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Inhibition of the hypercapnic ventilatory response by adenosine in the retrotrapezoid nucleus in awake rats

Bárbara Falquetto [a, 1], Luiz M. Oliveira [a, 1], Ana C. Takakura [a], Daniel K. Mulkey [b], Thiago S. Moreira [c, *]

[a] Dept. of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, 05508, Brazil
[b] Dept. of Physiology and Neurobiology, University of Connecticut, Storrs, CT, 06269, USA
[c] Dept. of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, 05508, Brazil

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ABSTRACT

The brain regulates breathing in response to changes in tissue CO_2/H^+ via a process called central chemoreception. Neurons and astrocytes in the retrotrapezoid nucleus (RTN) function as respiratory chemoreceptors. The role of astrocytes in this process appears to involve CO_2/H^+-dependent release of ATP to enhance activity of chemosensitive RTN neurons. Considering that in most brain regions extracellular ATP is rapidly broken down to adenosine by ectonucleotidase activity and since adenosine is a potent neuromodulator, we wondered whether adenosine signaling contributes to RTN chemoreceptor function. To explore this possibility, we pharmacologically manipulated activity of adenosine receptors in the RTN under control conditions and during inhalation of 7–10% CO_2 (hypercapnia). In urethane-anesthetized or unrestrained conscious rats, bilateral injections of adenosine into the RTN blunted the hypercapnia ventilatory response. The inhibitory effect of adenosine on breathing was blunted by prior RTN injection of a broad spectrum adenosine receptor blocker (8-PT) or a selective A1-receptor blocker (DPCPX). Although RTN injections of 8PT, DPCPX or the ectonucleotidase inhibitor ARL67156 did not affected baseline breathing in either anesthetized or awake rats. We did find that RTN application of DPCPX or ARL67156 potentiated the respiratory frequency response to CO_2, suggesting a portion of ATP released in the RTN during high CO_2/H^+ is converted to adenosine and serves to limit chemoreceptor function. These results identify adenosine as a novel purinergic regulator of RTN chemoreceptor function during hypercapnia.

1. Introduction

The retrotrapezoid nucleus (RTN) contains a subset of neurons that are intrinsically sensitive to changes in CO_2 and pH (Mulkey et al., 2004; Nattie et al., 1993; Nottingham et al., 2001; Smith et al., 1989; Wang et al., 2013) and relay responses to respiratory control regions, such as the ventral respiratory complex to control breathing automaticity (Connelly et al., 1989, 1990; Guyenet and Bayliss, 2015; Li et al., 1999; Smith et al., 1989). Besides the intrinsic property, RTN neuronal mechanisms also involve an indirect activation by purinergic signaling, most likely from CO_2/H^+-sensitive astrocytes (Barna et al., 2016; Gourine et al., 2005a, 2010; Huckstepp et al., 2010b; Wenker et al., 2010, 2012). Evidence also suggests that purinergic signaling is a unique feature of RTN chemoreception (Sobrinho et al., 2014). It was shown that CO_2/H^+ facilitates ATP release at discrete locations near the RTN (Gourine, 2005; Gourine et al., 2005a; Sobrinho et al., 2014; Thomas and Spyer, 2000) and that RTN astrocytes, but not cortical astrocytes, respond to H^+ with increased Ca^{2+}-dependent exocytosis of ATP-containing vesicles (Kasymov et al., 2013). As described above, it is generally well accepted that CO_2-evoked ATP release from ventral medullary surface astrocytes contributes to respiratory drive by activating chemosensitive RTN neurons (Gourine et al., 2010; Wenker et al., 2010). We demonstrated that blocking P2 receptors at the RTN level decreased the hypercapnia ventilatory response (HCVR) (Barna et al., 2016; Sobrinho et al., 2014; Wenker et al., 2012). These results strongly suggest that

* Corresponding author. Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 1524, 05508-000, São Paulo, SP, Brazil.
E-mail addresses: tmoreira@icb.usp.br, tmoreirausp@gmail.com (T.S. Moreira).
[1] Both authors contribute equally as first author.

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purinergic signaling through P2 receptors enhances CO_2/H^+-dependent output of the RTN. It is also well established in other brain regions that extracellular ATP can be rapidly metabolized to adenosine which can modulate neural activity by activation of adenosine receptors (A1-, A2A-B-, and A3-receptors) (Funk, 2013; Howell, 1993; Spyer et al., 1997; Thomas and Spyer, 1999, 2000). In the context of breathing, activation of A1 and A2 receptors generally results in suppression or enhancement of respiratory activity, respectively. Note that A3 receptors have relatively low expression in the central nervous system (Borea et al., 2015) and have not been implicated in control of breathing. Adenosine has been shown to contribute to the ventilatory response to hypoxia in mammals (Howell, 1993; Montandon et al., 2007). For example, exposure to hypoxia may trigger ATP release in respiratory centers including the Pre-Bötzinger complex and its subsequent conversion to adenosine has been shown to suppress respiratory output of this region and potentially contribute to central hypoxic respiratory depression (Angelova et al., 2015; Bissonnette, 2000; Bissonnette et al., 1991; Sheikhbahaei et al., 2018). In terms of central chemoreception, previous work in rats and sheep showed that A1 receptors are highly expressed in the rostral ventrolateral medulla (Bissonnette and Reddington, 1991) in the vicinity of the RTN, and consistent with a role in central chemosensitivity, systemic application of a specific A1 blocker (DPCPX) potentiated the CO_2 ventilatory response in juvenile awake rats (Montandon et al., 2007). However, despite evidence that CO_2/H^+-evoked ATP release occurs in the RTN, the effects of adenosine signaling in the RTN on breathing and the HCVR have not been characterized.

The goals of this study are to determine whether and how ADO signaling in the RTN affects baseline breathing and the HCVR in anesthetized and awake rats. We find that RTN injections of ADO inhibited breathing and the HCVR by an A1-recpetor dependent mechanism. We also found that blocking A1 receptors potentiated respiratory frequency responses to CO_2, suggesting endogenous ADO produced in the RTN during hypercapnia limits CO_2/H^+-dependent respiratory drive.

2. Methods

2.1. Animals

Animal use was in accordance with guidelines approved by University of São Paulo Institutional Animal Care and Use Committee (ICB/USP: 70/2012). *In vivo* experiments were performed on male Wistar rats weighing 250–330 g (3–5 months old; N = 52). All efforts were made to minimize animal discomfort and the number of animals used.

2.2. In vivo preparation and physiological recordings

2.2.1. Anesthetized rats

The surgical procedures and experimental protocols were done in bilaterally vagotomized, artificially ventilated urethane anesthetized rats prepared as described previously (Sobrinho et al., 2014, 2017; Takakura et al., 2011; Takakura and Moreira, 2011). Briefly, general anesthesia was induced with 5% isoflurane in 100% O_2. Artificial ventilation with 1.4–1.5% isoflurane in 100% O_2 was maintained throughout surgery. The surgical procedures (bilateral vagotomy, arterial cannulation, electromyography (EMG), and dorsal transcerebellar access to the ventrolateral medulla) were standard. Bipolar electrodes were coupled to record the activity of diaphragm (Dia_{EMG}), and abdominal (Abd_{EMG}) muscles, and the amplification used for the signal was 5000–10,000x. Rectal temperature (maintained at 37 °C) and end-tidal CO_2 were monitored throughout the experiment with a capnometer (Columbus Instruments) that was calibrated twice per experiment.

On completion of surgical procedures, isoflurane was gradually replaced by urethane (1.2 g/kg iv. over 30 min). After injection of the intravenous anesthetic, the anesthesia level was monitored by testing for absence of withdrawal response and lack of arterial pressure (AP) changes due to firm paw pinch. The rats were ventilated with 100% oxygen throughout the experiment in order to silence peripheral chemoreceptors. Activation of central chemoreflex was done by hypercapnia (10% of $etCO_2$).

All analog data (end-expiratory CO_2, EMG activities, and AP) were stored on a computer via a Micro1401 digitizer from Cambridge Electronics Design (CED, Cambridge, UK) and were processed with version 5 of Spike2 software (CED). Integrated electromyography activity (EMG) was obtained after rectification and smoothing ($\tau = 0.003$ s) (Oliveira et al., 2016). Diaphragm, and abdominal muscle activities were determined by averaging EMG over 30 s and normalizing the result by assigning a value of 0 to the dependent variable recorded at 3–4% of end-expiratory CO_2 and a value of 9–10% of end-expiratory CO_2.

2.2.2. Conscious rats

Rats were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) combined with xylazine (7 mg/kg) and placed in a stereotaxic frame (model 900; David Kopf Instruments). Stainless steel cannulas were placed bilaterally into the RTN using the following coordinates: 2.3 mm caudal to lambda, 1.8 mm lateral to the midline, and 5.5 mm below dura mater. The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. Rats received a prophylactic dose of penicillin (30,000 IU) given intramuscularly and a subcutaneous injection of the analgesic Ketoflex (1%, 0.03 ml/rat) postsurgically. After the surgery, the rats were maintained in individual boxes with free access of tap water and food pellets.

Respiratory rate (f_R, breaths/min) and tidal volume (V_T, ml/kg) in conscious, freely moving rats were measured by whole-body plethysmography as described previously (Barna et al., 2016; Silva et al., 2016; Totola et al., 2017). All experiments were performed at room temperature (24–26 °C). In brief, freely moving rats were kept in a Plexiglas recording chamber (5 L) that was flushed continuously with a mixture of 79% N_2 and 21% O_2 (unless otherwise required by the protocol) at a rate of 1 L/min. The pressure signal was amplified, filtered, recorded, and analyzed off-line using PowerLab software (ADInstruments). A volume calibration was performed during experiment by injecting a known air volume (1 ml) into the chamber. Animals were allowed ~30 min to acclimatize to the chamber environment at normoxia/normocapnia (21% O_2, 79% N_2, and <0.5% CO_2) before measurements of baseline AP and ventilation were taken. Hypercapnia was induced by titrating CO_2 into the respiratory mixture up to a level of 7% for 10 min. Measurements of f_R, and V_T were taken during the last 2 min before exposure to the stimulus and during the 2 min period at the end of drug stimulus, when breathing stabilized. Changes in the f_R, V_T, and minute ventilation (V_E) ($f_R \times V_T$; ml/min/kg) were averaged and expressed as mean ± SE.

2.3. Drugs

All drugs were purchased from Sigma Aldrich (Sigma Chemicals Co.) unless otherwise stated. For experiments in anesthetized animals, ADO (1–50 mM in sterile saline, pH 7.4), 8-PT (100 μM in sterile saline, pH 7.4) or DPCPX (5 μM in sterile saline, pH 7.4) were injected into the RTN using single barrel glass pipettes (tip diameter of 25 μm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp.) All drugs contained a 2% dilution of fluorescent latex microbeads (Lumafluor, New York, NY) for later histological