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Purinergic transmission in the rostral but not caudal medullary raphe contributes to the hypercapnia-induced ventilatory response in unanesthetized rats

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ABSTRACT

The medullary raphe (MR) is a putative central chemoreceptor site, contributing to hypercapnic respiratory responses elicited by changes in brain PCO_2/pH. Purinergic mechanisms in the central nervous system appear to contribute to central chemosensitivity. To further explore the role of P2 receptors within the rostral and caudal MR in relation to respiratory control in room air and hypercapnic conditions, we performed microinjections of PPADS, a non-selective P2X antagonist, in conscious rats. Microinjections of PPADS into the rostral or caudal MR produced no changes in the respiratory frequency, tidal volume and ventilation in room air condition. The ventilatory response to hypercapnia was attenuated after microinjection of PPADS into the rostral but not in the caudal MR when compared to the control group (vehicle microinjection). These data suggest that P2X receptors in the rostral MR contribute to the ventilatory response to CO_2, but do not participate in the tonic maintenance of ventilation under room air condition in conscious rats.

1. Introduction

It has been proposed that central chemoreception (CCR), the specialized property of detecting CO_2/pH changes within the brain, is a widely distributed function in the central nervous system and involves many sites (Nattie, 2000; Nattie and Li, 2009), such as the medullary raphe (MR) which includes raphe magnus (RMg), raphe pallidus (RPa), and raphe obscurus (ROb). It is well established, indeed, that serotoninergic (5-HT) MR neurons play an important role in CCR (Ray et al., 2011; Richerson, 2004). Of interest are the observations that there is heterogeneity in the MR function concerning CCR, when the rostral (RMg) and caudal (ROb) MR are compared (da Silva et al., 2011; Dias et al., 2008; Li et al., 2006). The rostral MR is of particular interest in CCR since it contains a very large percentage of serotonergic neurons (Gao and Mason, 2001) and there is physiological and anatomic evidence for its role in the control of respiration during baseline and hypercapnic conditions (Dias et al., 2007; Holtman et al., 1990; Hosogai et al., 1998). However, the mechanisms associated with the CCR in the MR are not fully understood.

It has been firmly established that ATP has an important role as a neuro- and gliotransmitter in the central nervous system, in addition to its known role as an intracellular energy source (Burnstock, 1997). Among its actions, there is increasing evidence that ATP is an important mediator of CCR (Funk, 2010). Consistent with this possibility, the microinjection of suramin, a P2 receptor antagonist, into the medullary ventral respiratory column (VRC), attenuated respiratory responses to hypercapnia in anesthetized rats (Thomas et al., 1999). Moreover, the blockade of ATP receptors in the same region blocked the CO_2-evoked increase in frequency discharge of respiratory neurons (Thomas and Spyer, 2000). There is compelling evidence that the source of ATP in medullary VRC may be glial cells, which sense changes in the CO_2/pH, and thus release ATP to activate nearby neurons by a P2-receptor-dependent mechanism (Gourine et al., 2010; Wenker et al., 2010). However, the involvement of medullary raphe purinergic neurotransmission in the CCR has not been evaluated.

Several subtypes of P2X (ligand-gated cationic channels) and P2Y (G protein-coupled receptors) receptors have been cloned and described (North, 2002; Ralevic and Burnstock, 1998). P2X receptors have been found to be pH sensitive (King et al., 1996) and therefore could be implicated in the CCR by medullary neurons that express these receptors. Indeed, there is evidence supporting the hypothesis that ATP-P2X signalling has a functional role in the control of respiration and CCR. Moreover, P2X receptors are found in brainstem regions involved in respiratory control including the nucleus tractus solitarii (NTS), ventrolateral medulla (VLM), locus coeruleus (LC) and MR (Close et al., 2009; Gourine et al., 2003;

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Kanjhan et al., 1999; Yao et al., 2000). With respect to CCR, there is evidence that the chemosensitivity of neurons in the pre-Bötzinger Complex is inhibited by PPADS, a non-selective P2X antagonist (Thomas and Spyer, 2000). Considering the MR, an earlier study in anesthetized rats showed that microinjection of ATP in RMg and RPa produced inhibition or facilitation of respiration respectively, while the microinjection of PPADS had no effect on respiratory activity but partially blocked the ATP effects (Cao and Song, 2007). Nevertheless, the role of P2X receptors within the MR in CCR has not been explored in conscious animals.

Therefore, in the present study we evaluated, in different antero-posterior aspects of MR (rostral and caudal) of conscious rats, the role of P2X receptors on the respiratory responses to hypercapnia (7% CO_2). To this end, we performed experiments in unanesthetized rats, in which PPADS was microinjected into the rostral or caudal MR and respiratory parameters measured in room air and hypercapnia conditions.

2. Materials and methods

2.1. Animals

Experiments were performed on unanesthetized adult male Wistar rats weighing 270–300 g. The animals had free access to water and food and were housed in a temperature-controlled chamber at 24–25 °C (model: ALE 9902001; Alesco Ltda., Monte Mor, SP, Brazil), with a 12:12 h light–dark cycle (lights on at 7 AM). All experiments were performed in the light phase between 9:00 AM and 4:00 PM. Animal care was carried out in compliance with the guidelines set by SBCAL (Sociedade Brasileira de Ciência em Animais de Laboratório/Brazilian Society of Animal Lab Science) and with the approval of the University of São Paulo Animal Care and Use Committee (protocol no. 040/2007).

2.2. Surgery

Animals were anesthetized by administration of ketamine (100 mg kg^{-1}; i.p.) and xylazine (15 mg kg^{-1}; i.m.). The head and a portion of the abdomen were shaved, the skin was sterilized with betadine solution and alcohol and the animals were placed in a stereotaxic apparatus (insight, Brazil). Once fixed in the stereotaxic frame, rats were implanted with a stainless steel guide cannula. The guide cannula (0.7 mm o.d. and 15 mm in length) was implanted 3 mm above the rostral MR, which includes the RMg and RPa (10.52 mm caudal from bregma, in the midline, and 7.5 mm below the surface of the skull), or the caudal MR, which comprises the ROb (12.0 mm caudal from the bregma, in the midline, and 7.5 mm below the surface of the skull) (Paxinos and Watson, 1998). The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion. Additionally, animals of all groups were submitted to paramedian laparotomy for the insertion of a temperature datalogger for body temperature measurements (SubCue, Calgary, AB, Canada). Body temperature readings were acquired at 5 min intervals. At the end of surgery, rats received 0.2 mL (1,200,000 units) of benzyl-penicillin administered intramuscularly. Surgical procedures were performed over a period of approximately 40 min and experiments were initiated seven days after surgery.

2.3. Measurements of respiratory variables and body temperature

Respiratory variables were obtained by the whole body plesthymography method (Bartlett and Tenney, 1970). Unanesthetized rats were placed into a 3.9 L Plexiglas chamber at 25 °C and allowed to move freely while the chamber was flushed with humidified air or with a hypercapnic gas mixture containing 7% CO_2 and 21% O_2 and N_2 balance. During each measurement of respiratory variables, the inlet airflow was interrupted for a short period of time (~1 min) while the chamber remained closed. Pressure oscillations caused by respiration were detected by a differential transducer and then amplified (MLT141 spirometer, Power Lab, AdInstruments, NSW, Australia). Recordings were saved and analysed using the PowerLab software (AdInstruments, NSW, Australia). Volume calibration was performed during each measurement throughout the experiments by injecting a known air volume (1 mL) inside the chamber. Respiratory variables such as respiratory frequency (fR) and tidal volume (V_T) were calculated described by Malan (1973). Ventilation ($\dot{V}_E$) was calculated as the product of V_T and fR and presented at ambient barometric pressure, at body temperature, saturated with water vapour at this temperature (BTPS). Body temperature was measured using an i.p.-implanted temperature datalogger (SubCue Dataloggers, Canada).

2.4. Drugs

The P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium (PPADS, Sigma Chemical, St. Louis, MO, USA) (Lambrecht, 2000), was freshly dissolved in pyrogen-free sterile saline (154 mM NaCl), and sodium bicarbonate was added to adjust the pH to 7.4. The concentration of PPADS (0.02 M) used in this study was selected on the basis of previous reports (Cao and Song, 2007).

2.5. Microinjection

For microinjections, a 1 μL syringe (Hamilton, Reno, NV, USA) connected to a PE-10 tubing and to a thin needle injector (33 gauge) was prefilled with PPADS, and then the needle injector was inserted into the rostral or caudal MR accordingly. The average accuracy of the 1 μL syringe is within ±1% of nominal volume and precision (coefficient of variation) within 1%, measured at 80% of total scale volume. The rostral MR contains the RMg while the caudal MR comprises the ROb. Prior to microinjection, animals were gently held in order to insert the needle injector into position in the guide cannula and once in the right position, the injections were manually initiated after a 30 s delay without handling or restraint of the rats. Animals did not undergo multiple injections. Each animal received only one microinjection and each experimental group was composed of different animals. The needle used for microinjection was 3 mm longer than the guide cannula. All microinjections were made with a volume of 50 nL, and in order to avoid reflux, a minute was allowed before removing the injection needle from the guide cannula.

2.6. Experimental protocols

Each animal was individually placed in a Plexiglas chamber (3.9 L) and allowed to move freely while the chamber was flushed with humidified room air. Following a 30 min acclimatization period, measurements of respiratory variables were taken. Subsequently, rats received microinjections of vehicle (saline) or the P2X receptor antagonist, PPADS, into the rostral MR or caudal MR, and a hypercapnic gas mixture (7% CO_2, 21% O_2, N_2 balance) was flushed into the chamber for 30 min. Respiratory variables were measured at 5, 10, 20 and 30 min after initiating hypercapnic condition. Finally, rats were returned to a period of normocapnia. Alternatively, in order to test the effects of PPADS in the baseline respiratory variables, the same procedures were performed, but instead of hypercapnia animals were maintained in normoxic, normocapnic condition after drug injection. All gas conditions were administered by a flow metre gas-mixing pump (Cameron Instruments GF-3/MP). O_2 (Raytech quadralyser 224A) and CO_2 (Beckman