. Animal procedures approved by the Ethical Committee of the Fudan University Shanghai Medical College (No. 20110307-060), and were in accordance with the recommendations in the ARRIVE Guidelines in the care and use of experimental animals.

2.1. Retrograde fluorescent labeling of AVPNs in the eNA

Halothane, a volatile anesthetic, was dripped onto a cotton pad placed at the bottom of a glass box (5 cm × 5 cm × 5 cm). Three- to 5-day-old Sprague-Dawley rats (Shanghai Institute for Family Planning) were put in the box for 30 s with the lid closed. This procedure anesthetized the rats but kept their breathing at a relatively normal state. When the rat no longer responded to pinching the limbs, the body was surrounded by ice-water-filled plastic bags to decrease the body temperature and slow the heart rate. After the automatic breathing stopped (usually within 2 min), the animal was put on an ice-water-filled plastic bag in a supine posture, and fixed with gauze. A ventral midline incision was made in the neck to expose the extrathoracic trachea, and rhodamine (XRITC, Molecular Probes, 1% solution, 0.2–0.5 μL) was injected into the trachea adventitia between the fourth and the eighth tracheal cartilage with a glass pipette (tip diameter 30 μm), which was attached to a syringe through polyethylene tubing. The incision was rinsed with saline containing 5 mg mL^{-1} streptomycin sulfate and 50,000 U mL^{-1} penicillin, and sutured. In all instances, great care was taken to avoid unwanted spread of the dye onto neighboring tissues. The animals were heated with a thermo-pad to help recovery. During the entire surgery period (about 5 min), the body temperature of the animal was below 10 °C and the animal had no automatic breathing or struggling. After the surgery, the animal usually resumed automatic breathing within 3 min and started moving freely within another 5 min. A single dose of morphine chloride (10 mg kg^{-1}) was injected intraperitoneally to relief the pain after the surgery. The animals were allowed 36–48 h to recover.

2.2. Slice preparation

The animals were anesthetized deeply with halothane and decapitated at the supracollicular level. The hindbrain was exposed, isolated and immerged in cold (4 °C) artificial cerebral spinal fluid (ACSF) with the following composition (in mmol L^{-1}): NaCl, 124; KCl, 3; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; D-glucose, 10; and sucrose, 10. The solution was constantly bubbled with gas (95% O_2–5% CO_2) and had a pH of 7.4. The cerebellum was removed from each brain and the brainstem was dissected under the aid of a dissection microscope. The brainstem was secured in the slicing chamber of a vibratome (Leica VT 1000S, Leica Microsystems, Wetzlar, Germany) filled with the same ACSF. The rostral end of the brainstem was set upwards and the dorsal surface was glued to an agar block facing the razor. The brainstem was sectioned serially at variable thicknesses (50–300 μm) in the transverse plane. The compact portion of the nucleus ambiguus (NA) was used as a landmark. Once it was visible under the microscope, a single 500–800-μm-thick medullary slice with one to two hypoglossal rootlets retained in each lateral side was taken for experimentation. The thick medullary slice preparation, which contains the pre-Bötzinger complex (pre-BötC), local circuits for motor output generation and respiratory hypoglossal motor neurons, generates inspiratory-phase motor discharge in the hypoglossal nerves (Smith et al., 1991). The slice was transferred to a recording chamber and superfused with flowing ACSF (flow rate, 8–11 mL min^{-1}). The rostral cutting plane of the slice was set upwards to allow fluorescent identification and patch-clamp recording of AVPNs in the eNA. The temperature was maintained at 23 ± 0.5 °C, and the concentration of KCl in the ACSF was increased to 10 mmol L^{-1} to allow steady recording of the respiratory rhythm.

2.3. Electrophysiological recording

Individual AVPN in the eNA was identified by the presence of the fluorescent tracer using an Olympus upright microscope (Olympus American Inc., Center Valley, PA, USA) through a 40× water immersion objective lens. The patch pipettes (2–4 MΩ) were filled with a solution consisting of (in mmol L^{-1}): K^+ gluconate, 150; $MgSO_4 \cdot 7H_2O$, 2; $CaCl_2$, 0.1; HEPES, 10; EGTA, 1; K_2ATP, 2; and Na_3GTP, 0.1. The pH was adjusted to 7.2. The osmolality of the ACSF and the pipette solution was adjusted to 320 mOsm L^{-1} before use.

In voltage clamp experiments, the membrane under the pipette tip was ruptured for whole cell configuration. Neurons with a stable membrane potential that was more negative than −40 mV were accepted for further study. The cells were normally clamped at −80 mV. Using this pipette solution and the holding voltage, the Cl^--mediated inhibitory synaptic currents were minimized and only excitatory synaptic events were detectable. However, when the holding voltage is switched to −50 mV, both excitatory synaptic events (inward) and inhibitory synaptic events (outward) can be detected. To obtain voltage-gated channel currents, 500 ms voltage steps from the holding potential of −80 mV to command potentials from −70 mV to +30 mV in 10 mV increments were tested. In some