

# Activation of 5-Hydroxytryptamine Type 3 Receptor-Expressing C-Fiber Vagal Afferents Inhibits Retrotrapezoid Nucleus Chemoreceptors in Rats

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**Moreira TS, Takakura AC, Colombari E, Guyenet PG.** Activation of 5-hydroxytryptamine type 3 receptor-expressing C-fiber vagal afferents inhibits retrotrapezoid nucleus chemoreceptors in rats. *J Neurophysiol* 98: 3627–3637, 2007. First published October 10, 2007; doi:10.1152/jn.00675.2007. 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 urethan-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 solitary tract nucleus (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 postinspiratory 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.

## 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 (Moreira et al. 2007; Takakura et al. 2006). 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 (McCrimmon and Alheid 2007; Moreira et al. 2007; Takakura et al. 2006).

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.

## METHODS

### Animals

The experiments were performed on 32 male Sprague-Dawley rats (Taconic; Germantown, NY) weighing 250–350 g. Procedures were in accordance with National Institutes of Health 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. 2005a; Takakura et al. 2006). A bilateral vagotomy was performed in three rats 1–2 cm caudal to the confluence of the superior laryngeal nerve.

On completion of surgical procedures, halothane was reduced to 1% (21 rats) or gradually replaced by urethan (1.2 g/kg iv over 20 min; 11 rats). The initial dose of urethan was adequate for ~1 h 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 ( $\leq 5$  h 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 CO<sub>2</sub> was monitored throughout the experiment with a microcapnometer (Columbus Instruments). After injection of the in-

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travenous 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 iv, and the adequacy of anesthesia 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 postfixed overnight in the paraformaldehyde solution. The brains were then sectioned in the coronal plane (30  $\mu$ m), and the sections were kept in cryoprotectant awaiting histological procedures.

Arterial blood pressure (AP), PND, tracheal CO<sub>2</sub>, and tracheal pressure were recorded as previously described (Moreira et al. 2007; Takakura et al. 2006). Single-unit recording and juxtacellular labeling of RTN neurons with biotinamide were done as described previously (Moreira et al. 2007; Mulkey et al. 2004; Takakura et al. 2006). RTN units were concentrated between 150 and 300  $\mu$ m below the lower edge of the facial motor nucleus and from 200  $\mu$ m caudal to 300  $\mu$ m 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% CO<sub>2</sub> and firing rate of 6–14 Hz at 10% CO<sub>2</sub>). Their high sensitivity to hypercapnia and insensitivity to changes in blood pressure distinguish these neurons from the blood-pressure regulating presympathetic 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 CO<sub>2</sub> to 4% at steady-state (60–80 cycles/s; tidal volume 1–1.2 ml/100 g). Variable amounts of pure CO<sub>2</sub> were then added to the breathing mixture to adjust end-expiratory CO<sub>2</sub> 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 CO<sub>2</sub>, 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 (Moreira et al. 2007; Mulkey et al. 2004; Takakura et al. 2006). Processing included action potential discrimination and binning, neuronal discharge rate measurement. PND “integration” (iPND) consisted of full-wave rectification and smoothing ( $\tau$ : 0.015s). Neural minute  $\times$  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 CO<sub>2</sub> (below PND threshold) and a value of 1 at the highest level of pCO<sub>2</sub> investigated (between 9.5 and 10%) before any injection of muscimol was made. CO<sub>2</sub> 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 perievent histograms of neuronal activity and peri-event averages of iPND, tracheal CO<sub>2</sub>, or tracheal pressure. The perievent histograms of neuronal single-unit activity were triggered either on iPND or on the tracheal pressure trace. Each histogram represents the summation of  $\geq 100$  central respiratory or ventilation cycles (350–800 action potentials per histogram). The steady-state relationship between RTN neuronal activity and end-expiratory CO<sub>2</sub> was obtained by stepping the inspired CO<sub>2</sub> level to various values for a minimum of 3 and  $\leq 5$  min (Guyenet et al. 2005a; Moreira et al. 2007; Takakura et al. 2006). The mean discharge rate of the neuron was measured during the last 30 s of each step at which time end-expiratory CO<sub>2</sub> and the discharge of the neuron appeared to have reached equilibrium. End-expiratory CO<sub>2</sub> 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 (NaCN) (50  $\mu$ g/kg iv). Under our experimental conditions, sodium cyanide activates brain stem 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 intravenous administration of the 5-hydroxytryptamine type 3 (5-HT<sub>3</sub>) receptor agonist phenylbiguanide (PBG, 50  $\mu$ g/kg iv). 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 three rats with bilateral cervical vagotomy. The cardiorespiratory effects caused by intravenous 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 s) from a resting level of +1 cm H<sub>2</sub>O to +6 cm H<sub>2</sub>O (Moreira et al. 2007).

### Muscimol injections

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

In eight rats, the electrode tip was directed to the 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 eight neurons to intravenous administration of PBG. The eight reported units (1 per rat) were kept for  $\geq 20$  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 NTS (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  $\mu$ m caudal to the calamus scriptorius. This experiment was carried out in six rats. The effect produced by muscimol injection into commNTS dissipated after 2 h, allowing the same six animals to be re-used 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  $\mu\text{m}$  rostral to the calamus scriptorius and 0.8 mm lateral to the midline) and the same RTN neuron was recorded for another 20 min.

### 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- $\mu\text{m}$ -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 h 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 14 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 (Rosin et al. 2006; Stornetta and Guyenet 1999). 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, Point Richmond, CA). Data are reported as means  $\pm$  SE. *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 NaCN, 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  $\text{CO}_2$ ) and silent in hypocapnia with a threshold between 4 and 5% end-expiratory  $\text{CO}_2$  (Fig. 1A). The  $\text{CO}_2$  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  $\text{CO}_2$  level was set at around 7.5%  $\text{CO}_2$ , concentration at which PND amplitude was 50–70% of its maximal value at 10%  $\text{CO}_2$ . Stimulation of carotid chemoreceptors with NaCN (50  $\mu\text{g}/\text{kg}$  iv boluses) increased AP (from  $126 \pm 3$  to  $140 \pm 3$  mmHg, 6 rats,  $P < 0.05$ ; Figs. 1B and 2, A1 and B), increased PND amplitude (Figs. 1B and 2, A1, F, and G), and vigorously activated every RTN neuron tested (from  $7.3 \pm 1$  to  $18.4 \pm 2.5$  Hz,  $n = 16$ ,  $P < 0.01$ ; Figs. 1B and 2, A1 and C). PBG administration decreased AP (from  $126 \pm 3$  to  $108 \pm 4$  mmHg,  $P < 0.05$ ; Figs. 1B and 2, A1 and B), reduced PND amplitude (Figs. 1B and 2A1, F, and G), and strongly inhibited every RTN neuron tested ( $n = 16$ , Figs. 1B and 2, A1 and D). Raising PEEP from a resting level of +1 to +6 cm of

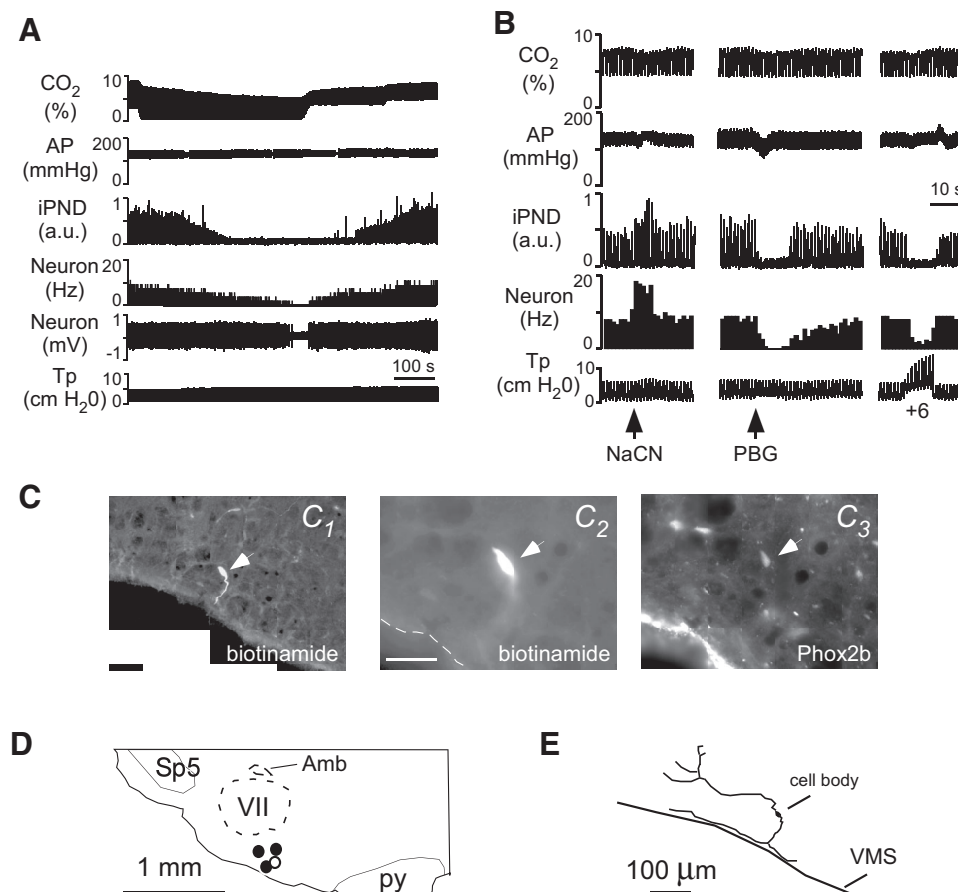


FIG. 1. Retrotrapezoid nucleus (RTN) triple-responders: definition, location and Phox2b expression. *A*:  $\text{CO}_2$ -sensitivity of an RTN neuron under urethan anesthesia in a rat with intact vagus nerves. *B*: effect of sodium cyanide (NaCN, 50  $\mu\text{g}/\text{kg}$  iv), phenylbiguanide (PBG, 50  $\mu\text{g}/\text{kg}$  iv), and lung inflation (+6 cm  $\text{H}_2\text{O}$  positive end-expiratory pressure, PEEP) on the neuron shown in *A* (representative neuron responding to all 3 stimuli, i.e., “triple responder”). *C*, 1–3: example of 1 biotinamide-labeled triple responder that expressed Phox2b. *C1*: biotinamide (single 30- $\mu\text{m}$ -thick coronal section; Alexa 488 fluorescence; scale: 50  $\mu\text{m}$ ) reveals the cell body ( $\rightarrow$ ) and a primary dendrite pointing toward the marginal layer. *C2*: biotinamide-labeled cell body at higher magnification. *C3*: 3 Phox2b-ir nuclei 1 of which (at  $\rightarrow$ ) belongs to the biotinamide-labeled RTN neuron (Cy-3 fluorescence; scale for *C*, 2 and 3: 30  $\mu\text{m}$ ). *D*: location of the 4 RTN triple responders labeled juxtacellularly with biotinamide. Each cell had a Phox2b-ir nucleus. The section is coronal at Bregma  $-11.5$  mm (Paxinos and Watson 1998). *E*: 2-dimensional (2-D) reconstruction of the biotinamide-filled neuron shown in *C*. Its location is shown by the red dot in *D*. 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.



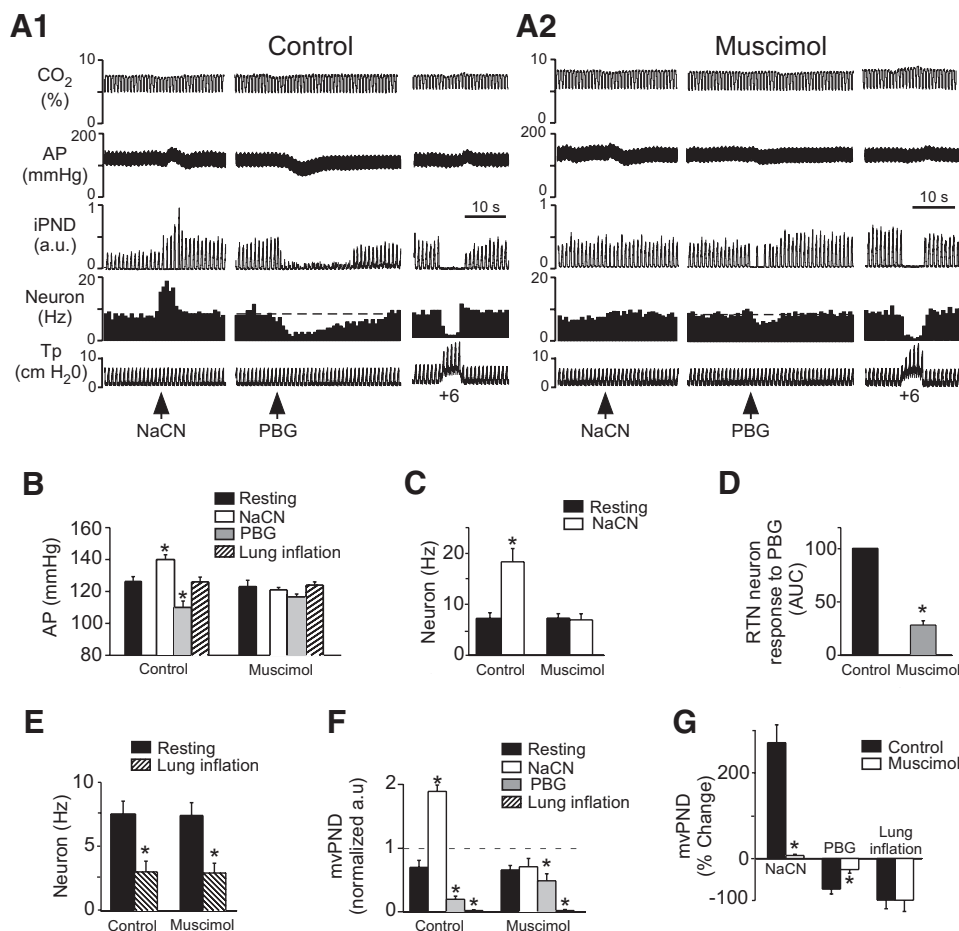


FIG. 2. Effect of muscimol injections into commissural solitary tract nucleus (NTS) on RTN neuron responses to cyanide, PBG, and lung inflation. *A*: effect of NaCN (50  $\mu$ g/kg iv), PBG (50  $\mu$ g/kg iv), and lung inflation (+6 cm H<sub>2</sub>O PEEP) on 1 RTN neuron before (*A1*) and after (*A2*) 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) 1-way ANOVA followed by Newman-Keuls multiple comparisons (6 rats). *C*: effect of muscimol on RTN discharge rate (all groups compared by RM 1-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 ... in *A*). The AUC before muscimol injection was normalized as 100% inhibition. *E*: effect of lung inflation (+6 cm H<sub>2</sub>O PEEP) on the discharge rate of RTN neurons (all groups compared by 1-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 1-way RM ANOVA). *G*: effect of NaCN, 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 with the control response before muscimol; all groups analyzed by 1-way RM ANOVA.

H<sub>2</sub>O eliminated PND (Figs. 1*B* and 2, *A1*, *F*, and *G*) and inhibited the RTN neurons selected for further study by more than 27% (27–100%; Figs. 1*B* and 2, *A1* and *E*). The CO<sub>2</sub>-activated neurons that responded to lung inflation with <25% inhibition (Moreira et al. 2007) were also uniformly inhibited by PBG (from  $8.8 \pm 0.6$  to  $4.6 \pm 0.6$  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 versus pulmonary C-fibers on RTN neurons.

Figure 1*B* 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. 1*B*). 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 CO<sub>2</sub>-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 CO<sub>2</sub>-sensitive neurons express the transcription factor Phox2b (Moreira et al. 2007; Stornetta et al. 2006). To complement these observations, we determined whether the CO<sub>2</sub>-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 juxtacellularly with biotinamide, and each labeled neuron had a Phox2b-immunoreactive (ir) nucleus (Fig. 1, C and D). As in prior work, the cell bodies of the recorded neurons were small (<15 μm in length) and were located under the caudomedial edge of the facial motor nucleus within 150 μm of the ventral surface (Fig. 1, D and 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 CO<sub>2</sub>-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. 2, A and B), resting PND (Fig. 2, A, B, and F) nor on the resting discharge rate of RTN neurons (1 triple re-

sponder per rat; Fig. 2, A, C, and E). More specifically, PND remained synchronized with ventilation, its amplitude was unchanged ( $96 \pm 4\%$  of control; NS), and its duration (Ti) was unaltered (from  $0.33 \pm 0.02$  to  $0.32 \pm 0.02$  s, NS). These injections eliminated the stimulatory effect of NaCN on blood pressure (Fig. 2, A2 and B), on PND (Fig. 2, A2, F, and G), and on RTN neuron activity (Fig. 2, A2 and C), but they did not change the inhibitory effect of lung inflation on PND (Fig. 2, A2, F, and G) and RTN neurons (Fig. 2, A2 and E). These muscimol injections reduced considerably the hypotension caused by PBG (Fig. 2, A2 and B), but some PND inhibition always persisted (Fig. 2, A2, F, and G). Likewise, RTN neuron inhibition by PBG was reduced but not eliminated by muscimol injection into commNTS ( $72 \pm 4\%$  reduction;  $P < 0.05$ ; paired *t*-test;  $n = 6$ ; Fig. 2, A2 and 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 ~200 μm 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 (~300 μm from the injection center) than laterally (~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 six animals described in the preceding text after the rats had completely recovered from

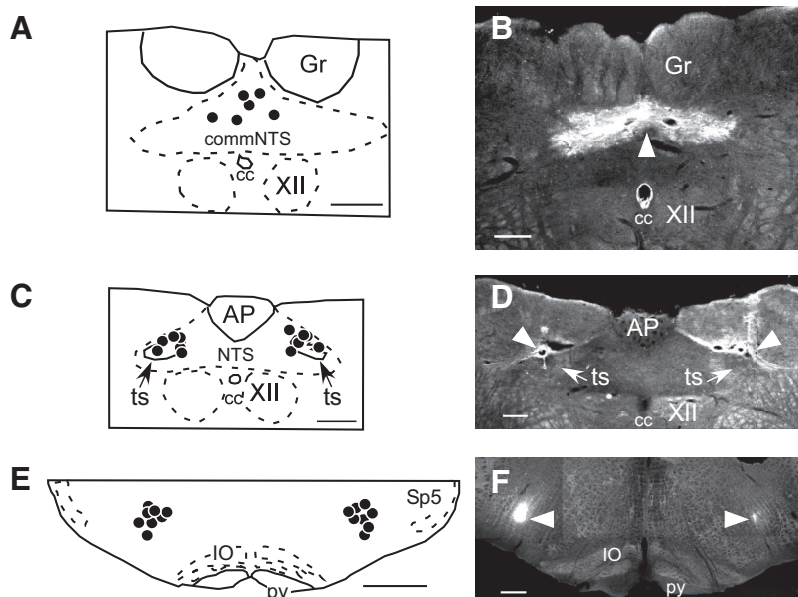


FIG. 3. Muscimol injection sites. A: computer-assisted plots of the center of the muscimol injection sites targeted to commNTS revealed by the presence of fluorescent microbeads. All sites are projected on a single section located at Bregma -14.3 mm according to Paxinos and Watson (1998). Scale: 0.5 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 brain stem showing a representative bilateral injection of muscimol into rVRG. Scale: 400 μm. AP, area postrema; cc, central canal; Gr, gracile nucleus; IO, inferior olive; ts, tractus solitarius; XII, hypoglossal nucleus.

the effect of muscimol injection into commNTS (2–2.5 h 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. 3, *C* and *D*). Bilateral injection of muscimol into the region of iNTS raised resting AP markedly (from  $127 \pm 6$  to  $155 \pm 4$  mmHg;  $P < 0.01$ ; Fig. 4, *A* and *B*) and increased the resting discharge rate of the selected RTN neurons by  $66 \pm 5\%$  ( $P < 0.01$ ; Fig. 4, *A*, *C*, and *E*). As described before (Moreira et al. 2007), muscimol had little effect on PND amplitude ( $103 \pm 5\%$  of control; NS; Fig. 4*F*), but the drug significantly increased Ti (from  $0.32 \pm 0.02$  to  $0.53 \pm 0.02$  s;  $P < 0.05$ ; Fig. 4*F*). 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. 4*F*).

Bilateral injection of muscimol in the iNTS region had no effect on the various effects of cyanide. AP still increased significantly (from  $155 \pm 4$  to  $168 \pm 3$  mmHg,  $P < 0.05$ ; Fig. 4, *A2* and *B*), and robust PND activation still occurred (Fig. 4, *A2*, *F*, and *G*). In fact, in proportion to baseline, the effect of cyanide on mvPND was the same before and after muscimol administration (Fig. 4, *F* 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  $11.94 \pm 1$  to  $28 \pm 3$  Hz,  $P < 0.01$ ; Fig. 4, *A2* and *C*). In contrast, muscimol injections centered on iNTS eliminated the effects of lung inflation on both PND (Fig. 4, *A2*, *F*, and *G*) and RTN neurons (Fig. 4, *A2* and *E*). These injections also significantly

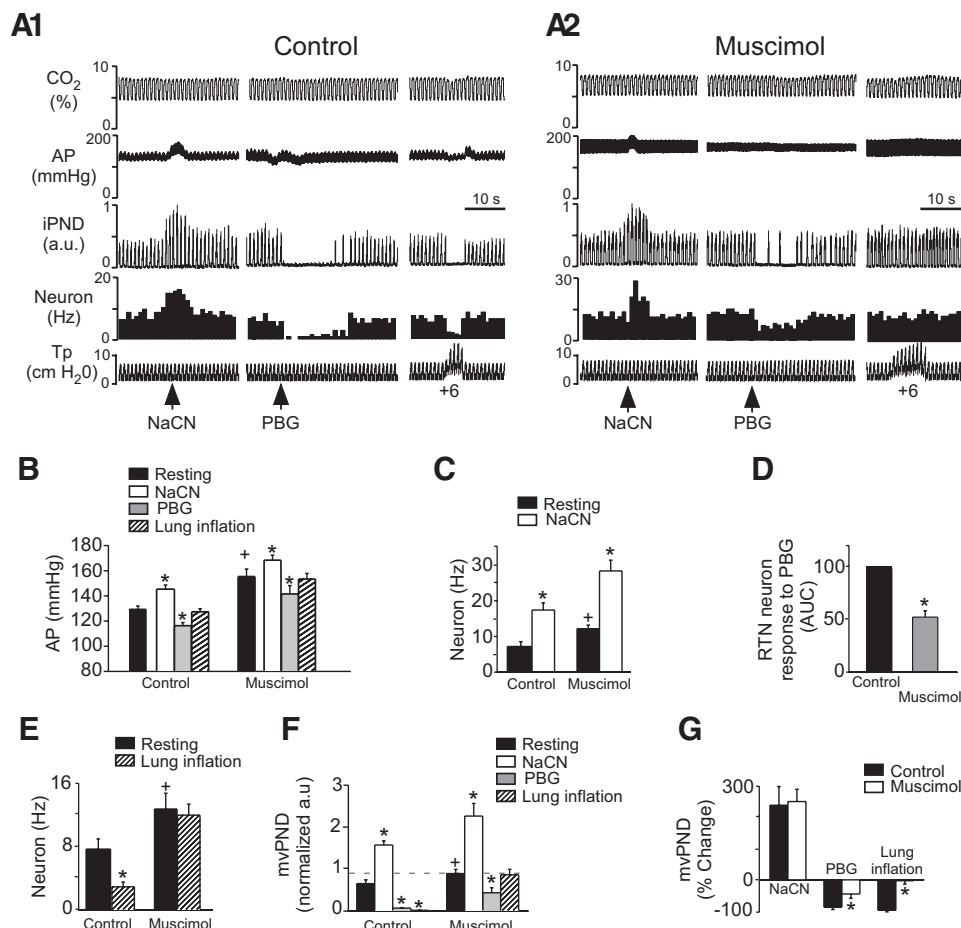


FIG. 4. Effects of muscimol injections targeted to the iNTS. *A*: effect of NaCN (50  $\mu$ g/kg iv), PBG (50  $\mu$ g/kg iv), and lung inflation (+6 cm H<sub>2</sub>O PEEP) on 1 RTN neuron before (*A1*) and after (*A2*) bilateral injection of muscimol (1.75 mM, 30 nl) into iNTS (*A2*). *B*: effect of muscimol on arterial pressure ( $*P < 0.05$  when comparing values achieved during lung inflation or administration of PBG or NaCN to resting AP before or after muscimol;  $+P < 0.05$  when comparing resting AP before and after muscimol; all groups compared by RM 1-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 with resting discharge before NaCN;  $+P < 0.05$  when compared with resting discharge rate before muscimol; RM 1-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 H<sub>2</sub>O PEEP) on the discharge rate of RTN neurons before and after muscimol ( $*P < 0.01$  when compared with resting discharge rate before muscimol;  $+P < 0.05$  when compared with resting discharge rate before muscimol;  $n = 6$  rats). *F*: effect of lung inflation (+6 cm H<sub>2</sub>O PEEP), PBG and NaCN on mvPND (neural minute  $\times$  volume) before and after muscimol ( $*P < 0.01$  relative to resting PND before or after muscimol;  $+P < 0.05$  when compared with resting mvPND before muscimol; 1-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 1-way RM ANOVA; significance determined by Newman-Keuls multiple comparisons test).

attenuated the effects of PBG including the transient hypotension (Fig. 4, A2 and B), the reduction in PND (Fig. 4, A2, F, and G) and the inhibition of RTN neurons by PBG ( $53 \pm 7\%$  reduction;  $P < 0.05$ ; paired  $t$ -test;  $n = 6$ ; Fig. 4, A2 and 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<sub>2</sub>O) and PBG on a variety of ventrolateral medullary neurons besides RTN chemoreceptors. The blood pressure-regulating neurons of the RVLN (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  $\leq 6$  cm H<sub>2</sub>O (Fig. 5A, 1 and 2).

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 postinspiratory neurons (Fig. 5, B 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<sub>2</sub>O PEEP (representative case in Fig. 5B1). Postinspiratory 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<sub>2</sub>O (Fig. 5C1). Postinspiratory 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 postinspiratory 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 postinspiratory 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).

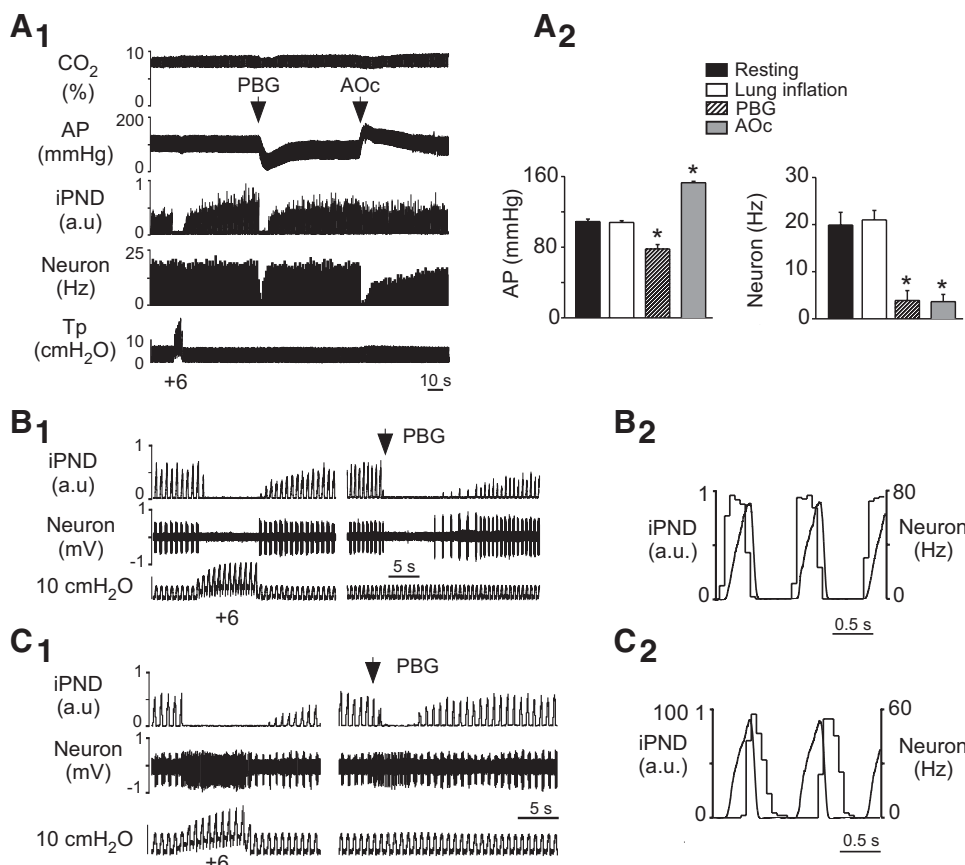


FIG. 5. Effect of PBG and lung inflation on other types of ventrolateral medullary neurons. A1: single RVLN blood pressure-sensitive neuron in a halothane-anesthetized rat, identified by its inhibitory response to a rise in blood pressure caused by brief aortic occlusion (AOc). The neuron was inhibited by PBG (50  $\mu$ g/kg, intravenous) but insensitive to lung inflation (+6 cm H<sub>2</sub>O). A2: effect of lung inflation (+6 cm H<sub>2</sub>O), PBG (50  $\mu$ g/kg iv), and AOc on mean AP and on discharge rate of RVLN neurons ( $*P < 0.05$  relative to resting; 10 cells in 6 rats; 1-way RM ANOVA). B1: throughout inspiratory neuron recorded in the rVRG. This cell was silenced by both lung inflation (+6 cm H<sub>2</sub>O PEEP) and PBG (50  $\mu$ g/kg). B2: PND-triggered activity histogram of the inspiratory neuron shown in B1. C1: postinspiratory neuron recorded in the rVRG. This cell was activated by both lung inflation (+6 cm H<sub>2</sub>O PEEP) and PBG (50  $\mu$ g/kg). C2: PND-triggered activity histogram of the postinspiratory neuron shown in C1.



### Inhibition of RTN neurons by PBG is attenuated by muscimol injection into rVRG

The postinspiratory interneurons located in the rVRG probably mediate a large part of the inhibitory effect of slowly adapting stretch receptor (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). Because postinspiratory 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 postinspiratory 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 intravenous 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  $6.16 \pm 1$  to  $6.33 \pm 0.9$  Hz;  $n = 8$ ; NS; Fig. 6, A 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 eight 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. 6, B 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  $32 \pm 4\%$  after bilateral administration of muscimol into rVRG ( $n = 8$ ;  $P < 0.05$ ; Fig. 6, A–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 (Moreira et al. 2007; Takakura et al. 2006), 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<sub>3</sub> 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<sub>3</sub> receptor-expressing vagal C-fiber afferents

PBG is a selective 5-HT<sub>3</sub> 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 intravenous PBG on PND and on RTN neurons, but the participation of other types of afferents,

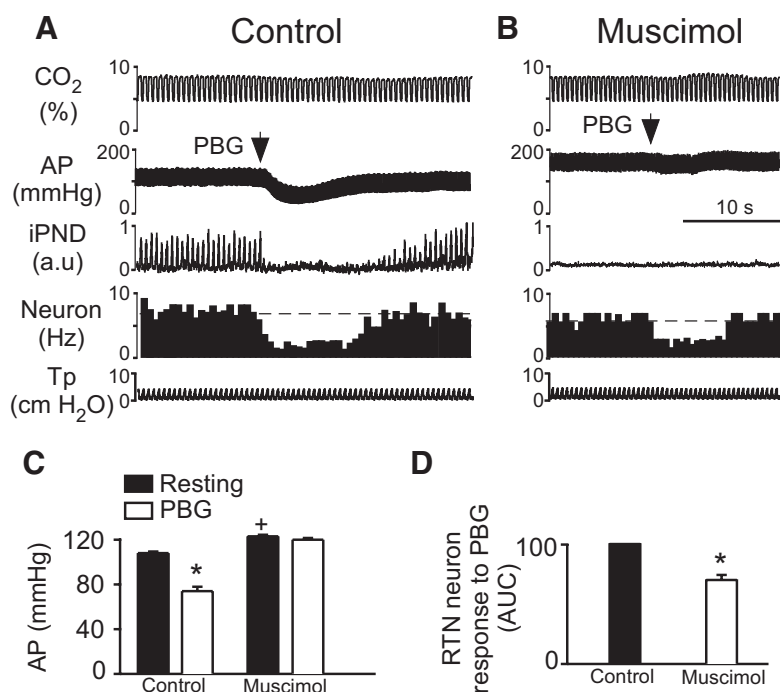


FIG. 6. Bilateral injection of muscimol into the rostral ventral respiratory group (rVRG) attenuates the effect of PBG on RTN neurons. A1: PBG (50  $\mu$ g/kg iv) decreased AP and inhibited PND and the RTN neuron. A2: bilateral injection of muscimol into the rVRG virtually eliminated the effect of PBG 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; 1-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.



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, 2001).

*RTN neurons receive convergent inputs from cardiopulmonary C-fiber afferents, SARs, and peripheral chemoreceptors*

According to the present study, the CO<sub>2</sub>-activated neurons of RTN receive convergent inputs from lung mechanoreceptors, peripheral chemoreceptors, and cardiopulmonary C-fiber afferents. Like previously identified CO<sub>2</sub>-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. 2005b; Li and Nattie 2002; Mulkey et al. 2004; Nattie and Li 2000; Onimaru and Homma 2003; Rosin et al. 2006). The rest of the discussion presupposes that this interpretation of the role of RTN neurons is correct.

Carotid body afferents and lung mechanoreceptors regulate RTN neurons, apparently via direct neuronal projections from the 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). RARs and carotid body afferents preferentially innervate caudal and medial structures such as the commissural portion of the NTS, whereas 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 McCrimmon 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 cardiopulmonary 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 versus the carotid bodies. However, the latter two types of commNTS neurons could in theory relay the effect of cardiopulmonary C-fibers to the ventral medullary respiratory network and RTN neurons.

Stimulation of cardiopulmonary C-fibers inhibits ventral medullary sympathoexcitatory neurons by activating GABAergic neurons located in the caudal ventrolateral medulla (CVLM) 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 cardiopulmonary 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, monosynaptic 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 (Moreira et al. 2007; Takakura et al. 2006). 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 postinspiratory 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-HT<sub>3</sub> 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 CO<sub>2</sub>, 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 CO<sub>2</sub> remains to be determined.

### GRANTS

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