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Respiratory signaling of locus coeruleus neurons during hypercapnic acidosis in the bullfrog, *Lithobates catesbeianus*

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ABSTRACT

The locus coeruleus (LC) in the brainstem senses alterations in CO_2/pH and influences ventilatory adjustments that restore blood gas values to starting levels in bullfrogs (*Lithobates catesbeianus*). We hypothesized that neurons of the bullfrog LC are sensitive to changes in CO_2/pH and that chemosensitive responses are intrinsic to individual neurons. In addition, we hypothesized putative respiratory control neurons of the bullfrog LC would be stimulated by hypercapnic acidosis within physiological ranges of P_{CO_2}/pH. 84% of LC neurons depolarized and increased firing rates during exposure to hypercapnic acidosis (HA). A pH dose response curve shows LC neurons from bullfrogs increase firing rates during physiologically relevant CO_2/pH changes. With chemical synapses blocked, half of chemosensitive neurons lost sensitivity to HA; however, gap junction blockade did not alter chemosensitive responses. Intrinsically chemosensitive neurons increased input resistance during HA. These data demonstrate that majority of neurons within the bullfrog LC elicit robust firing responses during physiological ΔCO_2/pH, likely enabling adjustment of acid–base balance through breathing.

1. Introduction

For amphibians, the transition from water to air-breathing during metamorphosis shifts respiratory control from being exclusively O_2 to primarily CO_2/pH driven (Gargaglioni and Milsom, 2007). Tadpoles increase gill ventilation during exposure to modest hypoxia whereas adult amphibians show blunted ventilatory responses to hypoxia, but marked increases in breathing during inhalation of hypercapnic gasses and acidified arterial pH (pH_a; Macintyre and Toews, 1976; Branco et al., 1992, 1993). When unidirectionally ventilated, elevating arterial P_{CO_2} stimulates ventilation, while large changes in P_{O_2} minimally alter breathing in bullfrogs (Kinkead and Milsom, 1994). Moreover, exposing the isolating bullfrog brainstem preparation to hypercapnic acidosis (HA) increases respiratory motor nerve output (Morales and Hedrick, 2002; Taylor et al., 2003a,b), demonstrating that the brainstem of bullfrogs intrinsically detects CO_2/pH and alters motor output independently of peripheral input during hypercapnia. Discrete regions in the amphibian brainstem including the caudal and rostral ventral lateral medulla (VLM) and locus coeruleus (LC) have been identified as CO_2/pH chemoreceptive sites because focal acidification of these areas increases ventilation and ablation attenuates the hypercapnic ventilatory response (Noronha-de-Souza et al., 2006; Taylor et al., 2003a,b). Collectively, these data show that maintenance of normal breathing in amphibians requires signaling from chemosensitive brainstem regions.

In both amphibians and mammals, CO_2/pH-sensitive brainstem areas involved in respiratory chemosensing have been located in regions surrounding the fourth ventricle (Coates et al., 1993; Torgerson et al., 2001). The relative contribution that each mammalian chemosensitive area makes towards respiratory control has been debated (Nattie and Li, 2005; Guyenet et al., 2008). Difficulty in determining the contribution of each brainstem area to respiratory control emanates, in part, from the varied percentage of neurons responding to acid challenges, as well as diverse neuronal sensitivities among regions (Putnam et al., 2004). For example, neurons of the medullary raphé elicit robust responses to CO_2/pH changes, yet only ~20% of these neurons are stimulated upon challenge (Wang et al., 1998). In contrast, LC neurons exhibit modest responses, although >80% of these neurons increase firing rates during acidification (Filosa et al., 2002). Because of the abundance of intrinsically chemosensitive neurons relative to other chemosensitive areas (Nichols et al., 2008), the mammalian LC has been of particular interest for the study of chemosensory mechanisms in respiratory control. Intrinsically chemosensitive LC neurons increase firing rates during acidification with chemical synapses blocked and electrical synapses uncoupled, providing a convenient model to study cellular mechanisms of CO_2/pH-sensing. Conversely, network-driven chemosensitive neurons increase discharge rates during acidification with synaptic connections intact, but do not exhibit chemosensitive responses when synapses are blocked (Nichols et al., 2008).

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In addition to the wide-spread distribution of chemosensitive regions, diversity of cell signaling mechanisms within individual regions introduces further complexity. For example, in mammalian LC neurons, acidification inhibits several different types of K^+ channels and activates TRP channels (Putnam et al., 2004; Jiang et al., 2005; Putnam, 2010; Gargaglioni et al., 2010; Cui et al., 2011), while bicarbonate activates L-type Ca^{2+} channels (Imber and Putnam, 2012) resulting in increase neuronal excitability. Specifics regarding these signal transduction events in LC neurons and other CO_2/pH-sensitive respiratory control neurons do not exist for amphibians.

Noronha-de-Souza et al. (2006) established that breathing a hypercapnic gas mixture increased *c*-fos expression within the LC of toads, suggesting that hypercapnia stimulated these neurons. Following ablation of the LC in toads, the hypercapnic ventilatory response significantly decreased when breathing hypercapnic air, while focal acidification (pH $\leq$7.6) of the intact LC increased minute ventilation. These data provide direct evidence that the LC of amphibians plays a significant role in sensing CO_2/pH and influencing changes in pulmonary ventilation during acid–base perturbations. Due to its noradrenergic content and projections to the spinal cord and telencephalon, the LC of amphibians has been considered homologous to the LC of mammals (Marin et al., 1996). Although the LC regions of mammals and amphibians have analogous functions in respiratory control, it is unknown whether bullfrog LC neurons share similar signaling properties. Previous studies have identified output from respiratory motor nerves and whole-animal ventilatory responses as CO_2/pH sensitive, but measurements directly assessing signaling properties of respiratory control neurons from any chemosensitive area in amphibians are lacking. We hypothesized that neurons of the bullfrog LC would be sensitive to changes in CO_2/pH and that chemosensitive responses would be intrinsic to individual neurons. In addition, we hypothesized putative respiratory control neurons of the bullfrog LC would be stimulated by hypercapnic acidosis within physiological ranges of P_{CO_2}/pH. To test these hypotheses we used the whole-cell patch clamp technique to measure changes in action potential firing frequency of LC neurons during acidification by elevated CO_2 with chemical synapses and gap junctions intact and blocked. In addition, we recorded changes in membrane resistance induced by acidification with CO_2.

2. Materials and methods

2.1. Brainstem slices

Adult bullfrogs (either sex; 98.04 ± 4.67 g; N = 43), *Lithobates catesbeianus*, were maintained at 22 °C water with access to wet and dry areas, 12:12 light dark cycles, and fed crickets ad libitum. Animals were handled following Wright State University Institutional Animal Care and Use Committee guidelines. Following rapid decapitation, the head was placed in 4 °C artificial cerebral spinal fluid (aCSF; for composition see Section 2.2) equilibrated with 97.5% O_2 and 2.5% CO_2. The frontoparietal bone was removed and the brainstem was dissected. 400 μm-thick, transverse brainstem slices were cut using a Vibratome tissue slicer (Leica Microsystems Inc., Buffalo Grove, IL, USA). The locus coeruleus (LC) area has been identified through tyrosine hydroxylase immunoreactive staining in amphibians including *Xenopus laevis*, *Rana ridibunda* (González and Smeets, 1993; González et al., 1994), *Rana perezi*, *Pleurodeles waltl* (Sánchez-Camacho et al., 2003; Marin et al., 1996), *Bufo schneideri* (Noronha-de-Souza et al., 2006), and *Lithobates catesbeiana* (Fournier and Kinkead, 2008), and is illustrated here in a cartoon (Fig. 1). Brainstem slices were equilibrated with control gas mixture (80% O_2, 1.3% CO_2, balanced N_2) at 22 °C and given 1 h to recover from slicing prior to experimentation. Slices containing the LC were placed in a polyethylene recording chamber with a glass coverslip base and stabilized using a nylon grid. LC slices were superfused with aCSF at a rate of ~2.5 mL/min via gravity-fed, stainless-steel drip lines. Solutions were maintained at 22 °C and bubbled with equilibrated gas mixtures (MFC-4 Mass Flow Controller, gas mixer, Sable Systems International, Las Vegas, NV, USA).

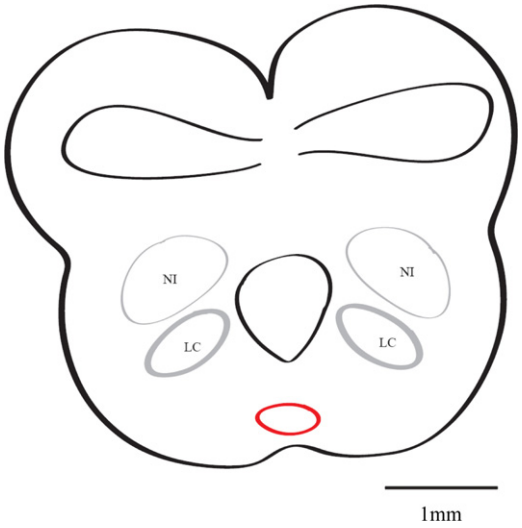

Fig. 1. Cartoon of the brain slice containing the region previously identified as the LC. Neurons were examined within the bold, gray oval (LC area). Neurons located on the ventral part of the slice, within the red oval (peri-LC neurons) served as negative controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Solutions

aCSF was composed (in mM) of 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 7.5 glucose, 40 $NaHCO_3$, 2.5 $CaCl_2$, and 1 NaH_2PO_4 (Taylor et al., 2003a,b, 2008). Control aCSF was equilibrated with 80% O_2, 1.3% CO_2, balanced N_2 (pH 8). 1.3% CO_2 was used as the control because bullfrogs typically experience resting arterial P_{CO_2} near 10 Torr at 20 °C (Howell et al., 1970; Reeves, 1972; Gottlieb and Jackson, 1976). Hypercapnic aCSF was identical to control aCSF except the CO_2 was elevated (percent CO_2 determined by experiment; see Section 2.4.1). Gases were mixed to the desired composition using infrared gas mixers. Synaptic blockade media (SNB; pH 8) was produced by lowering the $CaCl_2$ to 0.2 mM and raising $MgCl_2$ to 11.4 mM with balanced NaCl to maintain osmolarity (Dean and Boulant, 1989; Nichols et al., 2008). Gap junctions were uncoupled using 100 μM carbenoxolone (CBX; Sigma–Aldrich Co., St. Louis, MO, USA) in aCSF (Davidson and Baumgarten, 1988; Masaru and Williams, 1996a,b; Alvarez-Maubecin et al., 2000; Winmill and Hedrick, 2003; Nichols et al., 2009).

2.3. Whole-cell patch clamp recordings

The whole-cell patch clamp technique was used to record membrane potential (V_m) in individual neurons (n = 85) located within the region of the locus coeruleus (LC neurons) of bullfrog brainstem slices. Micropipettes were fabricated from borosilicate glass capillary tubes using a two-stage pipette puller (Model PC-10, Narishige, East Meadow, NY, U.S.A.) with resistances of 3–7 MΩ. Pipettes were back-filled with mock-intracellular solution (composition in mM: 110 K-gluconate, 2 $MgCl_2$, 10 HEPES, 1 Na_2-ATP, 0.1 Na_2-GTP, 2.5 EGTA; pH 7.2) (Martini et al., 2009) and placed over a $AgCl_2$-coated Ag wire connected to Axon instruments CV 203BU headstage (Molecular Devices, Sunnyvale, CA, USA). The LC area was visualized with a Nikon FN1 fixed stage microscope