Modulation of respiratory responses to chemoreflex activation by L-glutamate and ATP in the rostral ventrolateral medulla of awake rats

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Moraes DJA, Bonagamba LGH, Zoccal DB, Machado BH. Modulation of respiratory responses to chemoreflex activation by L-glutamate and ATP in the rostral ventrolateral medulla of awake rats. Am J Physiol Regul Integr Comp Physiol 300: R1476–R1486, 2011. First published March 16, 2011; doi:10.1152/ajpregu.00825.2010.—Presympathetic neurons in the different anteroposterior aspects of rostral ventrolateral medulla (RVLM) are colocalized with expiratory [Bötzinger complex (BötC)] and inspiratory [pre-Bötzinger complex (pre-BötC)] neurons of ventral respiratory column (VRC). Sympathetic activity during chemoreflex activation is not completely understood. The RVLM consists of a group of neurons extending from the caudal end of facial nucleus to the rostral ventrolateral medulla (CVLM) with an anteroposterior extension of ~700 μm in adult rats (12, 44). In all anteroposterior extension of RVLM, there are vasomotor presympathetic neurons essential for the generation and control of baseline and reflex-evoked sympathetic activity (24, 25, 38, 45). Intermingled with the presympathetic neurons in the different anteroposterior aspects of RVLM is also found the expiratory (rostrally) and part of inspiratory (caudally) neurons of Bötzinger (BötC) and pre-Bötzinger complexes (pre-BötC) of the ventral respiratory column (VRC), respectively, which are involved in the generation of respiratory pattern (4, 35, 45). Besides this anatomical overlapping, it has been proposed that the respiratory neurons of the VRC are an important source of respiratory modulation of sympathetic activity during chemoreflex activation (10, 11). Considering that in the chemoreflex activation there is an increase in the inspiratory and respiratory activities (1), these different anteroposterior aspects of RVLM appear to be critical sites for the processing of the respiratory responses and for integration of the cardiorespiratory responses to chemoreflex activation.

Experimental evidence supports the concept that L-glutamate and ATP in the RVLM are important for the control of respiratory parameters (26, 41). It has been demonstrated that microinjection of ATP and L-glutamate into the RVLM of anesthetized animals produced comparable changes in the arterial pressure and ventilation (Ve) (26, 41). Nonetheless, there is no evidence about the role of ATP and L-glutamate in the RVLM in the processing of respiratory response of peripheral chemoreflex. Therefore, considering that cardiovascular and respiratory adjustments induced by activation of peripheral chemoreceptors are tightly correlated (10, 23) as well as the possible involvement of L-glutamate and ATP in the processing of chemoreflex responses at the level of RVLM, in the present study we sought to evaluate the role of glutamate ionotropic and purinergic receptors at the different anteroposterior aspects of RVLM (RVLM/BötC and RVLM/pre-BötC) on the respiratory responses to chemoreflex activation in awake rats.

MATERIAL AND METHODS

Animals. The experiments were performed on male Wistar rats provided by the Animal Care Facility of the University of São Paulo, campus of Ribeirão Preto, Brazil and kept on 12:12-h light-dark cycle, with food and water ad libitum. All the experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and Ethical Principles for Animal Experimentation established by the Brazilian Committee for Animal Experimentation and...
approved by the Ethics Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo (protocol no. 093/2009).

Surgical procedures. Five days prior to the experiments, male Wistar rats weighing 270–290 g, under tribromoethanol anesthesia (2.5%, 1 ml/100 g ip; Sigma-Aldrich, St. Louis, MO), were placed in a stereotaxic apparatus (David Kopf, Tujunga, CA) and two guide cannulae (15 mm of extension) were implanted in the same side toward rostral RVLM [containing RVLM and Bötzinger complex (RVLM/BötC) and caudal RVLM, containing RVLM and part of pre-BötC neurons (RVLM/pre-BötC)] for the protocols involving unilateral microinjections of agonist or antagonists vs. agonists as shown in the Fig. 1A. In the protocols involving bilateral microinjections of antagonist vs. chemoreflex responses, bilateral guide cannulae were implanted toward RVLM/BötC or RVLM/pre-BötC as shown in the Fig. 1, B and C, respectively. Supplemental doses of tribromoethanol were given as required to maintain rats at a depth of anesthesia at which hindpaw pinch evoked no motor reflex. According to the Atlas by Paxinos and Watson (30), we used the following coordinates: 1) RVLM/BötC: 3.0 to 3.4 mm caudal to the lambda, 1.8 mm lateral to the midline, and 6.5 mm below the skull surface; 2) RVLM/pre-BötC: 3.5 to 3.8 mm caudal to the lambda, 1.8 mm lateral to the midline, and 6.5 mm below the skull surface. The cannulae were attached to the bone with acrylic cement and tight-fitting stylets were kept inside the guide cannulae to prevent occlusion until the time of experiments involving microinjection through these cannulae. Post-surgery, rats were treated with antibiotic injected intramuscularly (Hemabiotic Veterinarian; Fort Dodge, Campinas, Brazil).

Recordings of cardiovascular parameters. Twenty-four hours before the experiments, under tribromoethanol anesthesia, a polyethylene catheter (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ) was inserted into the abdominal aorta through the femoral artery for measurement of pulsatile arterial pressure (PAP). Mean arterial pressure (MAP) and heart rate (HR) were processed by data acquisition system (PowerLab 4/25, ML845; ADInstruments, Bella Vista, NSW, Australia). A second catheter was inserted into the femoral vein for systemic administration of KCN to activate the peripheral chemoreceptors. The distal ends of the catheters were tunneled subcutaneously, exteriorized through the back of the neck, and sutured to the skin. On the day after the surgery, when the rats had adapted over night to the environment of recording room, the arterial catheter was connected to a pressure transducer (model MLT0380; ADInstruments) and, in turn, to an amplifier (Bridge Amp, ML221; ADInstruments). The pulsatile arterial pressure signals were acquired by a data acquisition system (PowerLab 4/25, ML845; ADInstruments) and recorded on a hard drive of a computer using appropriate software (Chart Pro; ADInstruments).

Phlethysmographic chamber. The experimental protocols were performed with rats placed inside a phlethysmographic chamber for simultaneous recordings of cardiovascular and ventilatory parameters in accordance with the method described by Malan (22a) and previous studies from our laboratory (5, 6, 9, 18). After a period of 30 min for rats to adapt to the plethysmographic chamber (volume of 6 liters), recordings of pressure oscillations produced by the breathing were monitored with the use of a differential pressure transducer (ML141 Sprometer, PowerLab; ADInstruments). VE, tidal volume (VT), and respiratory frequency (fR) were determined in accordance with the method described by Bartlett and Tenney (7). In the experimental protocols involving unilateral microinjections of different concentrations of L-glutamate and ATP into the RVLM, microinjections were performed with the chamber closed to verify simultaneously the alterations in cardiovascular and respiratory parameters in response to microinjection of L-glutamate or ATP into the different anteroposterior aspects of RVLM. In the case of experimental protocol involving bilateral microinjections of the glutamatergic and purinergic antagonists, after the second microinjection, the plethysmographic chamber was closed and the cardiovascular and respiratory parameters were recorded. In the experiments using sequential microinjections of antagonists, the plethysmographic chamber was opened for subsequent bilateral microinjections after measuring the effects of microinjection of the first antagonist.

Unilateral and bilateral microinjections into the RVLM/BötC and RVLM/pre-BötC in awake rats. All microinjections into different anteroposterior aspects of RVLM were performed without any restraint of the rats in accordance with previous studies from our laboratory (6, 9, 18, 20). The extension of the needle (33-gauge; Small Parts, Miami Lakes, FL) used for microinjection was 18.8 mm, which the tip of the needle extended 3.8 mm beyond the tip of guide cannula (15 mm of extension) and the needle was connected to a 1-μl syringe (Hamilton, Reno, NV) through a PE-10 tubing. After removal of the plethysmographic chamber, the injection was manually initiated 30 s later. In the experimental protocols of bilateral microinjections, the time interval between microinjections was ~2 min. The volume of microinjections of agonists/antagonists was 50 nl. Considering that the diffusion sphere of 50 nl microinjections is ~400 μm (28), with a radius of 200 μm, when a microinjections were centered in the middle of the anteroposterior aspect of RVLM/BötC (0–300 μm from caudal tip of facial nucleus) or in the middle of the anteroposterior aspect of RVLM/pre-BötC (400–700 μm from caudal tip of facial nucleus) the spread of the injected volume was restricted to the desired anteroposterior aspect of RVLM.
Chemoreflex activation. Peripheral chemoreceptors were activated by intravenous injection of KCN (40 μg/rat; Merck, Darmstadt, Germany). The cardiovascular and respiratory responses to intravenous KCN injections are exclusively related to the activation of carotid chemoreceptors because ligature of the carotid body artery abolished the responses to KCN injection as described in the study by Franchini and Krieger (15) and in previous experiments from our laboratory (9, 18, 20).

Blood gases evaluation. In a distinct group of rats samples of arterial blood was collected in heparinized tubules (40–60 μl) and analyzed immediately in a multifunctional blood gas analyzer (Cobas b 121; Roche, Mannheim, Germany) for measurements of partial pressure of arterial O2 and CO2 (Pao2 and Paco2) before and after bilateral microinjections of KYN into the RVLM/BötC.

Experimental protocols. Dose-response curves to L-glutamate and ATP were obtained through unilateral microinjections in the RVLM/BötC (L-glutamate, n = 10; ATP, n = 16) and RVLM/pre-BötC (L-glutamate, n = 10; ATP, n = 14). To this, in separate groups of animals, different doses of L-glutamate or ATP were randomly microinjected unilaterally into these subregions and all changes in MAP, HR, FR, Ve, and Ve were evaluated. The same volume of saline was unilaterally microinjected into the RVLM/BötC (n = 5) or RVLM/pre-BötC (n = 5) as a vehicle control. The doses of L-glutamate and ATP corresponding to the ED50 were unilaterally microinjected to determine the effective dose of the antagonists kynurenic acid (KYN) and pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), respectively; into the RVLM/BötC (L-glutamate, n = 6; ATP, n = 6) and RVLM/pre-BötC (L-glutamate, n = 6; ATP, n = 6). These doses of KYN and PPADS were bilaterally microinjected in all experimental protocols involving the activation of the chemoreflex. Thirteen groups of awake rats were used in the experimental protocols involving chemoreflex activation. In groups 1 (n = 7), 2 (n = 4), 3 (n = 6), and 4 (n = 6) baseline and chemoreflex-induced respiratory and cardiovascular parameters were evaluated before and after bilateral microinjections of KYN into the RVLM/BötC (group 1), RVLM/pre-BötC (group 2) and outside these subregions (groups 3 and 4). Groups 5 (n = 6), 6 (n = 6), 7 (n = 6), and 8 (n = 6): in these groups, baseline and chemoreflex-induced respiratory and cardiovascular responses were evaluated before and after bilateral microinjections of PPADS into the RVLM/BötC (group 5), RVLM/pre-BötC (group 6) and outside these subregions (groups 7 and 8). Groups 9 (n = 6) and 10 (n = 6): in these control groups, baseline and chemoreflex-induced respiratory and cardiovascular responses were evaluated before and after bilateral microinjection of the vehicle (saline) into the RVLM/BötC and RVLM/pre-BötC, respectively. Groups 11 (n = 5) and 12 (n = 4): in these groups baseline and chemoreflex-induced respiratory and cardiovascular parameters were evaluated before and after bilateral microinjections of KYN + PPADS (group 11) and KYN + saline (group 12) into the RVLM/BötC. Group 13 (n = 4): in this group, measurements of Pao2 and Paco2 before and after bilateral microinjections of KYN into the RVLM/BötC were evaluated. The FR was quantified within a time window from 10 s before (baseline) and 20 s after the chemoreflex activation in accordance with Granjeiro and Machado (18). The femoral artery and vein catheters were exteriorized throughout a hole in the top of the plethysmography chamber, and this allowed chemoreflex activation with intavenous injections of KCN, while simultaneously recording FAP and FR. The solutions of L-glutamate, ATP, KYN, PPADS (Sigma, St. Louis, MO), and KCN (Merck/Darmstadt, Germany) were freshly dissolved in saline (154 mM NaCl), and sodium bicarbonate was added to adjust the pH to 7.4. The pH was determined using a pH indicator (Spezialindikator, pH 6.4–8.0; Merck, Darmstadt, Germany).

Histology. At the end of each experiment, the rats were killed with an overdose of thiopental sodium [0.01 ml/100 mg iv (Abbott Laboratórios do Brasil, São Paulo, Brazil)] and perfused with saline (154 mM NaCl) followed by 10% buffered formalin. Brains were removed and stored in buffered formalin for 2 days, and then serial transverse sections (18-μm thickness) were cut and stained by neutral red using the Nissl method. The anteroposterior extension of the RVLM, located between the CVLM and the caudal tip of facial nucleus, was measured from serial histological sections of brainstem of the 174 animals used in the present study. Only the rats in which the sites of microinjections were centered in the RVLM/BötC or RVLM/pre-BötC were considered for data analysis in the correspondent experimental protocol and rats in which microinjections were performed outside these subregions or in adjacent areas were considered as a negative control and used as an additional control group (misplaced microinjections).

Data analysis. Values are expressed as means ± SE. Results were analyzed by one-way ANOVA, and the differences between individual means were determined by Student’s t-test followed by Newman-Keuls posttest. Differences were considered significant when P < 0.05.

RESULTS

The anteroposterior length of the area identified as RVLM and located between the rostral aspect of CVLM and the caudal tip of facial nucleus (measured from serial histological sections of brainstem of 174 animals used in the present study) was ~700 μm. Figure 1A is a photomicrograph of a sagittal section of the medulla of one rat representative of the group showing the center of unilateral microinjections sites in RVLM/BötC and RVLM/pre-BötC. Figure 1, B and C presents photomicrographs of coronal sections of the brainstem of two rats, representatives of the groups, showing the sites of microinjection bilaterally in the RVLM/BötC and RVLM/pre-BötC, respectively. The center of injections in RVLM/BötC was ~150 μm caudal from the caudal tip of the facial nucleus and centered in RVLM/pre-BötC was ~550 μm caudal from caudal tip of the facial nucleus. Figure 2 shows typical tracings of two rats representative of the group illustrating the cardiovascular and respiratory responses elicited by L-glutamate and ATP microinjected unilaterally into RVLM/BötC or RVLM/pre-BötC. Based on the pattern of evoked responses to microinjections of L-glutamate (Fig. 2, A and B) and ATP (Fig. 2, C and D) observed and in the anteroposterior extension of microinjections, we subdivided functionally the RVLM in two subregions: 1) rostral aspect of RVLM (RVLM/BötC), in which the respiratory neurons of BötC are colocalized with presympathetic neurons; and 2) caudal aspect of RVLM (RVLM/pre-BötC) containing mostly the respiratory neurons of pre-BötC and presympathetic neurons (4, 12, 26, 44).

Dose-response curves to unilateral microinjections of L-glutamate or ATP into the RVLM/BötC or RVLM/pre-BötC. Pressor and ventilatory responses to unilateral microinjections of increasing doses of L-glutamate and ATP into the RVLM/BötC and RVLM/pre-BötC followed a dose-dependence pattern (Tables 1 and 2). The dose of 0.5 mmol/50 nl of L-glutamate and 0.25 mmol/50 nl of ATP produced changes in MAP and Ve corresponding to 50% of the maximal responses (ED50). Unilateral microinjection of ED50 of L-glutamate produced comparable increases in the MAP either in the RVLM/BötC or in the RVLM/pre-BötC (27 ± 3 vs. 28 ± 5 mmHg; n = 20; Fig. 2, A and B). However, L-glutamate into the RVLM/BötC and RVLM/pre-BötC elicited different patterns of respiratory responses. Unilateral microinjections of L-glutamate into the RVLM/BötC decreased the FR (−60 ± 15 cpm), Vt (−4 ± 1 ml/kg), and Ve (−421 ± 104 ml/kg·min⁻¹; Fig. 2A), while
an increase in fR (45 ± 15 cpn) and $\dot{V}E$ (294 ± 13 ml·kg$^{-1}$·min$^{-1}$) were verified at the RVLM/pre-BötzC level (Fig. 2B). With respect to ATP, unilateral microinjection of ED$_50$ of ATP produced an increase in MAP and decrease in fR, $\dot{V}T$, and $\dot{V}E$ of similar magnitude in RVLM/BötzC (46 ± 1 mmHg; −52 ± 2 cpn; −5 ± 1 ml/kg; −600 ± 24 ml·kg$^{-1}$·min$^{-1}$; Fig. 2C; n = 16) and RVLM/pre-BötzC (45 ± 6 mmHg; −48 ± 1 cpn; −4 ± 0.3 ml/kg; −593 ± 47 ml·kg$^{-1}$·min$^{-1}$; Fig. 2D; n = 14). Unilateral microinjections of the same volume of saline (vehicle) into RVLM/BötzC, and RVLM/pre-BötzC produced negligible changes in the MAP and $\dot{V}E$ (RVLM/BötzC: MAP: 3 ± 0.2 mmHg; $\dot{V}E$: 9 ± 3 ml·kg$^{-1}$·min$^{-1}$, n = 5; RVLM/pre-BötzC: MAP: 5 ± 0.3 mmHg; $\dot{V}E$: 13 ± 5 ml·kg$^{-1}$·min$^{-1}$, n = 5).

**Antagonism of ionotropic glutamate receptors and ionotropic purinergic receptors.** Antagonism of ionotropic glutamate receptors and ionotropic purinergic receptors reduced the pressor and ventilatory responses to unilateral microinjection of L-glutamate and ATP into RVLM/BötzC or RVLM/pre-BötzC, respectively. We evaluated the effects of the unilateral microinjections of KYN (8 nmol/50 nl) into the RVLM/BötzC and RVLM/pre-BötzC on pressor and respiratory responses to ED$_50$ of L-glutamate as well as PPADS (0.25 nmol/50 nl) on pressor and respiratory responses to ED$_50$ of ATP into the same regions. Unilateral microinjections of the KYN or PPADS into the RVLM/BötzC, as well as into the RVLM/pre-BötzC did not change the baseline cardiovascular and respiratory parameters. However, we observed that KYN reduced significantly pressor and respiratory responses to unilateral microinjection of L-glutamate into the RVLM/BötzC and RVLM/pre-BötzC (Table 3). Likewise, PPADS significantly reduced pressor and respiratory responses to unilateral microinjection of ATP in both regions (Table 3). Unilateral microinjection of saline into the same subregions produced negligible effects on pressor and respiratory responses to unilateral microinjection of L-glutamate or ATP (RVLM/BötzC: L-glutamate: ΔMAP: 29 ± 5 vs. 27 ± 8 mmHg; Δ$\dot{V}E$: −355 ± 27 vs. −341 ± 23 ml·kg$^{-1}$·min$^{-1}$, n = 5; ATP: ΔMAP: 51 ± 4 vs. 48 ± 6 mmHg; Δ$\dot{V}E$: −606 ± 44 vs. −598 ± 54 ml·kg$^{-1}$·min$^{-1}$, n = 4). RVLM/pre-BötzC: L-glutamate: ΔMAP: 26 ± 7 vs. 25 ± 4 mmHg; Δ$\dot{V}E$: 286 ± 15 vs. 254 ± 20 ml·kg$^{-1}$·min$^{-1}$, n = 5; ATP: ΔMAP: 46 ± 6 vs. 52 ± 1 ml·kg$^{-1}$·min$^{-1}$; Δ$\dot{V}E$: −598 ± 25 vs. −588 ± 44 ml·kg$^{-1}$·min$^{-1}$, n = 4).

Effects of bilateral glutamatergic receptor antagonism in the RVLM/BötzC or RVLM/pre-BötzC on baseline cardiovascular and respiratory parameters. Figure 3 illustrates tracings of one rat representative of the group, in which it is possible to verify the baseline cardiovascular and respiratory parameters after

**Table 1. Changes in mean arterial pressure (ΔMAP) and ventilation (Δ$\dot{V}E$) in response to unilateral microinjection of L-glutamate into RVLM/BötzC and RVLM/pre-BötzC**

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<tr>
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<th>RVLM/BötzC</th>
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<tr>
<td><strong>L-glutamate</strong></td>
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<td>0.1 nmol</td>
<td>ΔMAP: 14 ± 1 (mmHg)</td>
<td>Δ$\dot{V}E$: 14 ± 2 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<td>0.5 nmol</td>
<td>ΔMAP: 27 ± 3 (mmHg)</td>
<td>Δ$\dot{V}E$: 28 ± 5 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<td>1 nmol</td>
<td>ΔMAP: 39 ± 4 (mmHg)</td>
<td>Δ$\dot{V}E$: 46 ± 1 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<td>2 nmol/50 nl</td>
<td>ΔMAP: 41 ± 6 (mmHg)</td>
<td>Δ$\dot{V}E$: 41 ± 9 (ml·kg$^{-1}$·min$^{-1}$)</td>
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Values are means ± SE. RVLM, rostral ventrolateral medulla; BötzC, Bötzenger complex; pre-BötzC, Bötzenger complex.

**Table 2. ΔMAP and Δ$\dot{V}E$ in response to unilateral microinjection of ATP into RVLM/BötzC and RVLM/pre-BötzC**

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<th>RVLM/BötzC</th>
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<td><strong>ATP</strong></td>
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<td>0.0005 nmol</td>
<td>ΔMAP: 4 ± 1 (mmHg)</td>
<td>Δ$\dot{V}E$: 9 ± 1 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<tr>
<td>0.005 nmol</td>
<td>ΔMAP: 10 ± 1 (mmHg)</td>
<td>Δ$\dot{V}E$: −61 ± 8 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<tr>
<td>0.05 nmol</td>
<td>ΔMAP: 15 ± 2 (mmHg)</td>
<td>Δ$\dot{V}E$: −158 ± 37 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<tr>
<td>0.25 nmol</td>
<td>ΔMAP: 46 ± 1 (mmHg)</td>
<td>Δ$\dot{V}E$: −600 ± 24 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<tr>
<td>1.25 nmol</td>
<td>ΔMAP: 54 ± 2 (mmHg)</td>
<td>Δ$\dot{V}E$: −595 ± 45 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<tr>
<td>5 nmol</td>
<td>ΔMAP: 77 ± 3 (mmHg)</td>
<td>Δ$\dot{V}E$: −858 ± 224 (ml·kg$^{-1}$·min$^{-1}$)</td>
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<tr>
<td>25 nmol/50 nl</td>
<td>ΔMAP: 79 ± 3 (mmHg)</td>
<td>Δ$\dot{V}E$: −888 ± 24 (ml·kg$^{-1}$·min$^{-1}$)</td>
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Values are means ± SE.
bilateral microinjections of KYN (8 nmol/50 nl) into the RVLM/BötC. The results are summarized in the Fig. 4. We observed that microinjections of KYN into the RVLM/BötC produced a significant increase in baseline fR (98 ± 3 vs. 239 ± 4 cpm; Fig. 4B; n = 7; P < 0.001) but not in MAP (Fig. 4A) or HR (295 ± 9 vs. 303 ± 7 beats/min). These respiratory alterations resulted in an increase in PaO₂ (7.2 ± 1.3 mmHg; P < 0.05) and a decrease in PaCO₂ (-13.1 ± 2 mmHg; P < 0.05) (n = 4). Regarding microinjections into RVLM/pre-BötC, bilateral microinjections of KYN into this region in few rats (n = 4) produced a respiratory arrest and the animals were immediately killed with an overdose of thiopental sodium (0.01 ml/100 mg iv). This limitation precluded further studies with ionotropic glutamate receptors antagonism on this subregion of RVLM in awake rats. Microinjections of saline into the RVLM/BötC (n = 6) or RVLM/pre-BötC (n = 6) and KYN outside these subregions (n = 12) produced no changes on baseline cardiovascular and respiratory parameters (data not shown).

Effects of bilateral microinjections of KYN into the RVLM/BötC on cardiovascular and respiratory responses to chemoreflex activation. Fig. 3 shows tracings of one rat representative of the group illustrating the cardiovascular and respiratory responses to chemoreflex activation before and after bilateral microinjections of KYN into the RVLM/BötC. Figure 4 summarizes changes in MAP and fR in response to chemoreflex activation. Bilateral microinjections of KYN into the RVLM/BötC reduced the pressor response (55 ± 2 vs. 15 ± 1 mmHg; P < 0.001) and enhanced the tachypneic response (120 ± 9 vs. 180 ± 9 cpm; P < 0.01) to chemoreflex activation (Fig. 4, C and D; n = 7) with no changes in the bradycardic response (−256 ± 12 vs. −240 ± 12 beats/min). The antagonism of ionotropic glutamate receptors was reversible after 60 min, when cardiovascular and respiratory responses to chemoreflex activation were back to control values (Fig. 4, C and D). In relation to RVLM/pre-BötC, considering that bilateral microinjections of KYN at this level produced respiratory arrest, it was not possible to verify the role of ionotropic glutamate receptors in the RVLM/pre-BötC on the cardiovascular and respiratory responses to chemoreflex activation. Misplaced bilateral microinjections of KYN in areas adjacent to RVLM/BötC (n = 6) or RVLM/pre-BötC (n = 6) as well as bilateral microinjections of saline into these subregions (n = 12) produced no significant changes in the chemoreflex responses (data not shown).

Effects of bilateral microinjections of PPADS into RVLM/BötC or RVLM/pre-BötC on baseline cardiovascular and respiratory parameters. Figure 5 illustrates tracings of one rat representative of the group, showing the baseline cardiovascular and respiratory parameters after bilateral microinjections of PPADS (0.25 nmol/50 nl) into the RVLM/BötC. Bilateral microinjections of PPADS into the RVLM/BötC produced no changes on baseline cardiovascular and respiratory parameters (99 ± 3 vs. 104 ± 2 mmHg; 107 ± 6 vs. 97 ± 8 cpm; 295 ± 10 vs. 300 ± 12 beats/min; n = 6). In addition, bilateral microinjections of PPADS into RVLM/pre-BötC (100 ± 2 vs. 104 ± 1 mmHg; 102 ± 12 vs. 105 ± 9 cpm; 310 ± 14 vs. 293 ± 19 beats/min; n = 6) and outside these subregions (n = 12) (misplaced microinjections) produced no changes on baseline MAP, fR, and HR (data not shown).

Effects of bilateral microinjections of PPADS into the RVLM/BötC or RVLM/pre-BötC on cardiovascular and respiratory responses to chemoreflex activation. Figure 5 illustrates typical tracings of PAP and fR of one rat repre-

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**Table 3. Antagonism of ionotropic glutamate receptors and ionotropic purinergic receptors**

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<tr>
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<th>ΔMAP, mmHg</th>
<th>ΔfR, ml·kg⁻¹·min⁻¹</th>
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<td></td>
<td>Control</td>
<td>5 min</td>
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<tr>
<td>ATP</td>
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</tr>
<tr>
<td>RVLM/BötC</td>
<td>49 ± 1</td>
<td>24 ± 1*</td>
</tr>
<tr>
<td>RVLM/pre-BötC</td>
<td>47 ± 1</td>
<td>26 ± 1*</td>
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<tr>
<td>L-Glutamate</td>
<td></td>
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</tr>
<tr>
<td>RVLM/BötC</td>
<td>31 ± 4</td>
<td>13 ± 2*</td>
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<tr>
<td>RVLM/pre-BötC</td>
<td>28 ± 4</td>
<td>11 ± 5*</td>
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</table>

Values are means ± SE. Antagonism of ionotropic glutamate receptors by kynurenic acid (8 nmol/50 nl) and antagonism of ionotropic purinergic receptors by PPADS (0.25 nmol/50 nl) reduced the pressor and ventilatory responses to microinjection of L-glutamate (0.5 nmol/50 nl) or ATP (0.25 nmol/50 nl) into RVLM/BötC or RVLM/pre-BötC, respectively. *P < 0.05 compared to control responses.
sentative of the group, in which chemoreflex was activated before and 1 and 60 min after bilateral microinjection of PPADS into the RVLM/BötC. The results are summarized in the Fig. 6. We observed that bilateral microinjections of PPADS into the RVLM/BötC reduced significantly the tachypneic response to chemoreflex activation (127 ± 6 vs. 70 ± 5 cpm; Fig. 6B; *; n = 6; P < 0.001). On the other hand, PPADS into RVLM/BötC did not change the pressor (Fig. 6A), as well as the bradycardic responses (−259 ± 12 vs. −243 ± 14 beats/min) to chemoreflex activation. With respect to the RVLM/pre-BötC, no significant changes in the cardiovascular and tachypneic responses to chemoreflex activation were observed after bilateral antagonism of P2X receptors in this subregion (55 ± 2 vs. 60 ± 2 mmHg; 137 ± 14 vs. 126 ± 14 cpm; −243 ± 15 vs. 250 ± 10 beats/min; #; n = 6). Bilateral microinjections of PPADS outside RVLM/BötC (n = 6) or RVLM/pre-BötC (n = 6) did not affect the pressor and tachypneic responses to chemoreflex activation (data not shown).

Effects of sequential bilateral microinjections of KYN and PPADS into the RVLM/BötC on baseline cardiovascular and respiratory parameters and on chemoreflex responses. For a better evaluation of the role of L-glutamate and ATP in the RVLM/BötC on the tachypneic response to chemoreflex activation, we performed a protocol in which KYN and PPADS were sequentially microinjected into this subregion. Figure 7 shows tracings of one rat representative of the group illustrating the baseline PAP and fR as well the chemoreflex responses after sequential bilateral microinjections of KYN and PPADS into the RVLM/BötC. Bilateral microinjections of PPADS into the RVLM/BötC after KYN did not affect the alterations in baseline fR produced by previous bilateral microinjections of

Fig. 4. Effect of ionotropic glutamate receptors antagonism in the RVLM/BötC on baseline cardiorespiratory parameters and on chemoreflex responses. Changes in baseline mean arterial pressure (MAP, mmHg; A) and respiratory frequency (fR, cycles per min, cpm; B) before (C) and 1 and 60 min after bilateral microinjection of KYN (8 nmol/50 nl) into the RVLM/BötC. C and D: shows changes in MAP and fR, respectively, in response to chemoreflex activation with KCN (40 µg 0.1 ml/rat iv). Data are means ± S.E. *P < 0.001 and #P < 0.01 different in relation to the respective control; 1-way ANOVA followed by Newman-Keuls posttest.

Fig. 5. Baseline cardiorespiratory parameters and responses to chemoreflex activation before and after microinjection of PPADS into RVLM/BötC. Typical tracings of one rat representative of the group showing baseline PAP and pressure oscillations inside plethysmographic chamber produced by breathing. This figure also shows changes in these parameters produced by chemoreflex activation with KCN (40 µg 0.1 ml/rat iv) before (control) and 1 and 60 min after bilateral microinjections of PPADS (0.25 nmol/50 nl) into the RVLM/BötC.
enhanced the tachypneic response (120 ± 4 vs. 188 ± 2 cpm; 

\( P < 0.05 \) (Figs. 7 and 8, C and D; \( n = 5 \)) to chemoreflex activation. The sequential bilateral microinjection of PPADS (4 min after microinjection of KYN) into the RVLM/BötC reduced the magnitude of the KYN-induced increase of tachypneic response (188 ± 2 vs. 157 ± 3 cpm; \( P < 0.05 \); Fig. 8D) to chemoreflex activation. On the other hand, PPADS did not change the decrease in the magnitude of the pressor response produced by previous KYN microinjections in the same region (9 ± 6 vs. 10 ± 6 mmHg; Fig. 7 and 8C). Sequential bilateral microinjections of KYN and saline were performed into RVLM/BötC of a control group and the alterations induced by KYN were not altered by subsequent injection of saline (Fig. 8, A–D; \( n = 4 \)).

DISCUSSION

The data of the present study shows that L-glutamate and ATP in the RVLM/BötC play roles in opposite directions on the respiratory component of the chemoreflex in awake rats. The antagonism of ionotropic glutamate receptors in the RVLM/BötC of awake rats not only increased baseline fR, but also enhanced the tachypneic and attenuated the pressor responses to chemoreflex activation. These findings indicate that L-glutamate and its ionotropic receptors at the level of RVLM/BötC are important for the eupneic breathing pattern and for respiratory response to chemoreflex activation. On the other hand, the antagonism of glutamate ionotropic receptors in the RVLM/pre-BötC of awake rats produced respiratory arrest, indicating a critical role for L-glutamate in the generation of the respiratory pattern. Our data also show that the control and enhanced tachypneic response to chemoreflex activation produced by previous microinjection of KYN into the RVLM/BötC were attenuated by the antagonism of ATP receptor with PPADS in the RVLM/BötC, suggesting that ATP plays a facilitatory role at this subregion on the tachypneic response to peripheral chemoreflex activation.
Distinct pattern of cardiorespiratory responses to activation of RVLM/BötC and RVLM/pre-BötC with L-glutamate and ATP. Our results with microinjections of L-glutamate into the RVLM/BötC and RVLM/pre-BötC are the first functional evidence in awake rats showing that RVLM neurons are superimposed with the functionally distinct respiratory neurons of the VRC (4, 26, 35). In the RVLM/BötC (~0–300 μm caudal to the facial nucleus), are found mostly the expiratory neurons of BötC, which are important for the generation of respiratory pattern by suppressing the activity of inspiratory neurons, including those located at the pre-BötC (35). Accordingly, the pressor and apneic responses to activation of glutamatergic receptors in the RVLM/BötC are in agreement with the fact that presympathetic neurons are colocalized with expiratory neurons in this subregion, as suggested by previous studies (4). On the other hand, the pressor and tachypneic responses to microinjections of L-glutamate into the RVLM/pre-BötC indicate that at this level the presympathetic neurons are intermingled with inspiratory neurons. Our suggestion that the RVLM/pre-BötC encompasses the pre-BötC is consistent with previous anatomical examinations of the pre-BötC in adult rats showing that this region is located ~400–500 μm caudal to caudal tip of facial nucleus (12, 40, 44).

In the present study, we observed that microinjection of ATP into the RVLM/BötC and RVLM/pre-BötC of awake rats produces similar pressor and apneic response, as previously observed in rats under anesthesia (39, 41). The apneic response to microinjection of ATP into RVLM/BötC and RVLM/pre-BötC may be related to the fact that ATP is more effective to increase the activity of expiratory than inspiratory neurons, as demonstrated by previous electrophysiological studies (42). Although the inspiratory neurons of pre-BötC are found mainly at the RVLM/pre-BötC level, some expiratory neurons can also be located at this level (40), which may explain why ATP-induced apneic response in the RVLM/pre-BötC. In addition, the pressor response to ATP is in accordance with previous studies performed in anesthetized animals, showing that ATP excites vasomotor sympathetic neurons in the RVLM (31). Due to the fact that previous microinjections of P2X receptor antagonist PPADS into RVLM/BötC or RVLM/pre-BötC reduced the ATP-induced pressor and respiratory effects, we suggest that these responses are driven mainly by the activation of P2X receptors.

Effects of the antagonism of ionotropic glutamate receptors in the RVLM/BötC and RVLM/pre-BötC on baseline cardiorespiratory parameters and on the responses to chemoreflex activation. With respect to baseline fR our results are the first experimental evidence showing that the antagonism of ionotropic glutamate receptors in the RVLM/BötC and RVLM/pre-BötC produce distinct patterns of alterations. In RVLM/BötC we noticed an increase in fR after KYN microinjections, while in the RVLM/pre-BötC we observed a respiratory arrest indicating that L-glutamate plays a critical role at both RVLM subregions in the generation of baseline respiratory pattern. It has been proposed that respiratory pattern is generated by the coordinated activity of pontine-medullary expiratory and inspiratory neurons which are reciprocally connected by inhibitory pathways (8, 35). Although VRC seems to play a dominant role in the generation of respiratory rhythm (37), the coordinated activity of VRC neurons and the generation of three-phase eupneic respiratory pattern depend on excitatory afferent inputs from other nuclei, such as the pontine respiratory neurons (35). Studies performed in unanesthetized in situ preparations demonstrated that the mechanical disrup-
tion of pontine-VRC connections causes a dramatic alteration in the respiratory pattern (32). Moreover, the stimulation of dorsolateral pons, specifically of Parabrachial/Kölliker-Fuse nuclei, produced an activation of expiratory neurons in the BötC, mainly postinspiratory neurons (14, 34), which are important for phrenic on switch (8, 35). In this scenario, we suggest that the antagonism of ionotropic glutamate receptors in the RVLM/BötC produced an increased baseline fR possibly due to a reduction of the excitatory tonic drive from pons to expiratory neurons in the RVLM/BötC. This would decrease the inhibitory tone to RVLM/pre-BötC inspiratory neurons, allowing an increase in the inspiratory activity. Regarding the RVLM/pre-BötC, the observation that the antagonism of ionotropic glutamate receptors at this level produced a respiratory arrest in awake rats is in agreement with previous data showing that the antagonism of excitatory amino acid receptors at the pre-BötC in vitro abolished the respiratory rhythm (19), supporting the concept of an involvement of excitatory glutamatergic transmission in the generation of respiratory pattern at the pre-BötC level (33, 36). Therefore, these findings are the first demonstration in vivo and in unanesthetized animals showing that glutamatergic neurotransmission, even inside of RVLM, is required for the generation of eupneic respiratory pattern, either at the RVLM/BötC as well as RVLM/pre-BötC level.

Considering that in the present study we observed that bilateral microinjections of KYN into the RVLM/BötC produced a large increase in the baseline fR, we would expect a smaller tachypneic response to chemoreflex activation. However, we observed an enhanced of tachypneic response to chemoreflex activation after KYN microinjections. These results were different from those observed by Miyawaki et al. (24) in anesthetized rats in which the antagonism of ionotropic glutamate receptors in the RVLM/BötC did not change the respiratory response to chemoreflex activation. It is possible that the enhanced of tachypneic response to chemoreflex activation under this experimental condition is related to the fact that inspiratory neurons in the RVLM/pre-BötC become more excitable, because of reduction of inhibitory inputs from expiratory neurons in the RVLM/BötC and, consequently, during chemoreflex activation, excitatory inputs to inspiratory neurons in the RVLM/pre-BötC enhanced the tachypneic response. In this scenario, we suggest that during chemoreflex activation, glutamatergic inputs from the pons or retrotrapezoid/parafacial nucleus activate inhibitory respiratory neurons in the BötC, which are important to the expiratory response and to the control of magnitude of inspiratory response. However, the involvement of the glutamatergic neurotransmission in the RVLM/BötC and RVLM/pre-BötC on chemoreflex induced expiratory and inspiratory activities need further studies.

With respect to pressor response to chemoreflex activation, the present study indicates that the chemoreflex-mediated stimulation of bulbar presympathetic neurons of RVLM/BötC involves the activation of ionotropic glutamatergic receptors. The anatomical location of microinjections into RVLM/BötC in the present study (~0–300 μm caudal to the facial nucleus) are in agreement with previous studies performed in anesthetized animals (24, 38), demonstrating that the presympathetic neurons of the RVLM/BötC are involved in generation of pressor response to chemoreflex activation. Considering that during chemoreflex activation the sympathoexcitatory response is coupled with the expiration (10) and that in the RVLM/BötC there are mainly expiratory neurons of BötC, further studies to evaluate the involvement of respiratory activity in the reduction of sympathoexcitatory response after KYN in the RVLM/BötC are required. It is important to mention that the blockade of sympathoexcitatory response to chemoreflex activation after microinjections of KYN into RVLM observed by Koshiya et al. (22) and Moreira et al. (27) was obtained when the center of microinjections was located 500 μm caudal to the facial nucleus (RVLM/pre-BötC). In the present study, we did not examine the effects of the antagonism of ionotropic glutamate receptors in the RVLM/pre-BötC on the pressor response to chemoreflex activation in awake rats due to respiratory arrest. The involvement of glutamatergic mechanisms at the RVLM/
pre-BötC level on the pressor and respiratory responses to chemoreflex activation requires further studies in an appropriated experimental model, in which the respiratory arrest in response to the antagonism of glutamate receptors will not preclude an evaluation of these responses.

Effects of P2X receptor antagonism in the RVLM/BötC or RVLM/pre-BötC on baseline cardiovascular and respiratory parameters and on the responses to chemoreflex activation. We observed that the antagonism of P2X receptors in the RVLM/BötC and RVLM/pre-BötC did not alter baseline cardiovascular and respiratory parameters. Although studies by Thomas and Spyer (42) showed that PPADS reduced the baseline firing in the activity of RVLM neurons with inspiratory-related discharge, our data allow us to suggest that in awake rats, ATP and P2X receptors are not playing a major role in the control of baseline cardiovascular and respiratory parameters. On the other hand, we observed that at the RVLM/BötC level ATP is important for the processing of the tachypneic response to chemoreflex activation. These results are in accordance with the observation that the hypoxia-induced ATP release at the RVLM level contributes to the generation of tachypneic response (17). We suggest that ATP is released in RVLM/BötC after the activation of peripheral chemoreflex and acts probably on expiratory neurons (42) controlling the duration of inspiration, which ensures the tachypneic response to chemoreflex activation. However, the involvement of ATP in the RVLM/BötC in the inspiratory and expiratory responses to chemoreflex activation as well as the origin of the ATP released requires further experiments. Considering that an important source of ATP are glial cells and that glutamate from neurons can induce ATP release by glia (16), we suggest that glial cells may contribute to the release of ATP in response to chemoreflex activation at the level of RVLM/BötC.

The lack of evidence about the involvement of ATP in neurotransmission of pressor and respiratory components of peripheral chemoreflex in RVLM/pre-BötC of awake rats does not rule out the possibility that this purine may be involved in the control of baseline cardiovascular and respiratory parameters as well on the processing of this reflex directly or via interactions with glutamatergic neurotransmission, because ATP acting on P2Y receptors may also release glutamate from glial cells in RVLM/pre-BötC (21). The possible role of P2Y receptors located in RVLM/BötC and RVLM/pre-BötC on baseline cardiovascular and respiratory parameters and on the cardiovascular and respiratory responses to chemoreflex activation also requires further investigation.

To better evaluate the effects in opposite directions of L-glutamate and ATP at the RVLM/BötC in the control of respiratory activity, we performed an experimental protocol involving the sequential microinjections of KYN and PPADS into RVLM/BötC. The observation that bilateral microinjection of PPADS into the RVLM/BötC reduced the increase in the tachypneic response to chemoreflex activation, after bilateral microinjections of the KYN in the same subregion, confirm our hypotheses that ATP and L-glutamate are required for the processing of tachypneic response in awake rats in opposite directions.

Conclusion. Our data indicate that ionotopic glutamate and P2X receptors in the RVLM/BötC play roles in opposite directions in the control of respiratory response to chemoreflex activation in awake rats. Figure 9 is a schematic model of the possible glutamatergic and purinergic medullary mechanisms involved on baseline and chemoreflex-induced cardiovascular and respiratory responses based on the present and previous studies (22, 32, 35, 38). The pontine-medullary respiratory network that generates inspiratory and expiratory activities to coordinate activity of spinal and cranial motoneurons is shown. In the Fig. 9 we suggest the possible origin of glutamatergic inputs to the vasomotor presympathetic or expiratory neurons of the RVLM/BötC, as well the release of the ATP in the same subregion acting on expiratory neurons.

Perspectives and Significance

Our findings have important implications for a better understanding of the neurochemical mechanisms underlying central coupling of respiratory and sympathetic activities in physiological and pathophysiological conditions. It is well described that during eupnea (normoxia and normocapnia) sympathetic nerve exhibited a phasic increase of its activity coincident with phrenic burst, reaching a peak during late inspiration or the beginning of expiration (postinspiration). On the other hand, during acute (10, 11) or intermittent hypoxia (46), there is a burst of sympathetic activity during postinspiratory and late expiratory phases, respectively. Considering that central respiratory-sympathetic coupling involves a tight interaction between neurons located in VRc and RVLM regions, it is possible that this distinct pattern of coupling in these different conditions (eupnea and hypoxia) may be due to the recruitment of the neurons of the different anteroposterior aspects of RVLM correspondent to the sub-regions RVLM/BötC and RVLM/pre-BötC identified and explored in the present study. Therefore, the data of the present study obtained in conscious freely moving rats showing the roles of ATP and L-glutamate in opposite directions on respiratory component of chemoreflex contribute to a better understanding of how neurons in the different anteroposterior aspects of RVLM participates in the central coupling of respiratory-sympathetic activities and opens new and interesting possibilities for a better understanding of neural dysfunctions underlying the autonomic and respiratory changes in pathophysiological conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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