Tonic sympathetic chemoreflex after blockade of respiratory rhythmogenesis in the rat

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1. We sought to determine whether the increase in sympathetic nerve discharge (SND) caused by carotid chemoreceptor stimulation requires the integrity of ventrolateral medullary structures involved in generating respiratory rhythm and pattern. Experiments were done in urethane-anaesthetized, vagotomized, aortic deafferented, ventilated rats except when indicated (see paragraph 3).

2. Brief hypoxia (N_2 for 5–12 s) or I.V. NaCN (50–100 μg kg^{-1}) activated SND in bursts synchronized with the phrenic nerve discharge (PND). No effect was produced in chemodeafferented rats.

3. In unanaesthetized vagotomized decerebrated rats, ligation of the internal carotid arteries preserved peripheral chemoreceptor function but abolished baroreflexes. In this preparation, stimulation of peripheral chemoreceptors (N_2 for 2–6 s) also activated SND in bursts synchronized with PND.

4. Bilateral microinjection of the $GABA_A$ receptor agonist muscimol into the caudal ventrolateral medulla (CVLM) instantly blocked the sympathetic baroreflex, eliminated PND at rest and during chemoreceptor stimulation but did not change the mean increase in SND produced by chemoreceptor stimulation. Sympathoactivation in response to chemoreceptor stimulation became tonic after 1–13 min and was still totally dependent on the integrity of the carotid sinus nerves.

5. Muscimol injection instantly eliminated the respiratory outflow of the Xth and XIIth cranial nerves, both at rest and during chemoreceptor stimulation.

6. Muscimol eliminated the on–off respiratory pattern of neurons in the rostral ventrolateral medulla (RVLM). During chemoreceptor stimulation, these cells became activated or inhibited tonically.

7. Muscimol injection raised the resting discharge rate of vasomotor presympathetic cells in RVLM, blocked their baroreceptor inputs but did not change the magnitude of their excitation by chemoreceptor stimulation. Muscimol injection eliminated their respiratory modulation.

8. In conclusion, the sympathetic response to chemoreceptor stimulation may be due to convergence and integration in RVLM of two processes: respiration-independent excitatory input to RVLM neurons and respiratory patterning of their activities via inputs from the pre-Bötzinger complex.

Stimulation of carotid chemoreceptors increases both respiration and the activity of the sympathetic system (e.g. Kollai & Koizumi, 1977; Huang, Lahiri, Mokashi & Sherpa, 1988; Katona, Dembowsky, Czachurski & Seller, 1989). Because sympathoexcitation occurs in bursts synchronized with the central respiratory cycle during chemoreceptor stimulation, it has generally been concluded that sympathoactivation was a secondary consequence of the activation of the respiratory network (for references, see Guyenet & Koshiya, 1992).

This view has been challenged recently by the results of a series of experiments from this laboratory demonstrating that anatomically specific manipulations of the brainstem

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(lesions, drug microinjections) could produce major disruptions of the respiratory drive or pattern but little change in the magnitude of the sympathetic chemoreflex (Koshiya, Huangfu & Guyenet, 1993; Koshiya & Guyenet, 1994*a*). In particular, inhibition of neuronal activity in the caudal ventrolateral medulla (CVLM), an area located a mere 1–1·5 mm caudal to the rostral ventrolateral medulla (RVLM) where most bulbospinal sympathetic vasomotor neurons reside (Guyenet, Koshiya, Huangfu, Baraban, Stornetta & Li, 1996), exerts a minimal effect on the chemoreceptor-mediated excitation of SND, though it immediately silences phrenic nerve discharge (PND)(Koshiya *et al.* 1993). However, in these experiments we also found that sympathetic activation retained a slow respiratory-like rhythm during chemoreceptor stimulation, suggesting that muscimol may have inhibited PND but not central respiratory rhythm generation or, possibly, respiratory outputs other than that to the phrenic nerve. In recent preliminary experiments we have found that the remaining rhythmicity in the sympathetic chemoreflex after muscimol microinjection into CVLM might be transient and may be followed by a tonic response, suggesting a delayed but complete cessation of central respiratory activity (Guyenet & Koshiya, 1995).

Our present goal is to examine thoroughly the effect of muscimol injections into CVLM on the activity of the respiratory network to determine if this experimental procedure can indeed eliminate not only respiratory outputs (to the diaphragm and airway muscles) but also central respiratory rhythm generation while preserving the sympathetic chemoreflex. A positive answer to this question would provide the strongest evidence to date that RVLM vasomotor neurons and hence the sympathetic outflow receive their principal excitatory drive from peripheral chemoreceptors via a pathway that bypasses the core of the central respiratory network, and that activation of the bulbar respiratory network may result primarily in superimposing a rhythm on the sympathetic response.

METHODS

General procedures

These were similar to our prior work (Koshiya *et al.* 1993; Koshiya & Guyenet, 1994*a*,*b*) and need but a short summary. Male Sprague–Dawley rats (300–390 g) were used. Anaesthesia was induced with ether or halothane (4% in O_2). The rats were intubated, and artificially ventilated with O_2 containing 1·3–1·5% halothane for surgery. No sign of nociception was observed (criteria listed in Anaesthesia below). The femoral artery and vein were catheterized for arterial pressure (AP) measurement and for drug administration, respectively. The vagosympathetic trunks and aortic depressor nerves were cut bilaterally in the lower neck but the carotid sinus nerves were left intact. The occipital plate was removed and the underlying dura mater opened. End-tidal CO_2 was monitored by infrared spectroscopy (Microcapnometer, Columbus Instrument, Columbus, OH, USA, sampling rate of 20 ml min^{-1}) and maintained between 3·5 and 4·0% during surgery. All physiological parameters were monitored on a chart recorder and simultaneously stored on a PCM recorder (Vetter 3000A, frequency range: DC–22 kHz). Rectal temperature was maintained between 38 and 39 °C.

Recording of nerve activities

The following nerves were isolated in several combinations for multiunit recording (for details see Koshiya *et al.* 1993; Koshiya & Guyenet, 1994*a*,*b*): the right phrenic nerve and the Xth cranial (right or left vagus), isolated via a dorsolateral approach and cut distally in the lower neck; the right splanchnic sympathetic nerve, approached retroperitoneally and recorded, uncut, distal to the suprarenal ganglion; the left lumbar sympathetic trunk (few cases only) isolated via a ventral transperitoneal approach and recorded uncut; theXIIth cranial (hypoglossal) nerve of either side, isolated in the submandibular region. Nerve discharges were recorded with bipolar silver wire electrodes buried in a pool of Sil-Gel (Wacker Silicones Corp., Adrian, MI, USA). Nerve activities (recording bandpass, 100–3000 Hz) were amplified, full-wave rectified and analog integrated (time constant, 1 s; sample/hold reset interval, 30 ms). The processed signals were displayed on a chart recorder.

The steep negative slope of the phrenic burst at the inspiration–expiration (I–E) transition was used as a trigger for peri-event time averaging of nerve activities (Guyenet, Darnall & Riley, 1990; Guyenet, Koshiya, Huangfu, Verberne & Riley, 1993).

All nerve activities were expressed as a percentage of the averaged value at rest (in the absence of chemoreceptor stimulation). The recording noise was subtracted in all cases. Noise was identified for vagus and hypoglossal discharges after the death of the animals; for SND after I.V. administration of the sympatholytic drug clonidine (200 $\mu g\ kg^{-1}$) or the ganglionic blocker trimethaphan (30 mg kg^{-1}); for PND as the interburst silent period (Koshiya & Guyenet, 1994*a*).

Anaesthesia

For recording, intravenous urethane (1·2–1·4 g kg^{-1} given over 10 min) was replaced by halothane to restore AP and carotid body function (Seagard, Hopp, Donegan, Kalbfleish & Kampine, 1982). Supplemental doses of urethane (0·1–0·2 g kg^{-1} I.V.) were administered as required to satisfy the following criteria for anaesthesia: (i) when the rat was not paralysed: lack of corneal reflex, lack of withdrawal reflex and absence of rise in AP or PND upon strong toe pinch, and (ii) during paralysis with pancuronium bromide (1·5 mg kg^{-1} I.V., 0·5–1·0 mg kg^{-1} thereafter as needed): absence of pressor response or phrenic activation to strong nociceptive stimulus (toe pinch) (Schwarzacher, Wilhelm, Anders & Richter, 1991). Before recording, ventilation was readjusted to achieve the minimal end-tidal CO_2 necessary for production of a steady PND (4·5–5·0%). After each anaesthetic supplementation, a 10 min stabilization period was allowed before resuming recording. At the end of the experiment, the animals were killed with 5% halothane. When intraparenchymal microinjections and/or unit recordings were performed, the animals were perfused transcardially with buffered saline (pH 7·4) followed by 4% paraformaldehyde (pH 7·0), and the brains were processed for histological localization of sites of injections and recordings as described previously (Koshiya *et al.* 1993).

Decerebrate animals

In a few cases decerebration was performed under deep halothane anaesthesia (1·75–2%) by aspiration of the brain rostral to the superior colliculus. Before decerebration all the surgical steps described under 'general procedures' except the craniotomy were completed. In addition ligatures were tightened around the base of both internal carotid arteries a few millimetres rostral to the carotid bifurcation. The carotid bodies and their vascularization remained intact. Following decerebration, the halothane concentration was lowered to between 0·8 and 1·3%, and the rest of the surgical procedure was completed. Halothane was then withdrawn. No anaesthetic was administered and the usual dose of muscle relaxant was given. A recovery period of at least 1 h was allowed. Preparations in which PND was stable and could be silenced by reducing end-tidal CO_2 to 3–3·5% by hyperventilation were used.

Carotid chemoreceptor stimulation

Stimulation of carotid chemoreceptors was done by ventilating the rats with 100% N_2 for short and precisely controlled periods ranging from 4 to 12 s depending on the rat, or by bolus injection of NaCN (50–100 μg kg^{-1} I.V.) (Koshiya *et al.* 1993). These stimuli were applied at intervals of at least 2 min. Under these conditions, the reflex responses were stable and signs of central hypoxia, such as depression of respiration (instead of excitation) and desynchronized activation of SND were not observed. Carotid chemoreceptor denervation was performed in some cases either surgically or by microperfusion of a local anaesthetic (1·5% mepivacaine) into the carotid bifurcation area via catheters implanted bilaterally.

Unit recording in the rostral ventrolateral medulla (RVLM)

All procedures have been described in detail elsewhere (Brown & Guyenet, 1985; Koshiya *et al.* 1993). In brief, the region of the RVLM was targeted with precision following field potential mapping of the immediately adjacent facial motor nucleus. In each experiment, the last recording site was marked with an iontophoretic deposit of Pontamine Sky Blue, and the locations of other units were reconstituted from their stereotaxic location with respect to the marked cell.

Microinjections

Muscimol (1·75 mM, Sigma), dissolved in standard artificial cerebrospinal fluid (pH 7·30–7·35; Sun, Young, Hackett & Guyenet, 1988) that contained fluorescent latex microbeads (Lumafluor, New City, NY, USA), was used for histological identification of injection sites (Guyenet *et al.* 1990). Intraparenchymal injections were done bilaterally with a pair of micropipettes (tip diameter, 40–50 μm) pulled from microcapillaries with a small inner diameter (1 mm o.d., 250 μm i.d.; Fisher Scientific, Pittsburgh, PA, USA). Each micropipette was connected to a compressed airline (500–600 kPa) via a solenoid valve system (Picospritzer II). The injection volume was monitored by observing the movement of the fluid meniscus within the capillary (accuracy, 3·0 nl) through a dissecting microscope equipped with an eye-piece micrometer. Each injection (50 nl, 87·5 pmol) was made with short pressure pulses of 4–10 ms over a 30–40 s period. This procedure minimizes backflow of the injectate along the pipette.

RESULTS

Time-dependent effect of muscimol microinjections into CVLM on the sympathetic chemoreflex

Stimulation of carotid chemoreceptors

Brief inhalation of N_2 and/or I.V. injection of NaCN (100 μg kg^{-1}) reversibly increased PND, SND and AP (Fig. 1*A*). The effects on PND and SND were abolished by carotid sinus nerve denervation (SND recorded in the lumbar trunk, $n = 5$, or in splanchnic nerve, $n = 2$; denervation either by surgical resection, $n = 4$, or by local anaesthesia, $n = 3$; not shown) in agreement with previous studies (Koshiya *et al.* 1993; Guyenet *et al.* 1993; Sun & Reis, 1995). The excitatory effects of brief hypoxia or I.V. cyanide on SND and PND are therefore entirely due to activation of carotid chemoreceptors in the present conditions.

Peripheral chemoreflex in a decerebrate debuffered preparation

The purpose of this experiment was to demonstrate that the respiratory-synchronous bursts of sympathetic discharge produced by stimulation of carotid chemoreceptors are due mostly to central coupling between the respiratory and sympathetic networks and not to a secondary feedback from baroreceptors. As illustrated in Fig. 2*A*, ligating the internal carotid arteries in addition to sectioning the vagosympathetic trunks and aortic depressor nerves (ADNs) produced a preparation in which the sympathetic baroreflex was eliminated ($n = 5$). This effect was not due to impairment of the central component of the baroreflex since electrical stimulation of the central end of one of the ADNs produced the expected sympathoinhibition (Fig. 2*Aa*, right panel). In this preparation, the chemoreflex remained very vigorous (Fig. 2*B*), due to the preservation of the carotid bodies and their main vascular supply. Note in particular the very large increase in arterial pressure, typical of this preparation and the activation of SND in bursts synchronized with the activity of the phrenic nerve with maxima in the early expiratory phase. The same results were obtained in the other four rats.

Effect of CVLM inhibition on mean SND

The potent and long-lasting $GABA_A$ receptor agonist muscimol was injected bilaterally into the caudal ventrolateral medulla (CVLM, $n = 12$). The targeted area (injection sites depicted in Fig. 1*E*) corresponded to the region previously described by us and others as containing propriomedullary inhibitory interneurons involved in the sympathetic component of the baroreflex (Koshiya *et al.* 1993; for references see Kumada, Terui & Kuwaki, 1990). This region overlaps with the rostral ventral respiratory group and the pre-Bötzinger complex (Smith, Ellenberger,

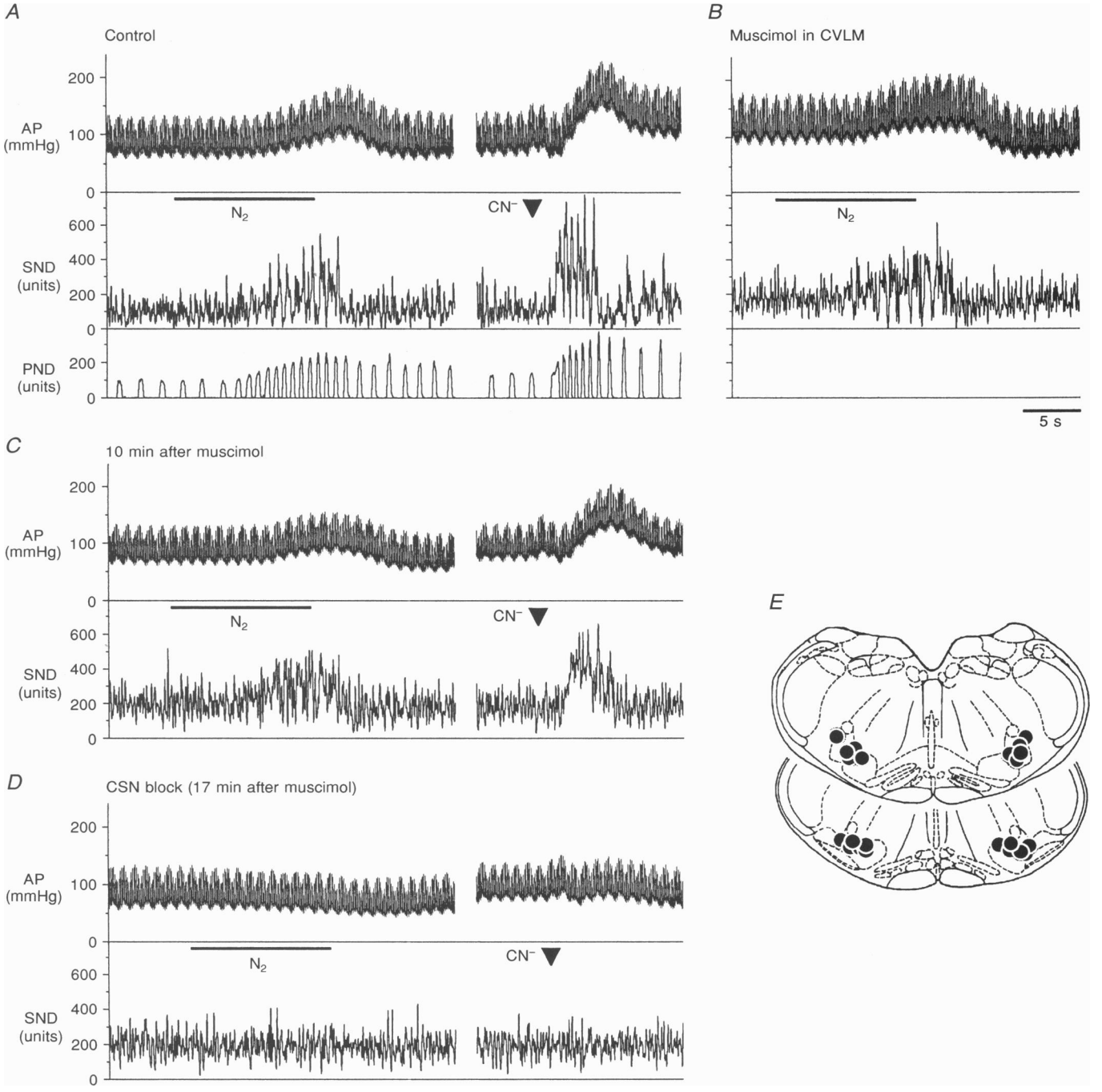

Figure 1. Sympathetic nerve response to carotid chemoreceptor stimulation is desynchronized after bilateral microinjection of muscimol into CVLM

A, control period: rhythmic excitation of SND during brief inhalation of N_2 (12 s, horizontal bars) or I.V. administration of NaCN (100 μg kg^{-1}, arrowheads). Arterial pressure (AP) and phrenic nerve discharges (PND) are also represented. *B*, response to hypoxia immediately after bilateral microinjection of muscimol (1·75 mM, 50 nl per side) into CVLM. Note loss of PND but persistence of rhythmic excitations of sympathetic nerve discharge. *C*, 10 min after injection, sympathetic excitation to hypoxia became tonic (PND still absent, not illustrated). *D*, 17 min after muscimol injection, tonic excitation of SND and pressor response were eliminated by bilateral application of mepivacaine (1·5%, 0·5 ml per side) to the carotid sinus regions. *E*, map of muscimol injection sites (●) for 11 experiments similar to that shown in *A–C*.

Ballanyi, Richter & Feldman, 1991). In all rats, PND was silenced immediately and resting SND increased significantly (Student's paired t test; $+46 \pm 6\%$, $n = 11$). The overall sympathetic response to hypoxia did not change significantly (hypoxia-induced SND increase before muscimol: $+80 \pm 7$ units from a resting level of 100 units; after muscimol: $+91 \pm 9$ units from a resting level of 146 ± 6 units, $n = 12$; not significant (n.s.) by paired t test). As expected, the baroreceptor reflex was abolished in all cases (not shown; for example see Koshiya *et al.* 1993). After muscimol injection into CVLM, hypoxia or I.V. cyanide failed in every case to activate the phrenic nerve (Fig. 1*B*).

Effect of CVLM inhibition on sympathetic oscillations during chemoreceptor stimulation

Before muscimol injection into CVLM, SND was activated by hypoxia or cyanide in bursts synchronized with the central respiratory cycle, with maxima during early expiration (post-inspiratory phase, Fig. 1*A*). Shortly after muscimol injection into CVLM, the pattern of SND activation by hypoxia (Fig. 1*B*) or cyanide (not shown) remained phasic and was therefore reminiscent of the predrug pattern, despite the disappearance of PND (Fig. 1*B*). This phase lasted from 1 to 12 min after CVLM injection of muscimol, depending on the rat (mean, 8 min; $n = 12$). Following this initial phase, SND activation by hypoxia or cyanide lost its slow oscillatory pattern, i.e. became tonic (Fig. 1*C*). This tonic activation of SND by both hypoxia and cyanide was also completely abolished by bilateral microperfusion of the carotid bifurcation area with the local anaesthetic mepivacaine (1·5%, 0·5 ml per side) in all cases tested ($n = 3$, e.g. Fig. 1*D*). As noted before, the effects of muscimol are long lasting and the experiments were terminated without waiting for recovery.

Respiratory outflows in the vagus and hypoglossal nerves: effect of CVLM inhibition

These experiments were designed to determine whether the loss of respiratory activity after microinjection of muscimol was generalized or whether it was restricted to the phrenic outflow.

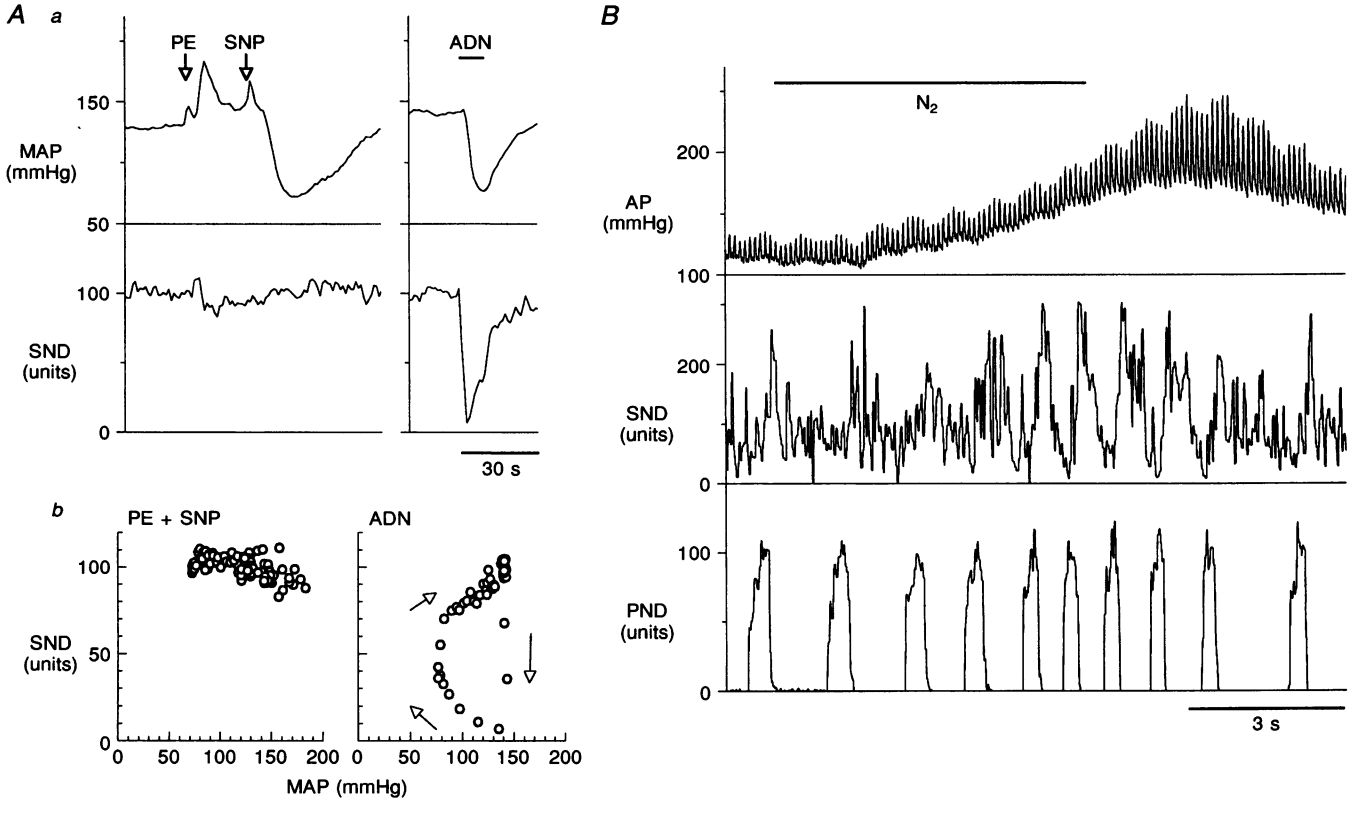

Figure 2. Sympathetic nerve response to carotid chemoreceptor stimulation in debuffered decerebrate rats

Aa, evidence of complete functional baroreceptor deafferentation. Left-hand traces illustrate lack of sympathetic nerve response to change in arterial pressure with I.V. phenylephrine (PE, 2 μg) followed by I.V. sodium nitroprusside (SNP, 4 μg). Right-hand traces illustrate sympathetic response to unilateral stimulation of aortic depressor nerve (ADN, 500 μA, 0·2 ms, 100 Hz for 10 s). *Ab*, relationship between SND and MAP during arterial pressure challenges shown in *Aa*. *B*, arterial pressure elevation and activation of SND and PND by hypoxia (N_2 at bar).

Respiratory discharges in the cranial nerves

PND was recorded in six rats, the discharge of a vagus nerve was recorded in three rats and that of the hypoglossal nerve in the other three. The vagus nerve discharge occurred during pre-inspiration (late expiration), inspiration and early expiration/post-inspiration (Fig. 3*A*). The amount of post-inspiratory activity in the vagus nerve varied among animals. The hypoglossal nerve discharge was present during late expiration, peaked during early inspiration and decreased abruptly with the same kinetics as PND (Fig. 3*B*). Brief hypoxia increased the cranial nerve activities in parallel with PND (increased burst rate and burst amplitude) (Fig. 3*C* and *D*).

Effect of CVLM inhibition

In four animals, bilateral microinjection of muscimol into CVLM was performed (sites in Fig. 3*G*). CVLM inhibition abolished PND and the activities of the simultaneously recorded cranial nerves in all cases (vagus, $n = 2$; hypoglossal, $n = 2$; Fig. 3*E* and *F*). Moreover, following

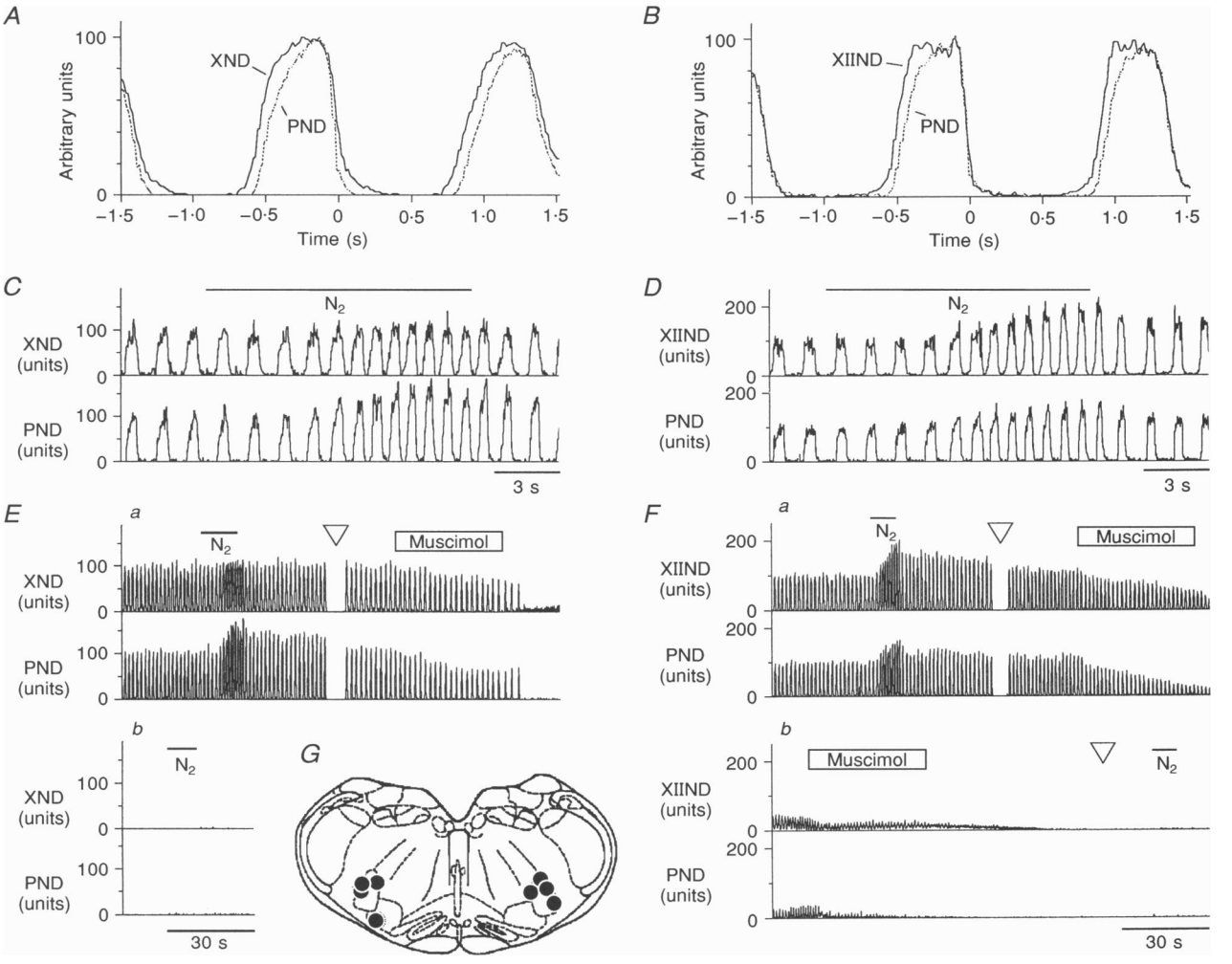

Figure 3. Muscimol injection into CVLM eliminates respiratory efferent activity in vagal and hypoglossal nerves

A, PND-triggered histogram of vagal efferent activity (XND, continuous line; phrenic discharge indicated by dotted line). *B*, PND-triggered histogram of efferent hypoglossal nerve activity (XIIND, continuous line). *C* and *D*, chemoreflex activation of vagus (*C*) or hypoglossal (*D*) nerves with brief inhalation of N_2 (horizontal bars); no moving average. *E* and *F*, effect of unilateral (*a*) and bilateral (*b*) microinjection of muscimol into CVLM on the nerve discharges. Period of application of muscimol indicated by boxes. Open triangles identify gaps in records of 2 min (*E*) or 1 min (*F*). *G*, injection sites of muscimol (nearest plane, −3·80 mm from interaural line; Paxinos & Watson, 1986).

muscimol injection, carotid chemoreceptor stimulation failed to elicit any activity in any of the nerves recorded ($n = 4$, e.g. panels *b* in Fig. 1*E* and *F*).

Neuronal activities in RVLM: response to hypoxia and effect of CVLM inhibition

We focused on units whose discharge pattern was notably altered by our hypoxia protocol ($n = 47$).

Classifications of the chemoreceptive RVLM neurons

Chemoreceptive units fell into two groups classified as respiratory or cardiovascular according to classic criteria. Units were defined as respiratory if they displayed a clear silent period during a particular phase of each respiratory cycle (defined by PND). Units were defined as cardiovascular using criteria established in prior work from this laboratory, namely inhibition during phenylephrine injection that is time-locked with the change in MAP and pulse-synchronous firing at elevated pressure. These cells never exhibit an on–off pattern of discharge during the respiratory cycle. We have shown previously that at least 75% of these RVLM cells can be antidromically activated from the spinal cord and therefore are presumably vasomotor presympathetic cells (Haselton & Guyenet, 1990). Because cord stimulation was technically incompatible with the remainder of the experimental procedures, we were unable to verify in the present study that the barosensitive cells recorded had spinal projections. These chemoreceptive units were recorded in the most rostral aspect of the RVLM (plotted in Fig. 4*A*).

Respiratory neurons. Forty chemoreceptive RVLM neurons were classified as respiratory. They belonged to seven known subtypes, previously defined by others on the basis of their firing pattern in relation to PND at rest (Schwarzacher *et al.* 1991; Bryant, Yoshida, Decastro & Lipski, 1993): early-I ($n = 7$) with peak activity in early inspiration; late-I ($n = 1$) with infrequent firing concentrated in late inspiration; throughout-I ($n = 1$) with even discharge throughout inspiration; decrement-E ($n = 10$), increment-E ($n = 7$) or throughout-E ($n = 10$) distinguished by their change in firing frequency during expiration; post-I ($n = 4$) firing immediately before and/or after the cessation of the PND burst. During chemoreceptor stimulation, 29/40 respiratory units (74%) increased their firing rate (up to +300%), while the remaining eleven (26%) were inhibited or silenced. All inspiratory and post-I neurons were activated. In contrast,

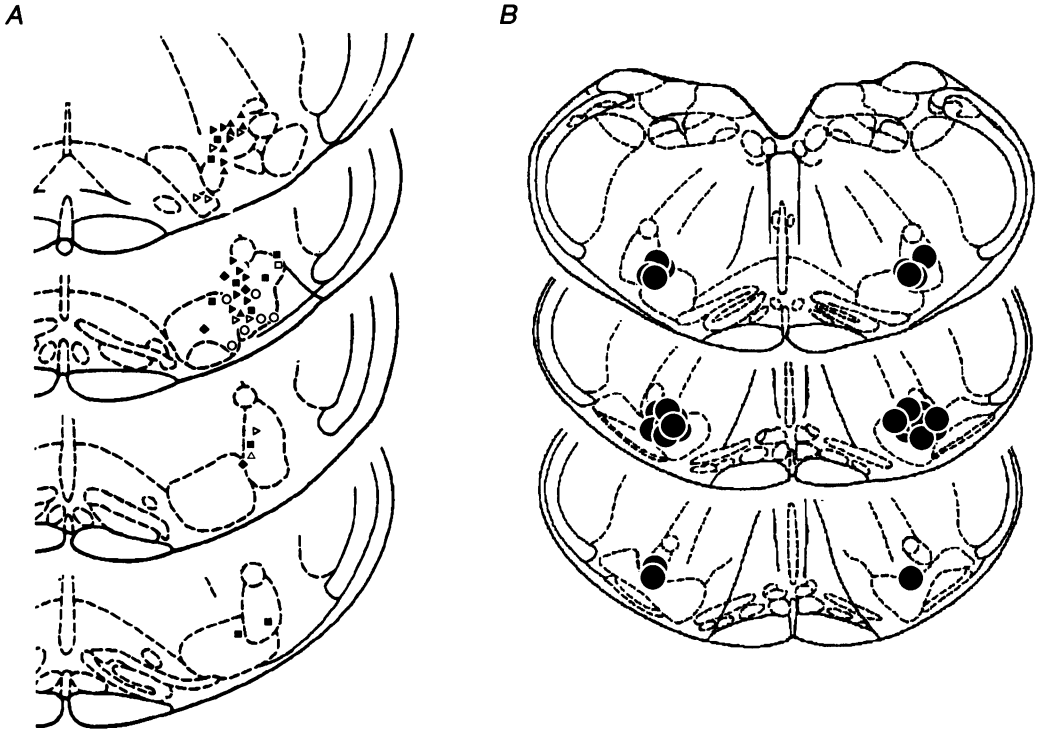

Figure 4. Location of recorded neurons and muscimol microinjections

A, sites of unit recordings, projected to nearest standard planes (Paxinos & Watson, 1986) from −2·60 to −3·30 mm from the interaural line. ○, cardiovascular units. ▷, early-I units. □, throughout-I units. △, late-I units. ◆, post-I units. ▶, decrement-E units. ■, throughout-E units. ▲, increment-E units. *B*, injections sites (●) of muscimol in unit recording experiments (interaural, −3·8, −4·24 and 4·30 mm).

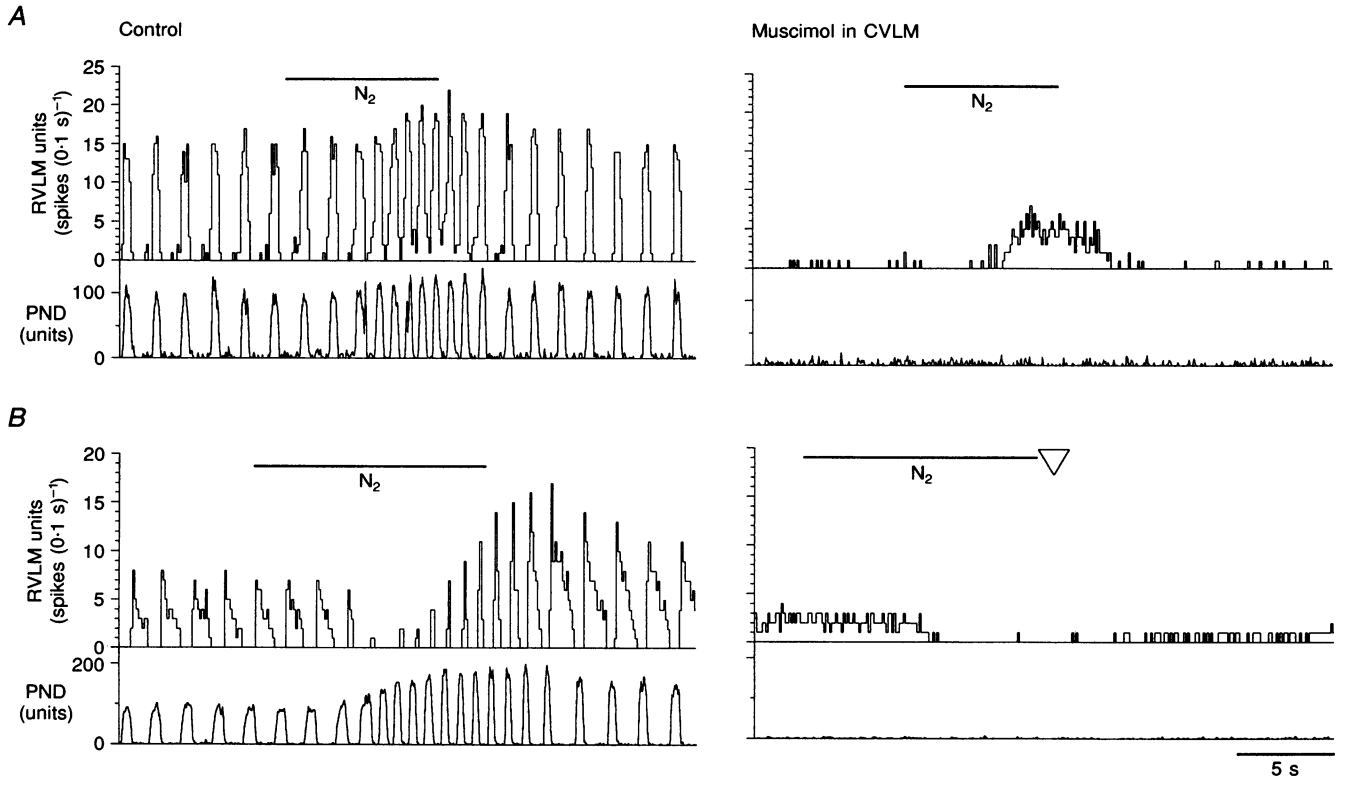

Figure 5. Muscimol injection into CVLM eliminates rhythmicity in RVLM respiratory neurons but not their responsiveness to hypoxia

Integrated rate histogram of the discharges of single RVLM units (0·1 s bins, upper trace) and integrated PND (lower trace; chemoreceptor stimulation with N_2 at bar). *A*, early-I neuron before (left) and after (right) muscimol. *B*, decrement-E neuron before (left) and after (right) muscimol. A period of 28 s is skipped at the level of the open triangle in *B* (right panel).

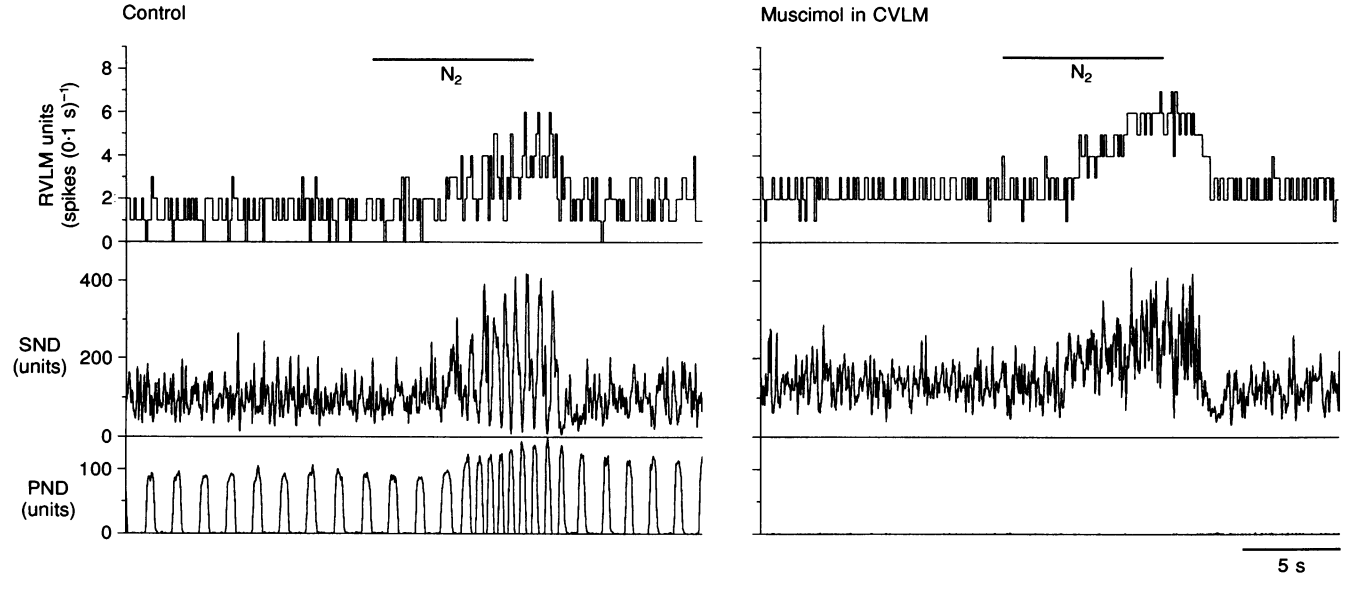

Figure 6. Response of RVLM cardiovascular units to hypoxia before and after muscimol injection into CVLM

From top to bottom: integrated rate histogram of unit activity of single RVLM barosensitive unit (0·1 s bins), SND and PND (stimulation with N_2 at bar). Left panel, before bilateral injection of muscimol into CVLM; right panel, after bilateral injection of muscimol into CVLM.

E neurons could be either excited or inhibited by chemoreceptor stimulation. Specifically eight decrement-E and three throughout-E were inhibited and the remainder ($n = 16$) were excited (2 decrement-E, 7 increment-E and 7 throughout E). In addition the inhibitory responses of decrement-E neurons were commonly followed by delayed excitation (see Fig. 5*B* for example).

Cardiovascular neurons. Seven tonically firing neurons that were excited by hypoxia (+85 ± 23% from the resting level) were identified as cardiovascular. During carotid chemoreceptor stimulation their firing pattern became noticeably entrained by the central respiratory cycle in a manner reminiscent of the SND oscillations (e.g. Fig. 6, left panel). All of them were subjected to the subsequent CVLM inhibition protocol described below.

Effect of CVLM inhibition on the discharges of RVLM neurons during hypoxia

In fifteen rats, single chemoreceptive RVLM units were recorded long enough to study the effect of muscimol injection into CVLM (sites illustrated in Fig. 4*B*) on their response to peripheral chemoreceptor stimulation. This sample consisted of eight respiratory neurons and seven cardiovascular units recorded along with PND and SND. As expected muscimol silenced PND in all cases.

Respiratory neurons. Muscimol eliminated the discharges of four respiratory units (1 early-I, 2 increment-E and 1 throughout-E) and rendered the remaining four tonically active (1 decrement-E and 3 throughout-E). During chemoreceptor stimulation, the neurons that had been silenced by CVLM inhibition ($n = 4$) were activated tonically despite the complete absence of phrenic discharge (example of one cell in Fig. 5*A*). Likewise, the cells that had become tonically activated after injection of muscimol into CVLM also remained sensitive to chemoreceptor activation, and the direction of the change (excitation or inhibition) remained the same. Specifically, two cells (throughout-E), which had been activated by hypoxia before muscimol, remained activated by hypoxia and two other E neurons (1 decrement-E, 1 throughout-E) that had been inhibited by hypoxia before muscimol remained inhibited by the stimulation (example of 1 cell in Fig. 5*B*). Note again the absence of respiration-like rhythmicity after muscimol injection.

Cardiovascular neurons. The resting firing rate of cardiovascular RVLM neurons increased significantly after CVLM inhibition by muscimol (mean, +22 ± 8%; $n = 7$; paired t test). After muscimol, these cells also lost their barosensitivity as expected (not shown) but their excitatory response to brief hypoxia persisted (+85 ± 23% increase in discharge rate from resting level before muscimol; +91 ± 30% after drug; n.s. by paired t test). A high time-resolution analysis of the effect of muscimol on PND, SND and the unit activity of a cardiovascular neuron of RVLM is shown in Fig. 6. The activation of this unit by chemoreceptor stimulation parallels that of SND. Note also that the respiratory rhythmicity exhibited by both unit and SND before muscimol disappeared after injection of muscimol. The behaviour of the other six cardiovascular units was identical.

DISCUSSION

Peripheral chemoreceptor stimulation triggers sympathetic bursts synchronized with the central respiratory cycle in debuffered rats

Decerebrate animals in which the internal carotid arteries had been ligated before decerebration were found to have virtually no baroreflex (less than 10% inhibition of SND at MAP of 180–200 mmHg, Fig. 2*A*) yet these rats had an intact central baroreflex pathway (Fig. 2*A*). We speculate that internal carotid ligation may eliminate carotid baroreceptor function by damaging baroreceptor afferents selectively and/or by preventing expansion of the carotid artery during arterial pressure elevation. In any case this serendipitous observation provided the opportunity to show definitively that the bursts of sympathetic activity triggered by carotid chemoreceptor stimulation are synchronized with the central respiratory activity even in the absence of baroreceptor feedback. This statement can be made because, during chemoreceptor stimulation, the respiratory oscillations of SND occurred in the presence of levels of AP (100–170 mmHg) where baroreceptor feedback was absent (Fig. 2*B*). In decerebrate rats, the timing of the sympathetic bursts relative to the phrenic nerve discharge was the same as in the urethane-anaesthetized rats (maxima during early expiration). The similarity of the two preparations suggests that, even in the incompletely debuffered urethane-anaesthetized rats, baroreceptor feedback does not play a significant role in the timing of the sympathetic oscillations produced by peripheral chemoreceptor stimulation. Of course we cannot exclude the possibility that the amplitude of the bursts might be attenuated somewhat by the baroreflex towards the end of the stimulus when arterial pressure has reached its peak. However, this effect is probably minor in these partially debuffered rats when hypoxia is used to stimulate chemoreceptors since the rise in arterial pressure is usually modest and the threshold of the baroreflex is high (around 120 mmHg).

Tonic activation of the sympathetic outflow by hypoxia after CVLM inhibition is mediated by activation of carotid chemoreceptors

As in our previous study (Guyenet & Koshiya, 1995), we observed that the average amount of increase in sympathetic nerve discharge elicited by brief hypoxia or by I.V. cyanide was unchanged after microinjection of the $GABA_A$ agonist muscimol into CVLM (from 80 to 90 units above baseline). This small but non-statististically significant increase may reflect the inhibition by muscimol

of the weak baroreflex present in the partially debuffered rat. The only change produced by muscimol was the disappearance of the slow oscillations of the sympathetic response which evolved (abruptly or gradually depending on the animal) from the classic respiration-locked pattern to a tonic desynchronized one. Desynchronized activation of SND was observed using either hypoxia or cyanide as stimulus and the response to both was still totally dependent on functional carotid sinus nerves (e.g. Fig. 1*D*). This evidence rules out the possibility that, after muscimol injection into CVLM, the desynchronized SND response to hypoxia or cyanide could have been due to other than peripheral chemoreceptor stimulation (e.g. central hypoxic response). In addition, the disappearance of the slow oscillations of SND cannot be attributed to the interruption of the baroreflex pathway since muscimol eliminated the baroreflex instantly in all cases while the loss of the SND oscillations could occur gradually over a 10 min period.

The injections of muscimol were centred in the rostral aspect of the CVLM (e.g. Figs 1*E* and 3*G*), an area that coincides with the reported location of the pre-Bötzinger complex, the presumed site of respiratory rhythm generation in rats (Smith *et al.* 1991). Conceivably, a varying degree of encroachment of muscimol on the pre-Bötzinger area may underlie the degree or rapidity with which SND activation by chemoreceptor activation became tonic.

Muscimol injection into CVLM produces a generalized blockade of respiratory outflows and blocked central respiratory rhythm generation

Muscimol injection into CVLM produced a generalized blockade of respiratory outflows as opposed to one restricted to the phrenic nerve. Indeed all three measured respiratory outflows (phrenic, vagal and hypoglossal) were eliminated both at rest and during chemoreceptor stimulation (Fig. 3). The hypoglossal outflow originates from motoneurons that are clearly out of reach of muscimol. The vagal outflow originates from motoneurons that lie either dorsal to the hypoglossal nucleus or in the nucleus ambiguus from the level of the facial motor nucleus to the caudal part of the ventral respiratory group, i.e. areas that are in large portion also out of reach of muscimol. Therefore, these experiments suggest that muscimol injection in CVLM blocked respiratory rhythm generation as well as respiratory efferent activity. This conclusion is supported by the observation that all RVLM neurons recorded also lost their respiratory pattern after muscimol injection into CVLM.

Chemoreceptor inputs to RVLM neurons persist after injections of muscimol into CVLM

Muscimol injection into CVLM did not inhibit the discharge of RVLM vasomotor cells. From this we conclude that the diffusion area of the toxin is limited and did not include RVLM. The most notable finding of the present study is that RVLM neurons retained their responsiveness to carotid chemoreceptor stimulation after inhibition of respiratory rhythm generation. This was observed regardless of neuronal type (respiratory or cardiovascular) and regardless of whether the responses were excitatory (most cases) or inhibitory (some respiratory neurons). Since the response of these neurons to chemoreceptor stimulation was of a comparable magnitude before and after injection of muscimol into CVLM, we conclude that RVLM neurons receive powerful inputs from carotid chemoreceptors that reach RVLM via pathways that do not relay in the CVLM and are independent of the respiratory rhythm generating circuit.

Central pathway of the sympathetic chemoreflex

Prior work from this laboratory has demonstrated that the reflex investigated in the present study does not require structures rostral to the pons (Koshiya & Guyenet, 1994*a*). Enhancement of the reflex following massive lesions of the dorsolateral pons also demonstrated that the parabrachial nuclei were involved solely in the respiratory modulation of the sympathetic response (Koshiya & Guyenet, 1994*a*). The present results offer a second example of total dissociation between sympathetic and respiratory effects of peripheral chemoreceptor stimulation. Combined, these studies strongly support our working hypothesis (Guyenet & Koshiya, 1995) that activation of the sympathetic outflow by stimulation of chemoreceptors involves the summation of two processes: (i) excitatory drive of RVLM neurons via inputs independent of the central respiratory network and (ii) respiratory modulation (possibly via periodic inhibition of RVLM neurons) via elements of the respiratory network. As in the case of all other sympathetic reflexes, the RVLM region (vasomotor cells themselves and/or antecedent interneurons) appears to be a critical site of integration between these two pathways. The key evidence to date is (i) the sympathetic chemoreflex is completely and selectively attenuated by microinjection of antagonists for excitatory amino acid receptors in the RVLM (Koshiya *et al.* 1993; Sun & Reis, 1995) and (ii) the unit discharge of many RVLM vasomotor cells mirrors that of SND before and after impairment of central respiratory networks (this study).

The pathway responsible for the respiration-independent activation of RVLM neurons by carotid chemoreceptor stimulation needs to be determined. The simplest and most probable pathway would be a direct projection from the nucleus of the solitary tract to RVLM. Such a projection, including a possible monosynaptic connection to C1 cells, is already documented anatomically (Hancock, 1988) but has yet to be verified neurophysiologically. Finally, it should be noted that the nucleus of the solitary tract contains a large number of neurons that receive chemoreceptor inputs and are devoid of central respiratory modulation (Mifflin, 1993). Neurons with such characteristics would be well suited to relay peripheral chemoreceptor information directly to various brain centres including the RVLM.

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