Activation of 5-hydroxytryptamine type 3 receptor-expressing C-fiber vagal afferents inhibits retrotrapezoid nucleus chemoreceptors in rats

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ABSTRACT: Retrotrapezoid nucleus (RTN) chemoreceptors are regulated by inputs from the carotid bodies (CB) and from pulmonary mechanoreceptors. Here we tested whether RTN neurons are influenced by 5-hydroxytryptamine type 3 receptor-expressing C-fiber vagal afferents. In urethane-anesthetized rats, selective activation of vagal C-fiber afferents by phenylbiguanide (PBG) eliminated the phrenic nerve discharge (PND) and inhibited RTN neurons (n = 24). PBG had no inhibitory effect in vagotomized rats. Muscimol injection into the solitary tract nucleus, commissural part, reduced inhibition of PND and RTN by PBG (73%), blocked activation of PND and RTN by CB stimulation (cyanide) but had no effect on inhibition of PND and RTN by lung inflation. Bilateral injections of muscimol into interstitial NTS reduced the inhibition of PND and RTN by PBG (53%), blocked the inhibitory effects of lung inflation but did not change the activation of PND and RTN neurons by CB stimulation. PBG and lung inflation activated post-inspiratory neurons located within the rostral ventral respiratory group (rVRG) and inhibited inspiratory and expiratory neurons. Bilateral injections of muscimol into rVRG eliminated PND and partially decreased RTN neuron inhibition by PBG (32%).

In conclusion, activation of cardiopulmonary C-fiber afferents inhibits the activity of RTN chemoreceptors. The pathway relays within a broad medial region of the NTS and involves the rVRG to a limited degree. The apnea triggered by activation of cardiopulmonary C-fiber afferents may be due in part to a reduction of the activity of RTN chemoreceptors.

Key words: phenylbiguanide, bronchiopulmonary chemoreceptors, central chemoreceptors, medulla oblongata, lung inflation, phrenic nerve activity.
INTRODUCTION

The retrotrapezoid nucleus (RTN) contains acid-sensitive glutamatergic interneurons with properties consistent with a central respiratory chemoreceptor function (Akilesh et al., 1997; Feldman et al., 2003; Guyenet et al., 2005b; Mulkey et al., 2004; Nattie and Li, 2006; Okada et al., 2002; Ritucci et al., 2005; Rosin et al., 2006; Stornetta et al., 2006). RTN neurons are also activated by carotid body stimulation and inhibited by lung inflation and these effects are probably mediated via direct projections from solitary tract nucleus (NTS) neurons (Takakura et al., 2006; Moreira et al., 2007). This circuitry suggests that the control of the respiratory pattern generator (CPG) by the carotid bodies and by lung mechanoreceptors operates in part by adjusting the excitatory drive that the respiratory network receives from central respiratory chemoreceptors such as RTN neurons (Takakura et al., 2006; McCrimmon and Alheid, 2007; Moreira et al., 2007).

In the present study, we test whether this concept can be extended to the control of inspiratory activity by cardiopulmonary C-fiber afferents. Activation of this type of afferent produces protective reflexes characterized by inhibition of the inspiratory motor output, bradycardia, sympathoinhibition and airway constriction (Coleridge and Coleridge, 2001; Verberne et al., 2003). Consistent with our hypothesis that regulation of central chemoreceptors contributes to the respiratory reflexes elicited by cardiopulmonary afferents, we show here that RTN neurons are uniformly inhibited by C-fiber vagal afferent stimulation. In addition, we provide some limited information on the pathway responsible for RTN inhibition by C-fiber vagal afferent stimulation.
MATERIALS AND METHODS

Animals

The experiments were performed on 32 male Sprague-Dawley rats (Taconic; Germantown, NY, USA) weighing 250-350 g. Procedures were in accordance with NIH Animal Care and Use Guidelines and were approved by the University of Virginia’s Animal Care and Use Committee.

In vivo recordings

General anesthesia was induced with 5% halothane in 100% oxygen. The rats received a tracheostomy. Artificial ventilation with 1.4-1.5% halothane in 100% oxygen was maintained throughout surgery. The surgical procedures (bladder catheterization, arterial cannulation, phrenic nerve dissection, dorsal access to the medulla oblongata through the atlanto-occipital membrane) have been described previously (Guyenet et al., 2005; Takakura et al., 2006). A bilateral vagotomy was performed in 3 rats 1-2 cm caudal to the confluence of the superior laryngeal nerve.

Upon completion of surgical procedures, halothane was reduced to 1% (21 rats) or gradually replaced by urethane (1.2 g/kg, administered i.v. over 20 min; 11 rats). The initial dose of urethane was adequate for about one hour and had to be supplemented hourly and at least twice with an injection of 0.1 g/kg. After a total of 1.4-1.5 g/kg the level of anesthesia was stable for the rest of the experiments (up to 5 hours after the initial anesthetic cross-over). The rats were ventilated with 100% oxygen throughout the experiment. Rectal temperature was maintained at 37°C and end-tidal CO2 was monitored throughout the experiment with a microcapnometer (Columbus Instruments, Ohio, USA).
After injection of the intravenous anesthetic or reduction of the halothane concentration, the adequacy of anesthesia was monitored during a 30 min stabilization period by testing for absence of withdrawal response, lack of BP change and lack of change in PND rate or amplitude to firm toe or tail pinch. Then, the muscle relaxant pancuronium was administered at the initial dose of 1 mg/kg i.v. and the adequacy of anaesthesia was thereafter gauged solely by the lack of increase in BP and PND rate or amplitude to firm toe or tail pinch. At the end of the physiological experiment the rats were deeply anesthetized with halothane (4% until AP reached 40 mmHg). Then they were perfused through the left cardiac ventricle with phosphate-buffered saline (pH 7.4; 150 ml) followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4, 500 ml). The brains were post-fixed overnight in the paraformaldehyde solution. The brains were then sectioned in the coronal plane (30 µm) and the sections were kept in cryoprotectant awaiting histological procedures.

Arterial blood pressure (AP), PND, tracheal CO₂ and tracheal pressure were recorded as previously described (Takakura et al., 2006; Moreira et al., 2007). Single-unit recording and juxtacellular labeling of RTN neurons with biotinamide were done as described previously (Mulkey et al., 2004; Takakura et al., 2006; Moreira et al., 2007). RTN units were concentrated between 150 and 300 µm below the lower edge of the facial motor nucleus and from 200 µm caudal to 300 µm rostral to the caudal boundary of this nucleus (Mulkey et al., 2004; Stornetta et al., 2006; Takakura et al., 2006). This region lies between coronal planes Bregma -11.8 and -11.3 mm of the Paxinos and Watson atlas (Paxinos and Watson, 1998). The defining property of RTN neurons is a strong activation by hypercapnia (discharge threshold at 4-4.5 % CO₂ and firing rate of 6-14 Hz at 10%
CO₂). Their high sensitivity to hypercapnia and insensitivity to changes in blood pressure
distinguish these neurons from the blood-pressure regulating pre-sympathetic neurons
that are the bulk of the other active neurons detected within this limited region of the
brain under our experimental conditions (Mulkey et al., 2004). Before searching for RTN
neurons, ventilation was adjusted to lower end-expiratory CO₂ to 4% at steady-state (60 -
80 cycles/s; tidal volume 1 - 1.2 ml/100 g). Variable amounts of pure CO₂ were then
added to the breathing mixture to adjust end-expiratory CO₂ to the desired level without
changing ventilation parameters. The barosensitive neurons of the RVLM were identified
as described before (Brown and Guyenet, 1985). They were recorded 0 - 0.4 mm caudal
to the facial motor nucleus (Bregma levels -11.6 to -12.0 mm after (Paxinos and Watson,
1998)). Transient occlusion of the descending aorta just below the diaphragm was used to
activate arterial baroreceptors (Brown and Guyenet, 1985). Rostral ventral respiratory
group (rVRG) respiratory neurons were recorded between Bregma levels –13.0 to –13.4
mm (Schwarzacher et al., 1991; Stornetta et al., 2003). Cell types are named according to
standard descriptive terminology referring to the timing of their discharge relative to that
of the phrenic nerve (Schwarzacher et al., 1991). We made no attempt to determine
whether the recorded respiratory neurons had a motor, premotor or some other function
within the respiratory network.

All analog data (end-expiratory CO₂, PND, unit activity, AP) were stored on a
microcomputer via a micro-1401 digitizer from Cambridge Electronics Design (CED,
Cambridge, UK) and were processed off-line using version 5 of the Spike 2 software
(CED) as described before (Mulkey et al., 2004; Takakura et al., 2006; Moreira et al.,
2007). Processing included action potential discrimination and binning, neuronal
discharge rate measurement. PND “integration” (iPND) consisted of full-wave rectification and smoothing (τ: 0.015s). Neural minute x volume (mvPND, a measure of the total phrenic nerve discharge per unit of time) was determined by averaging iPND over a fixed number of lung inflation cycles and normalizing the result by assigning a value of 0 to the dependent variable recorded at low levels of end-expiratory CO₂ (below PND threshold) and a value of 1 at the highest level of pCO₂ investigated (between 9.5 and 10%) before any injection of muscimol was made. CO₂ was set at the beginning of the experiment so that mvPND would be between 0.5 and 0.7 units. The CED software was also used for acquisition of peri-event histograms of neuronal activity and peri-event averages of iPND, tracheal CO₂, or tracheal pressure. The peri-event histograms of neuronal single-unit activity were triggered either on iPND or on the tracheal pressure trace. Each histogram represents the summation of at least 100 central respiratory or ventilation cycles (350 to 800 action potentials per histogram). The steady-state relationship between RTN neuronal activity and end-expiratory CO₂ was obtained by stepping the inspired CO₂ level to various values for a minimum of 3 min and up to 5 min (Guyenet et al., 2005; Takakura et al., 2006; Moreira et al., 2007). The mean discharge rate of the neuron was measured during the last 30 s of each step at which time end-expiratory CO₂ and the discharge of the neuron appeared to have reached equilibrium. End-expiratory CO₂ was measured by averaging the maximum values recorded from 10 consecutive breaths at the midpoint of the time interval sampled.

Stimulation of the carotid bodies was done with bolus injections of sodium cyanide (50 μg/kg, i.v.). Under our experimental conditions NaCN activates brainstem neurons exclusively via stimulation of peripheral chemoreceptors because denervation of
the carotid bodies eliminates the excitatory effect of cyanide on PND and RTN neurons (Takakura et al., 2006). Stimulation of unmyelinated cardiopulmonary receptors was done by i.v. administration of the 5-hydroxytryptamine type 3 (5-HT₃) receptor agonist phenylbiguanide (PBG, 50 µg/kg, i.v.). Under our experimental conditions the inhibitory effect of PBG on breathing depends entirely on the integrity of vagal afferents because the drug was ineffective in 3 rats with bilateral cervical vagotomy. The cardiorespiratory effects caused by i.v. PBG in the anesthetized rat are mediated by serotonin-3 receptors because they can be antagonized by the selective antagonist MDL-72222 (Verberne and Guyenet, 1992). Lung mechanoreceptors were activated by transiently elevating positive end-expiratory pressure (PEEP) (5-20 sec) from a resting level of +1 cm H₂O to +6 cm H₂O (Moreira et al., 2007).

**Muscimol injections.**

The GABA-mimetic drug muscimol (Sigma Chemicals Co., St-Louis, MO, USA; 1.75 mM in sterile saline pH 7.4) was injected intraparenchymally while a single RTN neuron was being recorded (Takakura et al., 2006; Moreira et al., 2007). The muscimol solution contained a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) for later histological identification of the injection sites (Moreira et al., 2006). Muscimol (30 nl; 50 picomoles) was pressure injected over 3-5 seconds through single-barrel glass pipettes with a 20 µm external tip diameter.

In 8 rats, the electrode tip was directed to the rostral ventral respiratory group (rVRG) by locating inspiratory-related field potentials (Takakura et al., 2006). These are the same rats in which we examined the effects of muscimol injection into rVRG on the
response of RTN neurons to lung inflation (Moreira et al., 2007). In the present study we report the effect of muscimol on the response of the same 8 neurons to i.v. administration of PBG. The 8 reported units (1 per rat) were kept for at least 20 min after the injections of muscimol after which the experiment was terminated.

The protocol used to test the effect of muscimol injection into the commissural or interstitial part of the nucleus of the solitary tract (commNTS, iNTS) was similar except that surface landmarks were used to identify the insertion point of the pipette. To inhibit commNTS, a single 30 nl injection of muscimol was placed at or near the midline, 0.5 mm below the surface of the medulla oblongata and 200-300 µm caudal to the calamus scriptorius. This experiment was carried out in 6 rats. The effect produced by muscimol injection into commNTS dissipated after two hours allowing the same 6 animals to be reused to test the effect of muscimol injection into iNTS. After isolating and characterizing a second RTN chemosensitive neuron, muscimol (30 nl) was injected into iNTS on both sides of the brain (0.5 mm below the surface of the medulla oblongata, 500 µm rostral to the calamus scriptorius and 0.8 mm lateral to the midline) and the same RTN neuron was recorded for another 20 minutes.

**Histology, cell mapping and imaging**

At the end of the experiment the rat was deeply anesthetized with halothane and perfused transcardially with PBS (pH 7.4) followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). All histochemical procedures were done using 30 µm-thick free-floating sections according to previously described protocols (Stornetta et al., 2006; Takakura et al., 2006). Cells labeled with biotinamide were identified by incubating the
sections with streptavidin conjugated with Alexa-488. The transcription factor Phox2b was detected as previously described using a rabbit polyclonal antibody (1:800 for 48-72 hrs followed by a Cy3-tagged donkey anti-rabbit IgG at 1:200, Jackson) (Stornetta et al., 2006). The antibody (a gift from J.-F. Brunet, Ecole Normale Supérieure, Paris, France) was raised against the fourteen amino acid C-terminal sequence of the Phox2b protein and its specificity has been previously established for both mouse and rat (Pattyn et al., 1997; Stornetta et al., 2006).

The relevant photographic techniques and the computer-assisted mapping method used to record the location of drug injection sites, labeled neurons and nerve terminals have been described in detail previously (Stornetta and Guyenet, 1999; Rosin et al., 2006). The neuroanatomical nomenclature and Bregma levels are according to the atlas of Paxinos and Watson (1998).

Statistics

Statistical analysis was done with Sigma Stat version 3.0 (Jandel Corporation, Point Richmond, CA, USA). Data are reported as means ± standard error of the mean. T-test (paired or unpaired) or one-way repeated measure parametric ANOVA followed by Newman-Keuls multiple comparisons test was used as appropriate. Significance was set at p < 0.05.

RESULTS

Effect of sodium cyanide (NaCN), phenylbiguanide (PBG) and lung inflation on RTN neurons.
Unless specifically mentioned, all experiments were done in rats with intact vagus nerves. According to our prior definition, RTN neurons (24 neurons in 14 rats with vagus nerves intact and 8 neurons in 3 vagotomized rats) were highly active in hypercapnia (6-14 Hz at 10% end-expiratory CO₂) and silent in hypocapnia with a threshold between 4 and 5% end-expiratory CO₂ (Fig. 1A). The CO₂ threshold of RTN neurons was always lower than that of the PND (Fig. 1A). Prior to testing the responses to NaCN, phenylbiguanide and lung inflation, end-expiratory CO₂ level was set at around 7.5% CO₂, concentration at which PND amplitude was 50-70% of its maximal value at 10% CO₂. Stimulation of carotid chemoreceptors with NaCN (50 μg/kg i.v. boluses) increased arterial pressure (AP) (from 126 ± 3 mmHg to 140 ± 3 mmHg, 6 rats, p<0.05) (Figs. 1B, 2A1 and B), increased PND amplitude (Figs. 1B, 2A1, F and G) and vigorously activated every RTN neuron tested (from 7.3 ± 1 Hz to 18.4 ± 2.5 Hz, n= 16, p<0.01; Figs. 1B, 2A1 and C). PBG administration decreased AP (from 126 ± 3 mmHg to 108 ± 4 mmHg, p<0.05) (Figs. 1B, 2A1 and B), reduced PND amplitude (Figs. 1B, 2A1, F and G) and strongly inhibited every RTN neuron tested (n = 16, Figs. 1B, 2A1 and D). Raising end-expiratory pressure (PEEP) from a resting level of +1 to +6 cm of H₂O eliminated PND (Figs. 1B, 2A1, F and G) and inhibited the RTN neurons selected for further study by more than 27% (27-100%; Figs. 1B, 2A1 and E). The CO₂-activated neurons that responded to lung inflation with less than 25% inhibition (Moreira et al., 2007) were also uniformly inhibited by PBG (from 8.8 ± 0.6 Hz to 4.6 ± 0.6 Hz, N=5; P<0.05). These neurons were not studied further because they were unsuitable to accomplish one of the main objectives of the study namely to differentiate the pathways mediating the effect of lung inflation vs. pulmonary C-fibers on RTN neurons.
Fig. 1B illustrates a typical RTN neuron that responded to all three stimuli (hereafter called a triple-responder). This RTN neuron was activated by NaCN, it was inhibited by PBG and was also robustly inhibited by a rise in PEEP. Although cardiopulmonary C-fiber activation ought to produce bronchoconstriction (Kubin et al., 2006), PBG produced no detectable effect on tracheal pressure in our experimental conditions (e.g. Fig. 1B). This negative finding is probably due to the vagolytic action of the muscle relaxant pancuronium.

To verify that the effects of PBG on RTN neurons were mediated by the activation of vagal afferents, control experiments were performed in three vagotomized rats (n = 8 cells; data not illustrated). As expected, the stimulatory effect of cyanide on PND and RTN neurons was of normal magnitude in these rats but we did not find any CO₂-activated neurons that could be inhibited by lung inflation. The inhibitory effect of PBG on PND and RTN neurons was either absent in vagotomized rats or replaced by a very small and transient excitation. The loss (lung inflation, PBG) or persistence (carotid body stimulation with cyanide) of the phrenic nerve responses following vagotomy are according to expectations. These control experiments also demonstrate that the effect of PBG on RTN neurons is selectively due to the activation of vagal afferents.

Most RTN CO₂-sensitive neurons express the transcription factor Phox2b (Stornetta et al., 2006; Moreira et al., 2007). To complement these observations we determined whether the CO₂-sensitive cells that respond vigorously to all three stimuli (NaCN, PBG and lung inflation; the triple responders) also express this transcription factor. Four such cells were labeled juxtacellularity with biotinamide and each labeled neuron had a Phox2b-immunoreactive (ir) nucleus (Fig. 1C and D). As in prior work, the
cell bodies of the recorded neurons were small (< 15 µm in length) and were located under the caudo-medial edge of the facial motor nucleus within 150 microns of the ventral surface (Fig. 1D, E). One of the biotinamide-filled neurons was labeled with sufficient intensity to allow extensive reconstruction of its dendritic arbor (Fig. 1E). This triple responder had the distinctive morphology of previously examined CO₂-sensitive RTN neurons (Mulkey et al., 2004). Its most notable feature was a basal dendrite that bifurcated at right angles within the marginal layer with the two branches spread out in a roughly transverse plane. As is the case of all other RTN neurons examined so far, this cell also had a second dendritic domain located dorsal to the marginal layer within the dorsal cap of the RTN.

Effect of muscimol injections into commissural NTS on RTN neuron responses to NaCN, lung inflation and PBG.

The next experiments were designed to identify which portion of the NTS mediates the inhibitory effect of PBG on RTN neurons and whether the same or different regions mediate the effect of NaCN and lung inflation. We first targeted the commissural portion of the NTS, a region that receives a heavy input from carotid body afferents (Blessing, 1997; Boscan et al., 2002; Paton et al., 2001) and rapidly-adapting lung mechanoreceptors but is sparsely innervated by the slowly adapting lung mechanoreceptors (Hayashi et al., 1996; Kubin et al., 2006).

As shown before (Takakura et al., 2006), muscimol injection into commNTS (six rats) had no effect on resting blood pressure (Fig. 2A and B), resting PND (Fig. 2A, B and F) nor on the resting discharge rate of RTN neurons (one triple responder per rat; Fig.
More specifically, PND remained synchronized with ventilation, its amplitude was unchanged (96 ± 4% of control; NS) and its duration (Ti) was unaltered (from 0.33 ± 0.02 to 0.32 ± 0.02 s, NS). These injections eliminated the stimulatory effect of NaCN on blood pressure (Fig. 2A2 and B), on PND (Fig. 2A2, F, and G) and on RTN neuron activity (Fig. 2A2 and C) but they did not change the inhibitory effect of lung inflation on PND (Fig. 2A2, F and G) and RTN neurons (Fig. 2A2, and E). These muscimol injections reduced considerably the hypotension caused by PBG (Fig. 2A2 and B) but some PND inhibition always persisted (Fig. 2A2 F and G). Likewise, RTN neuron inhibition by PBG was reduced but not eliminated by muscimol injection into commNTS (72 ± 4% reduction; P<0.05; paired t-test; n = 6) (Fig. 2A2, D).

In summary, muscimol injection into commNTS had no effect on any of the dependent variables measured at rest (blood pressure, PND, RTN). These injections blocked the effects of carotid body stimulation, they had no effect on the responses to lung inflation and they greatly but incompletely attenuated the effects of PBG.

These muscimol injections were located about 200 microns caudal to the calamus scriptorius as indicated in Fig. 3A. Although muscimol was administered as a single injection in the midline, the injectate spread bilaterally to cover most of the dorsal aspect of commNTS (representative case in Fig. 3B). Based on the area of distribution of the fluorescent microbeads, the injectate appeared to spread somewhat less in the rostrocaudal direction (approximately 300 µm from the injection center) than laterally (approximately 500 µm from the center). The effective spread of muscimol was not determined and could have been larger than that of the microbeads due to the difference in molecular mass.
Effects of muscimol injections targeted to the interstitial subnucleus of NTS.

These experiments were done in the 6 animals described above after the rats had completely recovered from the effect of muscimol injection into commNTS (2-2.5 hrs after the injection into commNTS). In each rat, a second RTN triple-responder was isolated and its response to NaCN, PBG and lung inflation was retested after injecting muscimol bilaterally at the level of interstitial NTS (Fig. 3C and D). Bilateral injection of muscimol into the region of iNTS raised resting AP markedly (from 127 ± 6 mmHg to 155 ± 4 mmHg; p<0.01) (Figs. 4A and B) and increased the resting discharge rate of the selected RTN neurons by 66 ± 5% (p<0.01; Fig. 4A, C and E). As described before (Moreira et al., 2007), muscimol had little effect on PND amplitude (103 ± 5% of control; NS; Fig. 4F) but the drug significantly increased Ti (from 0.32 ± 0.02 to 0.53 ± 0.02 s; p<0.05; Fig. 4F). Also as previously described (Moreira et al., 2007), muscimol completely desynchronized PND from the ventilation cycle, thereby reproducing a characteristic consequence of interrupting inputs from lung mechanoreceptors. Primarily due to Ti widening, the integrated PND per unit of time (mvPND) was significantly increased by muscimol (Fig. 4F).

Bilateral injection of muscimol in the iNTS region had no effect on the various effects of cyanide. AP still increased significantly (from 155 ± 4 mmHg to 168 ± 3 mmHg, p<0.05; Fig. 4A2 and B) and robust PND activation still occurred (Fig. 4A2, F, G). In fact, in proportion to baseline, the effect of cyanide on mvPND was the same before and after muscimol administration (Fig. 4F and G). Muscimol injection into iNTS caused an initial rise in the resting discharge of RTN neurons, probably due to the loss of
inhibitory input from the pump cells (Moreira et al., 2007). Despite this initial increase, RTN neurons were still vigorously activated by cyanide (from 11.94 ± 1 Hz to 28 ± 3 Hz, p<0.01; Fig. 4A2 and C). In contrast, muscimol injections centered on iNTS eliminated the effects of lung inflation on both PND (Fig. 4A2, F and G) and RTN neurons (Fig. 4A2 and E). These injections also significantly attenuated the effects of PBG including the transient hypotension (Fig. 4A2 and B), the reduction in PND (Fig. 4A2, F and G) and the inhibition of RTN neurons by PBG (53% ± 7% reduction; P<0.05; paired t-test; n = 6; Fig. 4A2, D).

In these six cases, the muscimol injections were centered on or very close to the tractus solitarius at mid-area postrema level (Fig. 3C). The fluorescent microbeads covered the tractus solitarius and the region surrounding it, including the interstitial nucleus and the dorsolateral NTS (Fig. 3D). The latter region contains large concentrations of second-order baroreceptive neurons (Chan and Sawchenko, 1998; Weston et al., 2003) whose inhibition by muscimol most likely caused the rise in blood pressure observed in the present experiments.

**Effect of PBG and lung inflation on other types of ventrolateral medullary neurons**

For comparative purposes, we examined the effect of lung inflation (+6 cm H$_2$O) and PBG on a variety of ventrolateral medullary neurons besides RTN chemoreceptors. The blood pressure-regulating neurons of the RVLM (10 neurons from 6 rats with intact vagus nerves recorded under halothane anesthesia; 2 neurons from 2 rats recorded under urethane anesthesia) were uniformly inhibited by PBG as expected (Verberne and
Guyenet, 1992) but these neurons were unaffected by lung inflation up to 6 cm H$_2$O (Fig. 5A1, A2).

We also recorded from a variety of respiratory-related cells located in the rVRG (24 cells in 7 rats under halothane anesthesia; 6 neurons in 2 urethane-anesthetized rats). The sample consisted of 16 inspiratory cells (6 inspiratory-augmenting, 4 inspiratory throughout, 4 early-inspiratory and 2 late-inspiratory), 6 expiratory cells (4 expiratory-augmenting and 2 expiratory throughout) and 8 post-inspiratory neurons (Fig. 5B and C).

Every inspiratory or expiratory neuron recorded within the rVRG region was profoundly inhibited by both lung-inflation and PBG (Fig. 5B1). Notably, both expiratory and inspiratory neurons were 100% inhibited by 6 cm H$_2$O PEEP (representative case in Fig. 5B1). Post-inspiratory neurons are typically activated by lung inflation (Hayashi et al., 1996). We confirmed this observation and found that the activity of these neurons remained phasic at low levels of PEEP and usually became tonic at 6 cm H$_2$O (Fig. 5C1). Post-inspiratory neurons are also activated by administration of PBG in the right atrium (Paton, 1997; Wilson and Bonham, 1997). Intravenous administration of PBG produced the same effect and we found that rVRG post-inspiratory neurons (n=8) were always activated by both stimuli (Fig. 5C1).

In summary, intravenous PBG changed the discharge of ventrolateral medullary respiratory neurons in the same manner as right atrium injection of a lower dose of the drug assumed to specifically activate bronchiopulmonary receptors (Paton, 1997; Wilson and Bonham, 1997). The present experiments also confirmed that the post-inspiratory neurons of the rVRG region are activated both by vagal C-fiber afferents stimulation and by lung inflation (Hayashi et al., 1996; Wilson and Bonham, 1997).
Inhibition of RTN neurons by PBG is attenuated by muscimol injection into rVRG

The post-inspiratory interneurons located in the rVRG probably mediate a large part of the inhibitory effect of SAR activation on the central respiratory pattern generator (Ezure et al., 2003; Hayashi et al., 1996; Kubin et al., 2006; Okazaki et al., 2001; Richter et al., 1987). Since post-inspiratory neurons are also activated by PBG ((Wilson and Bonham, 1997), present results), these cells presumably also contribute to PND inhibition when PBG-sensitive vagal afferents are activated. We therefore asked whether the activation of rVRG post-inspiratory neurons by PBG could also possibly mediate the inhibition of RTN neurons. This hypothesis was tested by determining whether bilateral injection of muscimol into rVRG changes the inhibition of RTN neurons caused by i.v. injection of PBG (8 neurons in 8 rats).

Bilateral injection of muscimol into rVRG eliminated PND and elevated blood pressure but muscimol did not change the resting discharge rate of RTN neurons (from 6.16 ± 1 Hz to 6.33 ± 0.9 Hz; n = 8; NS; Fig. 6A and B) and the drug had no effect on their response to raising end-expiratory pressure (data already reported in (Moreira et al., 2007)). Prior to muscimol injection, PBG inhibited PND as usual in these 8 rats (Fig. 6A). As shown before (Verberne and Guyenet, 1992), the effect of PBG on arterial pressure was virtually eliminated by injection of muscimol bilaterally into rVRG (Fig. 6B and C). This result is due to the fact that the rVRG region also contains the neurons that relay the effect of PBG to the blood pressure-regulating neurons of the rostral ventrolateral medulla (RVLM). RTN neuron inhibition by PBG was only reduced by 32 ± 4% after bilateral administration of muscimol into rVRG (n = 8; p < 0.05; Fig. 6A-C).
The same injections had no effect on RTN neuron inhibition by lung inflation (data already reported in (Moreira et al., 2007). These results demonstrate that although the rVRG plays no role in transmitting the effects of lung inflation or carotid body stimulation to RTN neurons under our experimental conditions (Takakura et al., 2006; Moreira et al., 2007), the rVRG does contribute to the inhibition of RTN neurons by C-fiber vagal afferent stimulation.

DISCUSSION

The present study demonstrates that RTN neurons are inhibited by activating 5-HT_3 receptor-expressing vagal C-fiber afferents. Given prior evidence that RTN neurons are respiratory chemoreceptors, we interpret the present results as a further indication that respiratory reflexes probably operate in part by adjusting the central chemoreceptor drive to the respiratory pattern generator.

Selectivity of phenylbiguanide for 5-HT_3 receptor-expressing vagal C-fiber afferents

PBG is a selective 5-HT_3 receptor agonist that activates a variety of cardiopulmonary vagal C-fiber afferents (Hainsworth, 1991; Verberne and Guyenet, 1992; Widdicombe, 2006). PBG has no effect on myelinated pulmonary stretch afferents or arterial baroreceptors in rats (Ho et al., 2001; Verberne et al., 2003). Under the present experimental conditions, the effects of intravenous PBG on the recorded cardiorespiratory variables were, as expected, predominantly caused by activation of vagal afferents because they were not observed after bilateral section of the vagal nerves just below the level of the superior laryngeal nerve. Thus, neither glossopharyngeal (including arterial baroreceptors and chemoreceptors) nor superior laryngeal afferents
could have contributed significantly to the effects of PBG examined presently. We presume that bronchio-pulmonary chemosensitive afferents (formerly J receptors; see historical perspective by (Widdicombe, 2006)) contributed most to the effects of i.v. PBG on PND and on RTN neurons but the participation of other types of afferents, particularly cardiac receptors, is likely (Hainsworth, 1991; Linz and Veelken, 2002; Verberne et al., 2003). Activation of these various C-fiber afferents produces a very similar cardiorespiratory reflex characterized by respiratory depression, hypotension and bronchoconstriction (Coleridge and Coleridge, 1994; Coleridge and Coleridge, 2001).

**RTN neurons receive convergent inputs from cardiopulmonary C-fiber afferents, slowly adapting stretch receptors (SAR) and peripheral chemoreceptors**

According to the present study, the CO$_2$-activated neurons of RTN receive convergent inputs from lung mechanoreceptors, peripheral chemoreceptors and cardiopulmonary C-fiber afferents. Like previously identified CO$_2$-sensitive cells of RTN, neurons responding to all three stimuli expressed the marker Phox2b and they were located in very close proximity of the ventral medullary surface (Mulkey et al., 2004; Stornetta et al., 2006). Current evidence that these RTN chemosensitive cells function as central respiratory chemoreceptors is based on three types of results: the selectivity of their projection pattern, the consistency between their electrophysiological properties and their postulated role, and the fact that inhibition or stimulation of the brain region in which they are located respectively depresses or activates breathing (Feldman et al., 2003; Guyenet et al., 2005a; Li and Nattie, 2002; Mulkey et al., 2004; Nattie and Li,
Carotid body afferents and lung mechanoreceptors regulate RTN neurons, apparently via direct neuronal projections from the nucleus of the solitary tract (NTS) (Moreira et al., 2007; Takakura et al., 2006). The existence of direct projections from NTS to RTN has led us to propose that the control of the central respiratory pattern generator by the carotid bodies and by lung mechanoreceptors operates in part by regulating the “chemical drive” of respiration i.e. by regulating the excitatory input that the CPG receives from central respiratory chemoreceptors such as RTN neurons (McCrimmon and Alheid, 2007; Moreira et al., 2007; Takakura et al., 2006). We interpret the present results along the same lines and suggest that withdrawal of the excitatory drive from RTN chemoreceptors contributes to the inhibition of breathing produced by activation of C-fiber cardiopulmonary afferents.

Further evidence that SARs, not RARs, mediate the inhibitory effect of lung inflation on RTN neurons

Rapidly-adapting pulmonary stretch receptors (RARs), carotid chemoreceptors and SARs innervate largely distinct subnuclei of the intermediate and caudal NTS (Kalia and Richter, 1985; Kubin et al., 1991). Rapidly-adapting lung stretch receptors (RARs) and carotid body afferents preferentially innervate caudal and medial structures such as the commissural portion of the NTS whereas slowly-adapting lung stretch receptors (SARs) target more rostral and lateral regions of the NTS located close to the interstitial nucleus, iNTS (Kubin et al., 2006). The iNTS region contains the greatest density of
SAR second-order neurons (the pump cells), which in turn innervate the NTS, the ventrolateral medulla and the pons (Bonham and McRimmon, 1990; Ezure et al., 2002; Kubin et al., 2006). This well-known topography accounts for our observation that bilateral muscimol injection near iNTS blocked selectively the effect of lung inflation on RTN neurons whereas injection into the commissural nucleus blocked selectively the effect of peripheral chemoreceptor stimulation. Given that most RAR second-order neurons reside in commNTS (Kubin et al., 2006), the fact that muscimol injection into this subnucleus did not change the effect of lung inflation on RTN neurons provides additional evidence that, under anesthesia, RTN chemoreceptors are regulated by SARs rather than RARs (Moreira et al., 2007).

Pathway responsible for RTN inhibition by phenylbiguanide

With rare exceptions, one of which was discussed in the previous paragraph, little functional specificity can be achieved by injecting even very small amounts of muscimol into NTS because of the elongated shape of the subnuclei and the layering and interdigitation of the various vagal afferents and their second-order cells (Altschuler et al., 1989; Kubin et al., 2006). Injections into iNTS, for example, also produced large increases in blood pressure, undoubtedly caused by the close proximity of arterial baroreceptor second-order neurons to iNTS (Weston et al., 2003). Cardiopulmonary C-fiber afferents have extensive projections to midline regions of the NTS both rostral and caudal to the calamus scriptorius (Kubin et al., 2006). Our present results are generally consistent with this anatomical data because the response of RTN neurons to PBG administration was attenuated to a similar extent by injecting muscimol into commNTS.
or more rostrally in the vicinity of iNTS. Our conclusion also agrees with prior experiments in which cobalt was administered into commNTS and produced attenuation but not elimination of the response to PBG (Bonham and Joad, 1991).

Relatively little is known of the properties and axonal projections of the NTS neurons that receive bronchiopulmonary C-fiber input. A frank majority of the commNTS neurons that are activated by cardio-pulmonary C-fiber stimulation receive convergent input from the carotid bodies but a minority have opposite responses to the two inputs and the region also contains neurons that respond selectively to only one of these stimuli (Paton, 1998). The former type of commNTS neuron is unlikely to mediate the effects of PBG on the respiratory network, the RTN, or the sympathetic vasomotor outflow because these targets always respond in opposite directions to stimulation of cardiopulmonary C-fibers vs. the carotid bodies. However, the latter two types of commNTS neurons could in theory relay the effect of cardio-pulmonary C-fibers to the ventral medullary respiratory network and RTN neurons.

Stimulation of cardio-pulmonary C-fibers inhibits ventral medullary sympathoexcitatory neurons by activating GABAergic neurons located in the caudal ventrolateral medulla at rVRG level (Schreihofer and Guyenet, 2003). These CVLM cells are also activated by arterial baroreceptors and they mediate the sympathetic baroreflex (Schreihofer and Guyenet, 2003). In theory, information from baroreceptor and cardiopulmonary C-fiber origin could reach CVLM neurons via separate neurons or these inputs could already be integrated at the level of the NTS. The latter possibility is supported to some extent by evidence that the NTS contains neurons that are activated by both types of inputs (Paton 1998).
The way in which information from cardio-pulmonary C-fibers is relayed from the NTS to RTN neurons is speculative. A direct inhibitory input from NTS to RTN is plausible but does not fully account for the present data. In support of this hypothesis, mono-synaptic transmission of inputs between vagal afferents and NTS neurons with axonal projections to the ventrolateral medulla is extremely common (Bailey et al., 2006) and RTN neurons already receive separate and most likely direct inputs from two other types of presumed second-order cells i.e. NTS pump cells and carotid body-receptive neurons (Takakura et al., 2006; Moreira et al., 2007). Also in favor of a direct input from NTS to RTN, a large fraction of the effect of PBG (68%) persisted after muscimol injection into the rVRG, a procedure expected to inhibit the neurons located in the injected area and to strongly depress the activity of the CPG. However, the fact that muscimol injection into rVRG attenuated the response of RTN neurons (by 32%) indicates that the pathway between NTS and RTN cannot be entirely direct and that the effect of PBG on RTN neurons must also involve interneurons located in the rVRG region. The present study does not permit to elaborate further on these ventrolateral relay neurons except to suggest that these cells are unlikely to be post-inspiratory interneurons. Although such cells are highly represented in the rVRG of the rat (Schwarzacher et al., 1991), consistently activated by stimulating cardiopulmonary C-fibers with PBG (Paton, 1997; Wilson and Bonham, 1997; present results) and inhibit numerous types of respiratory neurons (Ezure et al., 2003; Richter et al., 1987; Schwarzacher et al., 1991), they are also vigorously activated by lung inflation. Thus, if they were implicated in the effect of PBG on RTN neurons, they should also mediate some of the inhibitory effect of
lung inflation on these neurons. This hypothesis can be rejected because muscimol injection into rVRG did not change the effect of lung inflation on RTN neurons.

**Conclusion**

Activation of 5-HT₃ receptor-expressing C-fiber vagal afferents inhibits RTN neurons. The pathway relays through a broad region of the NTS and, for its full expression, the response requires the integrity of the rVRG. We interpret the inhibition of RTN neurons by C-fiber vagal afferents as further evidence that respiratory reflexes operate in part by regulating the intensity of the excitatory drive that the respiratory pattern generator receives from central chemoreceptors (McCrimmon and Alheid, 2007, Moreira et al., 2007). The present results were obtained under halothane anesthesia in the presence of a high level of CO₂, conditions required to overcome the powerful lung stretch reflex present under these experimental conditions. The extent to which RTN contributes to respiratory reflexes in an awake animal under normal levels of CO₂ remains to be determined.

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FIGURE LEGENDS

**Figure 1: RTN triple-responders: definition, location and Phox2b expression.**

A: CO₂-sensitivity of an RTN neuron under urethane-anesthesia in a rat with intact vagus nerves.

B: effect of NaCN (50 µg/kg, i.v), PBG (50 µg/kg, i.v) and lung inflation (+6 cm H₂O positive end-expiratory pressure, PEEP) on the neuron shown in A (representative neuron responding to all 3 stimuli, i.e. “triple responder”).

C1-3: example of one biotinamide-labeled triple responder that expressed Phox2b. Panel C1: biotinamide (single 30 µm-thick coronal section; Alexa 488 fluorescence; scale: 50 µm) reveals the cell body (arrow) and a primary dendrite pointing towards the marginal layer. Panel C2: the biotinamide-labeled cell body at higher magnification. Panel C3 shows three Phox2b-ir nuclei one of which (at arrow) belongs to the biotinamide labeled RTN neuron (Cy-3 fluorescence; scale for C2-3: 30 µm).

D: location of the four RTN triple responders labeled juxtaellularly with biotinamide. Each cell had a Phox2b-ir nucleus. The section is coronal at level Bregma -11.5mm (Paxinos and Watson, 1998).

E: 2-D reconstruction of the biotinamide-filled neuron shown in C. Its location is shown by the red dot in D.

Abbreviations: Amb, rostral compact portion of nuc. ambiguus; AP, arterial pressure; iPND, integrated phrenic nerve discharge; py, pyramidal tract; Sp5, spinal trigeminal tract; Tp, tracheal pressure; VMS, ventral medullary surface; VII, facial motor nucleus.

**Figure 2: Effect of muscimol injections into commissural NTS on RTN neuron responses to cyanide, phenylbiguanide (PBG) and lung inflation.**
A: Effect of NaCN (50 µg/kg, i.v), PBG (50 µg/kg, i.v) and lung inflation (+6 cm H2O PEEP) on one RTN neuron before (panel A1) and after (panel A2) a single midline injection of muscimol (1.75 mM, 30 nl) into commNTS. B: effect of muscimol on arterial pressure, AP. * P<0.05 level when compared with resting value before muscimol injection; repeated measures (RM) one-way ANOVA followed by Newman-Keuls multiple comparisons; 6 rats. C: effect of muscimol on RTN discharge rate (all groups compared by RM one-way ANOVA followed by Newman-Keuls multiple comparisons test; *P<0.01 relative to resting discharge before muscimol; 6 neurons). D: effect of muscimol on inhibition of RTN neurons by PBG (* P < 0.05; paired t-test). The inhibition of RTN neurons was measured as area under the curve (AUC). The AUC was defined as the area between the skyline of the histogram and the mean neuronal discharge before PBG and after recovery from the drug (represented as a dotted line in A). The AUC before muscimol injection was normalized as 100% inhibition. E: effect of lung inflation (+6 cm H2O PEEP) on the discharge rate of RTN neurons (all groups compared by one-way RM ANOVA followed by Newman-Keuls multiple comparisons test; *P<0.01 relative to resting discharge before or after muscimol; 6 neurons). F: effect of NaCN, PBG and lung inflation on mvPND before and after muscimol injection into commNTS (*P<0.01 relative to resting during control period; all groups analyzed by one-way RM ANOVA). G: effect of cyanide, PBG and lung inflation on mvPND before and after muscimol injection into commNTS. Changes are expressed as percentage increase or decrease from baseline. *P<0.01 when compared to the control response before muscimol; all groups analyzed by one-way RM ANOVA.
Figure 3: Muscimol injection sites

A: computer-assisted plots of the center of the muscimol injection sites targeted to commNTS revealed by the presence of fluorescent microbeads. All sites are projected on a single section located at Bregma −14.3 mm according to (Paxinos and Watson, 1998). Scale: 0.5 mm.  
B: Photomicrograph of a typical midline injection of muscimol into commNTS. Scale: 200 µm.  
C: location of bilateral muscimol injection sites targeted at the interstitial nucleus of NTS (iNTS). All sites projected on a single section (Bregma –13.8 mm). Scale: 0.4 mm.  
D: Photomicrograph of a typical bilateral injection of muscimol targeted to iNTS. Scale: 200 µm.  
E: muscimol injection sites targeted to the rVRG. All sites projected on a single section (Bregma –13.3 mm). Scale: 1 mm.  
F: Photomontage of a coronal section of the brainstem showing a representative bilateral injection of muscimol into rVRG. Scale: 400 µm. Abreviations: AP, area postrema; cc, central canal; Gr, gracile nucleus; IO, inferior olive; ts, tractus solitarius; XII, hypoglossal nucleus.

Figure 4: Effects of muscimol injections targeted to the interstitial subnucleus of NTS (iNTS).

A: Effect of NaCN (50 µg/kg, i.v), PBG (50 µg/kg, i.v) and lung inflation (+6 cm H\text{\textsubscript{2}}O PEEP) on one RTN neuron before (panel A1) and after (panel A2) bilateral injection of muscimol (1.75 mM, 30 nl) into iNTS (A2). B: effect of muscimol on arterial pressure (*) P<0.05 when comparing values achieved during lung inflation or administration of PB or,
CN to resting AP before or after muscimol; + P<0.05 when comparing resting AP before and after muscimol; all groups compared by RM one-way ANOVA followed by Newman-Keuls multiple comparisons; 6 rats). C: effect of NaCN on RTN discharge rate before and after muscimol (* P<0.01 when compared to resting discharge before NaCN; + P<0.05 when compared to resting discharge rate before muscimol; RM one-way ANOVA followed by Newman-Keuls multiple comparisons; n = 6 neurons). D: effect of muscimol on inhibition of RTN neurons by PBG (* P < 0.05; paired t-test). The inhibition was measured as area under the curve (AUC) as described in Fig. 2. E: effect of lung inflation (+6 cm H₂O PEEP) on the discharge rate of RTN neurons before and after muscimol (*P<0.01 when compared to resting discharge rate before muscimol; + P<0.05 when compared to resting discharge rate before muscimol; n = 6 rats). F: effect of lung inflation (+6 cm H₂O PEEP), PBG and cyanide on mvPND (neural minute x volume) before and after muscimol (* P<0.01 relative to resting PND before or after muscimol; + P<0.05 when compared to resting mvPND before muscimol; one-way RM ANOVA; n = 6 rats). G: effect of lung inflation, PBG and NaCN on mvPND expressed as percent change from resting level before muscimol (control) and after muscimol (* P<0.01 relative to control; all groups compared by one-way RM ANOVA; significance determined by Newman-Keuls multiple comparisons test).

**Figure 5: Effect of PBG and lung inflation on other types of ventrolateral medullary neurons**

*AI*: single RVLM blood pressure-sensitive neuron in a halothane-anesthetized rat, identified by its inhibitory response to a rise in blood pressure caused by brief aortic
occlusion (AOc). The neuron was inhibited by PBG (50 µg/kg, i.v.) but insensitive to lung inflation (+6 cm H$_2$O). $A2$: effect of lung inflation (+6 cm H$_2$O), PBG (50 µg/kg, iv) and AOc on mean arterial pressure, AP, and on discharge rate of RVLM neurons (*P < 0.05 relative to resting; 10 cells in 6 rats; one-way RM ANOVA). $B1$: throughout inspiratory neuron recorded in the rVRG. This cell was silenced by both lung inflation (+6 cm H$_2$O PEEP) and PBG (50 µg/kg). $B2$: PND-triggered activity histogram of the inspiratory neuron shown in B1. $C1$: post-inspiratory neuron recorded in the rVRG. This cell was activated by both lung inflation (+6 cm H$_2$O PEEP) and PBG (50 µg/kg). $C2$: PND-triggered activity histogram of the post-inspiratory neuron shown in C1.

**Figure 6: Bilateral injection of muscimol into the rostral ventral respiratory group (rVRG) attenuates the effect of PBG on RTN neurons.**

$A1$: PBG (50 µg/kg, i.v.) decreased arterial pressure (AP), inhibited PND and the RTN neuron. $A2$: Bilateral injection of muscimol into the rVRG virtually eliminated the effect of PBG on AP and attenuated the inhibition of RTN neurons by this drug. $B$: effect of PBG on AP before and after muscimol injection into the rVRG. (* or +: P<0.05 relative to resting level before muscimol; one-way RM ANOVA; n = 8). $C$: inhibition of RTN by PBG was reduced by muscimol injection; * P < 0.05; paired t-test). The neuronal inhibition caused by PBG was measured as AUC (for definition of AUC see Fig. 2). The AUC before muscimol injection was normalized as 100% inhibition.

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RTN neuron response to PBG (AUC)

Control  Muscimol

Neuron (Hz)

Lung inflation

PBG

NaCN

Resting

TP (cm H2O)

A1

B

C

D

E

F

G

B

C

D

E

F

G