Respiratory changes induced by kainic acid lesions in rostral ventral respiratory group of rabbits

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Mutolo, Donatella, Fulvia Bongianni, Marco Carfì, and Tito Pantaleo. Respiratory changes induced by kainic acid lesions in rostral ventral respiratory group of rabbits. Am J Physiol Regulatory Integrative Comp Physiol 283: R227–R242, 2002. First published February 28, 2002; 10.1152/ajpregu.00579.2001.—The role played by the Bötzinger complex (BötC), the pre-Bötzinger complex (pre-BötC), and the more rostral extent of the inspiratory portion of the ventral respiratory group (iVRG) in the genesis of the eupneic pattern of breathing was investigated in anesthetized, vagotomized, paralyzed, and artificially ventilated rabbits by means of kainic acid (KA, 4.7 mM) microinjections (20–30 nl). Unilateral KA microinjections into all of the investigated VRG subregions caused increases in respiratory frequency associated with moderate decreases in peak phrenic amplitude in the BötC and pre-BötC regions. Bilateral KA microinjections into either the BötC or pre-BötC transiently eliminated respiratory rhythmicity and caused the appearance of tonic phrenic activity (“tonic apnea”), whereas injections into the rostral iVRG completely suppressed inspiratory activity. Rhythmic activity resumed as low-amplitude, high-frequency oscillations and displayed a progressive, although incomplete, recovery. Combined bilateral KA microinjections (BötC and pre-BötC) caused persistent (>3 h) tonic apnea. Results show that all of the investigated VRG subregions exert a potent control on both the intensity and frequency of inspiratory activity, thus suggesting that these areas play a major role in the genesis of the eupneic pattern of breathing.

Bötzinger complex; breathing control; respiratory neurons

Breathing in mammals is reliant on a neuronal network located in the lower brain stem. Neuronal mechanisms responsible for generating the eupneic pattern of breathing are localized within the pons and the medulla oblongata (for reviews, see Refs. 5, 57, 62). In the pons, respiratory neurons are mainly concentrated in a region encompassing the nucleus parabrachialis medialis and the Kölliker-Fuse nucleus, which corresponds to the “pneumotaxic center” that controls the inspiratory off switch and, more generally, the timing of the respiratory phases (5, 31, 62). This pontine region has also been termed the pontine respiratory group, although other populations of respiration-modulated neurons have been encountered at scattered locations in the pons (5, 57, 62). Evidence has been provided that the pons plays an important role in respiratory-rhythm generation and eupneic pattern formation, as only gasping can be generated at the medullary level after complete removal of the pons (see, e.g., Ref. 57 also for further references). However, the results of several studies (for reviews, see Refs. 5, 41, 43, 47, 62, 63) have led to the proposal that the basic mechanism responsible for rhythm generation, the so-called central pattern generator (CPG), may be located within the medulla oblongata, where medullary respiratory neurons are concentrated in two distinct regions: the dorsal (DRG) and ventral (VRG) respiratory groups.

Extensive multiple electrolytic lesions placed in the DRG and VRG of anesthetized or decerebrated cats have caused a gradual decline in the amplitude of inspiratory activity without affecting the respiratory rhythm (54, 55). These studies suggest that these medullary areas are not crucial for respiratory-rhythm generation. In fact, gradual declines in the magnitude of inspiratory activity, but not in respiratory frequency, are usually considered to reflect blockade of tonic drive and not an impairment of rhythm generation (12, 39, 55, 62). Unilateral focal cold blocks of structures located in the rostral ventrolateral medulla (10) caused strong depression of respiratory rhythmicity or apnea, the latter being characterized by the complete absence of inspiratory activity or by low levels of tonic phrenic discharges (“tonic apnea”). A prominent feature of focal cold blocks in the rostral ventrolateral medulla was an increase in respiratory rate accompanying depressant effects on inspiratory activity. This “apnea region” extends in the ventrolateral aspects of the rostral medulla from ~2 mm rostral to the obex to the level of the facial nucleus and overlaps (at least in part) the inspiratory portion of the VRG, the retrofacial region (Bötzinger complex; BötC), and the more superficially located chemosensitive regions (10). The neural structures corresponding to the apnea region were not interpreted as possible sites for respiratory-rhythm generation but, with great caution, were suggested to be important for chemoceptive and other drive inputs necessary for respiratory rhythmogenesis (10). These experiments focused attention also on structures located outside the DRG and VRG as...
Figure 4: The respiratory rhythm is maintained by a complex network of neuronal groups. The inspiratory portion of the ventral respiratory group (iVRG), which is rostral to the obex, 2.3–3.5 mm below the dorsal medullary surface, is crucial for eupneic breathing. This region was at first identified in an in vitro brainstem-spinal cord preparation of the neonatal rat (48; for reviews, see Refs. 5, 41) and subsequently also in adult animals in vivo (13, 40, 50, 56; for reviews, see Refs. 5, 41). This subregion, named the pre-Bötzinger complex (pre-BoTc), corresponds to the transition zone between the rostral expiratory neurons of the BoTc and the inspiratory portion of the VRG (iVRG), which is often reported in the literature as the rostral VRG (5, 62). The rostral and caudal boundaries of the pre-BoTc are not defined with absolute precision, but in the adult this region of the VRG can be characterized by the presence of a mix of different types of inspiratory and expiratory neurons, which are for the most part propriobulbar neurons (13, 41, 45, 56, 60). Results obtained with electrolytic, radio-frequency, and kainic acid (KA) lesions (29, 53, 58) have suggested that the BoTc and rostral iVRG may be crucial in the generation and/or maintenance of the respiratory rhythm. All of these VRG subregions (see also Refs. 41 and 57) may be components of the apnea region described by Budzinska et al. (10).

However, considering the studies performed with different types of lesions and blockades of neuronal activity in the rostral portions of the VRG, including the BoTc (29, 53, 58) and the pre-BoTc (2, 10, 18, 21–24, 40, 56; for review, see Ref. 57), discrepancies in the results are obvious. Thus the role played by these different VRG subregions in the control of respiration is far from being definitely settled. Most of the above-mentioned studies have been carried out in cats and rats. The rabbit is widely used as an experimental model in the study of the neural control of breathing (e.g., Refs. 5, 8, 32, 62); however, very little information is available on the respiratory function of the rostral VRG subregions in this animal species.

The present study was undertaken in an attempt to get further insights into the role played by the BoTc, the pre-BoTc, and the immediately adjacent rostral part of the iVRG in the genesis of the eupneic pattern of breathing of anesthetized, vagotomized, paralyzed, and artificially ventilated rabbits. For this purpose, we have made use of microinjections of the neurotoxin KA (14) to produce lesions systematically placed in different subregions of the rostral VRG. Preliminary accounts of the present work have already been presented in abstract form (30).

**METHODS**

**Animal preparation.** Experiments were carried out on 58 male New Zealand White rabbits (2.8–3.6 kg) anesthetized with a mixture of α-chloralose (40 mg/kg iv; Sigma Chemical, St. Louis, MO) and urethane (800 mg/kg iv; Sigma Chemical) supplemented when necessary (4 and 80 mg/kg, respectively). The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw. All animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Communities Council on the use of laboratory animals (directive 86/609/EEC). The experimental protocol was approved by the Animal Care and Use Committee of the University of Florence.

After cannulation of the trachea, polyethylene catheters were inserted into a femoral artery and vein for the measurement of arterial blood pressure and systemic administration of drugs, respectively. Both C5 phrenic roots were dissected free, cut distally, and prepared for recordings. Both cervical vagus nerves were separated from the sympathetic trunks for subsequent vagotomy. The animal was placed in a prone position and fixed by a stereotactic head holder and vertebral clamps; the head was ventroflexed to facilitate recordings from the medulla. The dorsal surface of the medulla was widely exposed by occipital craniotomy, and the dura and arachnoid membranes were removed. The posterior part of the cerebellum was removed by gentle suction to provide access to the rostral part of the medulla. All exposed tissues were covered with warm paraffin oil (37–38°C). Body temperature was maintained within the range of 38.5–39.5°C by a heating blanket controlled by a rectal thermistor probe. The animals were vagotomized, paralyzed with gallamine triethiodide (4 mg/kg iv, supplemented with 2 mg/kg every 30 min; Sigma Chemical), and artificially ventilated. End-tidal CO2 partial pressure (P CO2) was maintained approximately at the level of spontaneous breathing (28.5–36.5 mmHg) by adjusting the frequency and stroke volume of the respiratory pump. In paralyzed animals, the depth of anesthesia was assessed by monitoring a stable and regular pattern of phrenic activity as well as the absence of fluctuations in arterial blood pressure, whether spontaneous or in response to somatic nociceptive stimulation.

**Recording procedures.** Efferent phrenic nerve activity was recorded with bipolar platinum electrodes from desheathed C5 phrenic roots, amplified, full-wave rectified, and passed through a leaky integrator (low-pass resistor capacitor filter, time constant 100 ms) to obtain a “moving average” of the activity usually referred to in the literature as integrated activity. Extracellular recordings from medullary neurons were made with tungsten microelectrodes (5–10 MΩ impedance as tested at 1 kHz). We defined the obex at the most rostral extent of the area postrema for use as a standard point of anatomic reference. Neuronal activity was recorded from rostral expiratory neurons of the BoTc (3.0–4.5 mm rostral to the obex, 2.4–3.4 mm lateral to the midline, and 3.5–4.6 mm below the dorsal medullary surface), from the iVRG (from 0.7 caudal to 2.0 mm rostral to the obex, 2.3–3.2 mm lateral to the midline, and 3.0–3.5 mm below the dorsal medullary surface), and from the transition zone between the BoTc and the iVRG where a mix of inspiratory and expiratory neurons is present (2.1–2.9 mm rostral to the obex, 2.4–3.3 mm lateral to the midline, and 3.5–4.2 mm below the dorsal medullary surface). The latter region has already been studied in the rabbit (32) and may correspond to the so-called pre-BoTc described in the adult cat and rat (see, e.g., Refs. 13,
Recordings were also performed from the expiratory neurons of the caudal VRG (cVRG; 0.8–2.6 mm caudal to the obex, 2.0–2.6 mm lateral to the midline, and 2.0–3.0 mm below the dorsal medullary surface). Stereotaxic coordinates of the different VRG subregions have been provided, taking into account animal variability; in each individual preparation, the rostrocaudal extent of each investigated region was smaller than that defined by the above-reported coordinates (see results). A schematic representation of medullary respiration-related regions in the rabbit is provided in Fig. 5A. Strain-gauge manometers were used for monitoring intrathoracic pressure and arterial blood pressure. PCO2 was monitored by an infrared CO2 analyzer (Datex CD-102, Normocap, Helsinki, Finland).

Integrated phrenic nerve activity as well as the signals of the other variables studied were recorded on an eight-channel rectilinearly writing chart recorder (model SK20, NEC San-ei, Tokyo, Japan). In some experiments, phrenic neurogram and neuronal activity were also acquired and analyzed by using a personal computer equipped with an analog-to-digital interface (Digitdata 1200, Axon Instruments, Forster City, CA). For histological procedures (Axon and, therefore, indirectly provide an estimate of the extent of the inactivated area. Theoretical calculations by Nicholson (35) suggest that volumes of 20–30 nl should spread <350 μm in any direction from the injection site. In addition, recordings of neuronal activity within the injected area and in close vicinity to it were performed 60 min or more after the injections to estimate the extent of KA-induced lesions.

To evaluate chemical sensitivity during KA-induced respiratory responses, hypercapnic and hypoxic stimuli were employed. Hypercapnia was produced by allowing the animal to inspire an appropriate mixture of CO2 and O2 from a large (150 liter) bag; PCO2 was adjusted to ~30 mmHg higher than the level of spontaneous breathing (range of 55–65 mmHg). After a stable PCO2 level was achieved, hypercapnic stimulation was maintained for at least 3 min. Hypoxia was induced by allowing the animal to inspire a gas mixture containing 6% O2 in N2 for ~2 min.

Histology. At the end of the experiment, the brain was perfused with 0.9% NaCl solution and then with 10% formalin solution via a carotid artery. After at least a 48-h immersion in 10% formalin solution, the brain was placed in a hypertonic sucrose solution, frozen, and cut to obtain coronal sections (20 μm thick). Unstained sections were used to evaluate the spread of the Pontamine sky blue dye. Sections stained with cresyl violet were used for the histological control of pipette tracks and injection sites. The atlas of Shek et al. (46) was used for comparison.

Data collection and analysis. Measurements were made on paper recordings (paper speed was usually 5–10 mm/s and less frequently 25 mm/s). From the trace of integrated phrenic nerve activity, we derived respiratory frequency (breaths/min), inspiratory time (TI), expiratory time (TE), and peak amplitude (arbitrary units). When a level of tonic phrenic nerve activity developed in response to KA microinjection (see results), it was included in the measurement of peak phrenic amplitude. The rate of rise of integrated phrenic nerve activity was evaluated with accuracy on higher speed recordings (paper speed of 25 mm/s). The slope of the straight line drawn from the onset to 90% of the maximum level of the phrenic ramp was considered a reliable estimate of the inspiratory rate of rise (see, e.g., Refs. 6, 8). Respiratory variables were measured for 10 s both in the period immediately preceding each trial (control period) and at scheduled times during KA-induced effects. After the completion of KA unilateral and bilateral microinjections, 30 min were taken before changes in respiratory activity were con-
sidered to be reliable and were, therefore, evaluated. This time was necessary to allow complete inactivation of neurons in the injected area (14, 16) and to avoid confounding effects on respiration due to changes in blood pressure (see RESULTS). In the same periods, systolic and diastolic blood pressure were assessed at 2-s intervals. Mean arterial pressure (MAP) was calculated as the diastolic pressure plus one-third of the pulse pressure. Owing to the small variations in respiratory variables and MAP within a measurement period, average values for each period were taken as single measurements for the purpose of analysis. Statistical analysis of data obtained for each VRG subregion was performed by using Wilcoxon signed-rank tests. Kruskal-Wallis nonparametric analysis of variance, followed by Dunn's multiple comparison test, was used in the comparisons of the effects observed in response to KA microinjections into the different VRG subregions. Changes in respiratory variables were expressed as percent variations of control values. All values are presented as means ± SE; P < 0.05 was considered as significant.

RESULTS

Neuronal recordings. The distribution of respiratory neurons showed a consistent rostrocaudal organization. In the most rostral medulla, the BötC could be easily identified by recordings of almost purely multiunit expiratory activities with prevailing augmenting discharge patterns (see Ref. 8). This region had an extent ≤1.2 mm in each individual animal. Immediately caudal to the BötC, a mix of inspiratory and expiratory neuronal activities was recorded (Fig. 1A). A total of 72 single units was recorded from this area in 24 preparations. We encountered different types of respiratory neurons, i.e., expiratory neurons with augmenting discharge patterns (17/72, 23.6%), expiratory neurons with decrementing discharge patterns or postinspiratory neurons (13/72, 18.1%), inspiratory neurons with augmenting discharge patterns (22/72, 30.5%), and phase-spanning neurons (20/72, 27.8%). Some phase-spanning neurons started firing during the expiratory phase (expiratory-inspiratory; n = 13, 18.1%) and attained a maximum rate during inspiration [in most cases (n = 10) at or close to the transition from expiration to inspiration]. Other phase-spanning neurons started firing during inspiration (inspiratory-expiratory; n = 7, 9.7%) and reached a firing peak at the transition from inspiration to expiration; these could extend firing into midexpiration (n = 4) or late expiration (n = 3). The patterns of discharge of these different types of neurons are shown in Fig. 1, B-F. This transition zone, which extends ≤0.7 mm in individual preparations, appears to correspond to the pre-BötC region as defined by neuronal recordings in the

Fig. 1. Discharge patterns of respiratory neurons encountered in the pre-BötC region. Each record shows phrenic neurogram (PN) and extracellular neuronal activity (NA). A: mix of inspiratory and expiratory neuronal activities. B: expiratory augmenting discharge pattern. C: expiratory decrementing discharge pattern. D: inspiratory augmenting discharge pattern. E: discharge pattern of an expiratory-inspiratory phase-spanning neuron beginning to fire during expiration with maximum firing rate at the transition between expiration and inspiration. F: discharge pattern of an inspiratory-expiratory phase-spanning neuron starting firing during inspiration with firing peak at the transition between inspiration and expiration and extending its firing into late expiration.
cat and the rat (see, e.g., Refs. 13, 41, 45, 56, 60). Caudal to this region, a high concentration of inspiratory neurons with prevailing augmenting discharge patterns was encountered (multiunit recordings); this area, which corresponds to the iVRG, extended $\pm 2.5$ mm in each preparation. In the most caudal part of the VRG, expiratory neurons with prevailing augmenting discharge patterns were encountered for a rostrocaudal extent $\pm 1.6$ mm (cVRG).

**Unilateral KA microinjections.** The main purpose of the present study was to investigate the respiratory responses induced by bilateral KA microinjections. Nevertheless, on some occasions, respiratory changes induced by unilateral KA microinjections (performed as a first step) were evaluated for $\sim 60$ min; at that time, the corresponding contralateral microinjection was executed. Unilateral KA microinjections into either the BötC ($n = 6$) or the transition zone, where a mix of inspiratory and expiratory neurons was encountered (i.e., the pre-BötC region; $n = 5$), produced similar effects. Respiratory changes observed 30 min after KA microinjections (Table 1) were characterized mainly by increases in respiratory frequency accompanied by moderate decreases in peak phrenic amplitude without significant changes in the inspiratory rate of rise. The increases in respiratory frequency were largely due to reductions in $T_E$ associated with small decreases in $T_I$ (Fig. 2); they were accompanied by the development of variable but relatively low levels of tonic phrenic activity. Respiratory effects decreased over time but were still significant 60 min after the injection (Table 1). Similar unilateral KA microinjections ($n = 5$) performed into the rostral part of the iVRG ($\sim 1.5$ mm rostral to the obex) induced moderate increases in respiratory frequency within 30 min that were mainly due to reductions in $T_E$ and, to a lesser extent, in $T_I$ (Fig. 2); small, not significant decreases in peak phrenic amplitude and inconsistent changes in the inspiratory rate of rise were also observed. Significant increases in respiratory frequency were still present 60 min after the injection (Table 1).

Table 1. Changes in respiratory variables after unilateral 4.7 mM kainic acid microinjections

<table>
<thead>
<tr>
<th>Respiratory Variable, %control</th>
<th>Respiratory frequency</th>
<th>Peak phrenic activity</th>
<th>Inspiratory rate of rise</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bötzing complex ($n = 6$)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control ($n = 6$)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30 min</td>
<td>$160.2 \pm 8.7^*$</td>
<td>$73.3 \pm 2.2^*$</td>
<td>$107.6 \pm 5.4$</td>
</tr>
<tr>
<td>60 min</td>
<td>$139.1 \pm 3.0^*\dagger$</td>
<td>$89.5 \pm 1.5^\dagger$</td>
<td>$113.8 \pm 2.1$</td>
</tr>
<tr>
<td><strong>Pre-Bötzing complex ($n = 5$)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control ($n = 5$)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30 min</td>
<td>$139.6 \pm 5.8^*$</td>
<td>$77.9 \pm 3.9^*$</td>
<td>$111.0 \pm 4.5$</td>
</tr>
<tr>
<td>60 min</td>
<td>$129.7 \pm 4.1^*$</td>
<td>$90.2 \pm 3.5^\dagger$</td>
<td>$109.2 \pm 3.1$</td>
</tr>
<tr>
<td><strong>Inspiratory VRG ($n = 5$)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control ($n = 5$)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30 min</td>
<td>$132.3 \pm 7.1^*$</td>
<td>$84.5 \pm 2.3$</td>
<td>$106.9 \pm 5.8$</td>
</tr>
<tr>
<td>60 min</td>
<td>$124.8 \pm 5.8^*$</td>
<td>$89.8 \pm 1.8$</td>
<td>$100.4 \pm 2.1$</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE; $n$, no. of animals/group. Values in parentheses are breaths/min. VRG, ventral respiratory group. $^*P < 0.05$ compared with control values; $^\dagger P < 0.05$ compared with 30 min.
Bilateral KA microinjections. Bilateral KA microinjections caused similar results independently of the interval scheduled between them. As shown in Fig. 3, bilateral KA microinjections into the BoTc (n = 15) and the pre-BoTc (n = 12) caused loss of respiratory rhythmicity within 30 min and the progressive development of a relatively intense tonic phrenic nerve activity (tonic apnea). The level of tonic phrenic nerve activity was significantly higher after KA microinjections into the BoTc; the mean amplitudes of tonic phrenic nerve activity were 39.3 ± 3.1 and 23.5 ± 2.5% of the peak phrenic amplitude in control breaths after bilateral KA microinjections into the BoTc and the pre-BoTc, respectively (P < 0.05). In both cases, rhythmic activity resumed within 50 min after the injections (Fig. 3) as low-amplitude, high-frequency irregular oscillations superimposed on tonic activity and then displayed a progressive recovery. Owing to the presence of tonic activity and the irregular pattern of breathing, it was possible to provide an accurate evaluation of respiratory variables only ~60 min after KA microinjections. At that time, significant increases in respiratory frequency and the rate of rise of inspiratory activity, which are associated with marked reductions in peak phrenic amplitude, were observed. The increases in respiratory frequency were due to marked reductions in Tc combined with less-pronounced decreases in Ti. An evaluation of changes in some respiratory variables 60 and 120 min after KA microinjections is provided in Table 2.

Bilateral KA microinjections (n = 12) into the iVRG from ~1.0–2.0 mm (usually 1.5 mm) rostral to the obex (Fig. 3) induced apnea without any appearance of tonic phrenic activity within 30 min. However, 35–50 min after the injections, low-intensity, high-frequency phrenic bursts progressively appeared. Also in this case, a reliable evaluation of respiratory variables could be performed only 60 min after KA microinjections (Table 2). Respiratory frequency showed marked increases (mainly due to reductions in Tc) accompanied by strong decreases in the peak amplitude of phrenic nerve activity and no significant changes in the inspiratory rate of rise. Respiratory activity displayed a progressive and relatively slow recovery (Table 2); comparisons between changes in respiratory variables observed after KA microinjections into each of these three VRG subregions revealed on some occasions the presence of small but significant differences (Table 2).

Control injections of equal volumes of the vehicle solution performed at the same locations were without significant effects. Bilateral KA microinjections (n = 12) at sites >0.5 mm away from these responsive sites of the BoTc, pre-BoTc, and iVRG, where intense multiunit respiratory neuronal activity was encountered, failed to induce the characteristic respiratory responses reported above and caused either no change or only transient (<5-min duration), inconsistent changes in respiratory activity. For comparison, bilateral KA microinjections were performed also in more caudal regions of the VRG. Changes in respiratory variables in response to bilateral KA microinjections (n = 6) from ~0.5 mm rostral to 0.7 mm caudal to the obex were less consistent and did not reach the level of statistical significance. Respiratory responses could be completely absent. When present, they were similar to those described for the more rostral portion of the iVRG but characterized by much shorter durations; complete recovery occurred within <15 min. No appreciable variations in respiratory activity were observed in response to bilateral KA microinjections (n = 6) into the cVRG extending from ~0.8 to 2.6 mm caudal to the obex.

An attempt was also made to evaluate CO2 and O2 sensitivity after bilateral KA microinjections into ei-
either the BötC (n = 3) or the pre-BötC (n = 2) regions. The complete absence of responsiveness to hypercapnic or hypoxic stimuli was observed during tonic apnea; i.e., neither the tonic level of phrenic nerve activity changed, nor did rhythmic activity resume during chemical stimulations. However, both types of chemical sensitivity were present as soon as some rhythmic phrenic nerve activity resumed. Examples of respiratory responses to chemical stimuli after KA lesion in the iVRG, phrenic nerve activity resumed. Examples of respiratory responses to chemical stimuli after KA lesion in the iVRG, rhythmic respiratory patterns but not apnea turned out to be affected by hypercapnic or hypoxic stimuli (n = 2).

Combined bilateral KA microinjections were performed into the BötC and the pre-BötC in the same preparation. The injections were performed either at first into the BötC and subsequently into the pre-BötC (n = 3) or vice versa (n = 2); they were made just in the middle of each region so that the distance between them was ≈0.8 mm. Bilateral microinjections into the first region caused respiratory changes similar to those already described. Bilateral microinjections in the second region caused loss of respiratory rhythmicity and the appearance of tonic phrenic nerve activity, which, however, did not show any tendency to change over time (Fig. 5). The absence of recovery of rhythmic respiratory activity was ascertained at least for 3 h.

Histological examination of coronal sections of the medulla oblongata revealed the bilateral presence of the Pontamine sky blue marking dye in the injected areas. Each dye spot covered an approximately spherical area with a diameter that ranged from 0.6 to ~0.75 mm. Bilateral microinjections (20–30 nl) of 160 mM DLH (see Ref. 8) into the BötC region (n = 5) or in the region immediately caudal to it (n = 3), performed 60 min after KA microinjections, were completely ineffective. Similar DLH microinjections into these two regions have been proven to cause depressant and excitatory respiratory responses, respectively (8, 50). These findings confirmed the presence of inactivated or lesioned areas with diameters comparable to those of the dye spots (see Ref. 35). Similarly, bilateral KA microinjections (20–30 nl) performed into each of the investigated VRG subregions 60 min after the completion of similar injections at the same sites did not affect the ongoing respiratory activity or the recovery process. Finally, recordings of neuronal activity performed in 15 experiments within KA-injected areas 60 min or more after the completion of the injections approximately along the injection track and along tracks located in close vicinity to it (≈0.4 mm) revealed either the complete absence of neuronal activity or the presence of very sparse, low-frequency, nonrespiration-related discharges. Recordings at different depths along each track enabled a determination of the dorsoventral extent of the silent area; the dorsoventral extent showed a maximum range from ~0.6 to ~0.8 mm at the level of the injection track and decreased at progressively increasing distances from it. Neuronal activities similar to those encountered in control trials were recorded along tracks performed at rostrocaudal distances ≈0.5 mm from the injection site. An example of these recordings has been reported in Fig. 6. Taken together, the results of these trials indicate that the inactivated regions have diameters ranging from 0.6 to 0.8 mm; i.e., very similar to those revealed by the marking dye or expected on the basis of theoretical calculations (35).
Examples of typical placements of the micropipette within three different medullary regions are shown in Fig. 7. Series of representative coronal sections of the medulla oblongata of the rabbit, showing the distribution of sites where bilateral KA microinjections induced changes in respiratory activity, are represented in Fig. 8.

Cardiovascular effects. Although our interest was mainly focused on respiratory effects, we will briefly deal with changes in MAP observed in our experiments. Both unilateral and bilateral KA microinjections into rostral sites of the VRG, which correspond to the BötC and pre-BötC, caused similar marked increases in MAP ranging from 21 to 90 mmHg. These pressor responses occurred immediately after the injections and reached a maximum within ~10 min. Maximum pressor responses were 27.6 ± 3.3 and 44.4 ± 4.1 mmHg after unilateral and bilateral KA microinjections, respectively (P always < 0.001). Arterial blood pressure progressively decreased and completely recovered within <30 min. Transient decreases in MAP, which reached a maximum (20.3 ± 4.5 mmHg; P < 0.005) within ~10 min, were observed after bilateral KA microinjections into the iVRG. Transient and inconsistent decreases in MAP were also seen after unilateral KA microinjections into the same area.
observed in the period immediately after the injections may also reflect transient activation of adjacent neuronal systems, as subtoxic levels of KA could diffuse beyond the lesion site (14). Accordingly, KA-induced changes in blood pressure are conceivably due to the transitory activation of neighboring medullary neuronal pools implicated in cardiovascular regulation, such as the rostral and caudal vasomotor areas (15, 28); these faded out within <30 min from the injection. Therefore, the role of baroceptive reflexes in the genesis of the KA-induced responses can be ruled out (15).

Respiratory responses induced by KA microinjections in the three rostral VRG subregions are rather similar; the main differences concern the presence and the level of tonic inspiratory activity during KA-induced loss of respiratory rhythmicity as well as the intensity of changes in some respiratory variables during the recovery period (see Table 2). Owing to the close proximity of these subregions, the spread of the injectate could have played a role in determining these similarities. However, we are confident that the respiratory effects were confined to circumscribed areas localized within the VRG. Injection sites were selected by using stereotaxic coordinates and especially extracellular recordings of respiratory neurons that are well known to be concentrated according to type in rather well-defined subregions of the VRG (5, 41, 62). Our extracellular recordings of neuronal activities allowed us to define accurately not only the BöC and the iVRG (5, 62), but also the pre-BöC region (see Fig. 1), where we encountered patterns of neuronal activities similar to those described in other animal species (see, e.g., Refs. 13, 41, 45, 56, 60). Furthermore, the localization of the injection sites was confirmed by the histological control (Fig. 7). Microinjections were performed, as far as possible, just in the middle of each selected subregion to avoid the spread of the injectate to adjacent structures. To restrict the spread of the injectate and thereby the number of neurons affected, relatively small volumes (20–30 nl) of KA solutions were injected. Theoretical calculations (35) suggest that volumes of 30 nl should spread <350 μm in any direction from the injection site. Accordingly, KA injections into neighboring regions >0.5 mm away from responsive sites, where intense multiunit respiratory activity was recorded, failed to induce the characteristic respiratory effects reported above. Our histological observations on the spread of the marking dye are in keeping with theoretical suggestions and provide evidence that we have lesioned circumscribed areas (35). Furthermore, the results of DLH or subsequent KA microinjections into KA-lesioned areas, and especially those of extracellular recordings performed within or close to the injected regions (see Fig. 6), indicate the presence of lesions with an extent comparable to that expected on the basis of the spread of the injectate. As already mentioned, subtoxic levels of KA could diffuse beyond the injection site and cause transient activation of adjacent neurons (14); only the respiratory responses observed immediately after the injections may reflect these excitatory phenomena, as also suggested by the

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**DISCUSSION**

The results of this study indicate that important components of the neural network underlying respiratory-rhythm generation are located in the rostral ventrolateral medulla. However, they do not allow us to ascribe to any lesioned area of the VRG an exclusive role in the generation of the eupneic pattern of breathing. In fact, present data suggest that the BöC, the pre-BöC, and the rostral portion of the iVRG have similar and potent influences on the respiratory frequency and intensity of the inspiratory motor output. KA produces profound metabolic alterations of cell bodies within 30 min while sparing axons of passage; affected neurons have been reported to cease firing within 10 min after the injections (14). However, we waited 30 min before evaluating the effects of KA microinjections (see, e.g., Refs. 16, 58). The effects

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**Fig. 5.** Respiratory changes induced by combined bilateral microinjections (30 nl) of 4.7 mM KA performed into the BöC and pre-BöC in the same preparation. A: dorsal view of the medulla oblongata of the rabbit indicating the regions where multiple KA microinjections (●) have been performed. AP, area postrema; cVRG, caudal ventral respiratory group; DRG, dorsal respiratory group. B: integrated Phr under control conditions, 60 min after bilateral KA microinjections into the BöC, and 120 min after subsequent bilateral KA microinjections into the pre-BöC region.

(13.8 ± 4.7 mmHg; P > 0.05). In all instances, blood pressure displayed stable values quite similar to those observed during control periods when evaluation of respiratory effects was commenced, i.e., 30 min after KA injections. No obvious or consistent changes in MAP were observed with KA microinjections into more caudal sites of the VRG.
time course of KA-induced changes in blood pressure observed in the present study. However, we started to evaluate changes in respiratory activity only 30 min after the completion of the injections.

The technique of KA microinjections has widely been used as a useful and reliable tool in the study of the respiratory network in vivo preparations (4, 18, 21, 22, 29, 56, 64). KA-induced respiratory effects were specific because control injections of equal volumes of the vehicle solution performed at the same responsive site did not alter respiratory activity. In addition, specificity is strengthened by the absence of significant respiratory responses to KA injections into neighboring regions 0.5 mm away from the three investigated VRG subregions, as well as by the lack of responsive sites in the cVRG. In fact, although some studies have shown that strong activation of caudal expiratory neurons may cause respiratory depression and alter the pattern of breathing (e.g., Ref. 6), the bulk of data from electrophysiological, morphological, and lesion studies indicate that neurons of this region are not involved in the respiratory rhythmogenesis (see, e.g., Ref. 54; for reviews, see Refs. 5, 62). Nevertheless, each injected area could lead to respiratory responses via synaptic interactions with the adjacent VRG subregions (for reviews, see Refs. 5, 57, 62). If these responses were due to the ablations of neurons in the circumscribed injected subregion, then they would exactly reflect the results that we were aiming for and, therefore, would be specific. Furthermore, neurons within the injected area are at first strongly activated and subsequently cease firing within 10 min after the injection (14). Thus respiratory effects could also be due to synaptic interactions of activated neurons with neighboring regions, where either inhibitory or excitatory short-lasting phenomena might be produced. In particular, excitatory phenomena in adjacent regions may produce local depolarization blocks of neurons. The duration of these blocks cannot be long lasting, because even depolarization blocks caused by microinjections of large volumes of excitatory amino acids at high concentrations have relatively short durations (26). We believe that respiratory responses induced by these synaptic interactions, if present, should have faded out well before the time at which the evaluation of KA-induced respiratory effects was commenced.

Lesions produced by KA microinjections have been reported to be irreversible (4, 14, 16). However, a progressive albeit partial recovery of eupneic patterns of breathing was observed in our experiments. At
present, the mechanisms underlying this behavior are not clear. The hypothesis can be advanced that readjustments, compensation, or plasticity phenomena in the respiratory network may subserve the recovery process of respiratory activity. In agreement with St. John (57), a general aspect of blockades or lesions of medullary regions leading to respiratory depression or apnea is that the magnitude of respiratory effects is...
greatest immediately after these experimental maneuvers (Ref. 56 also for further reference). Furthermore, because KA lesions were relatively small, they could have affected only part of each investigated subregion; the remaining neurons may have contributed to the recovery process. However, taking into account the relative extent of each investigated area, it seems conceivable that this may be relevant, especially to the iVRG and the BOtC, but rather unlikely to the pre-BOtC, owing to its very small size. We cannot at present exclude that an irreversible cessation of rhythmic respiratory activity could be induced by completely lesioning each of the investigated medullary subregions.

As we have stated above, we are confident that in most cases the whole pre-BOtC region was ablated by our KA microinjections. This is in obvious conflict with the view that the pre-BOtC is crucial for respiratory-rhythm generation in in vivo preparations of adult mammals (41). However, this contrast seems to be strongly attenuated by the fact that even authors who support the hypothesis that pre-BOtC pacemaker neurons constitute the kernel for respiratory-rhythm generation (47) have recently proposed that purely reciprocal inhibitory interactions could generate a stable respiratory rhythm in the absence of active pacemaker cells; under these circumstances, rhythmogenesis has been suggested (47) to occur as originally proposed by Richter et al. (42) and demonstrated by a number of computational models (see, e.g., Refs. 3, 5, 36, 44). Recently it has been suggested that the normal breathing rhythm in mammals is generated within the pre-BOtC by neurokinin 1 receptor (NK1R)-expressing neurons, which have been estimated to represent <10% of all pre-BOtC neurons (20). Bilateral destructions of >80% of NK1R neurons within the pre-BOtC and possibly other neighboring regions of unrestrained awake adult rats did not result in fatal apnea, but in an “ataxic” breathing pattern generally characterized by increases in frequency associated with an irregular sequence of inspiratory efforts of near normal amplitude interspersed with apnea or very low-amplitude inspiratory activity (20). Lesioned rats displayed altered blood gases and pH as well as abnormal responses to hyperoxia, hypercapnia, and hypoxia. Interestingly, no gasping was observed in response to severe hypoxia (for a discussion on the neurogenesis of gasping, see, e.g., Refs. 25, 40, 56, 57). On the basis of these results, Gray et al. (20) have suggested that normal breathing in mammals requires the presence of NK1R-expressing neurons of the pre-BOtC. In the absence of these neurons, an irregular rhythm sufficient to maintain life can still be produced in awake rats by undetermined structures, which may include, for example, non-NK1R-expressing neurons in the pre-BOtC as well as the VRG or other brain stem respiratory structures. Thus in agreement with the suggestions of Smith et al. (47), these conclusions seem to imply the possibility that a respiratory rhythm can be generated by interactions within the neuronal network in which kernel neurons are embedded, even when these neurons have been silenced or destroyed. The reasons of the discrepancies between present results and those of Gray et al. (20) are obscure but are unlikely due to differences in the animal species employed, because bilateral ablations of the pre-BOtC in decerebrate rats (56) proved to cause results consistent with those observed in the present study. The discrepancies may be at least partially accounted for by the great differences in the type of preparation employed and by the very selective lesions of NK1R neurons within the pre-BOtC and possibly neighboring regions performed by means of relatively large-volume microinjections (100–150 nl) in the study by Gray et al. (20). However, how these differences may have contributed to produce contrasting results is only a matter of speculation.

The finding of apneic responses, albeit transient, in all of the investigated subregions of the rostral VRG may indicate that we have operated on important components of the neuronal network subserving eupneic breathing (see Fig. 3). As far as the BOtC region is concerned, present results are in general agreement with those previously obtained in the cat with different types of lesions (53, 58) or focal cold blocks (10). However, the medullary areas involved in some of these studies extended well beyond the boundaries of the BOtC (10, 58). Persistent or transient apnea was induced by blockades or ablations of neuronal activities within the pre-BOtC or possibly corresponding medullary regions of cats and rats in some previous studies (10, 21–24, 29, 40, 56; see also Ref. 41), but contrasting results were obtained in other investigations (2, 18). The reasons for these discrepancies are not clear; they are probably related to the differences in the preparations and the experimental techniques used, as well as in the localization and extent of the lesioned or inactivated area. We believe that our results could contribute to disclose the role of these rostral VRG subregions because they have been obtained in the same animal species, with the same type of preparation, and the same technique. Apnea observed in response to KA injections into the iVRG may be due to the involvement not only of proprio-bulbar neurons of the medullary respiratory network but also of bulbospinal premotor inspiratory neurons (5, 62).

Despite the similarities in the results obtained in the different subregions, the arrest of the respiratory rhythm was combined with the appearance of relatively intense tonic phrenic nerve activity after KA lesions in the BOtC and pre-BOtC (see Fig. 3). The development of tonic activity has been reported to occur in the cat in response to focal cold blocks in the rostral regions of the ventrolateral medulla, including the BOtC (10). Although not specifically described, tonic inspiratory activity after KA or electrolytic lesions in these subregions can be recognized in the results of some other studies (see, e.g., Ref. 29, Fig. 4; Ref. 53, Fig. 3; Ref. 58, Fig. 4). A plausible interpretation of these findings is that the development of tonic inspiratory activity is due to the lack of the potent inhibitory influences on inspiratory activity arising from rostral expiratory neurons (7, 8; for a review see Ref. 5) that
are mainly concentrated in the BötC but are also encountered in the pre-BötC as shown in the present results (see Fig. 1) and described in previous reports (13, 41, 45, 56, 60). Accordingly, it has been shown that blockade of inhibitory synaptic activity within the pre-BötC of the cat causes the cessation of rhythmic respiratory activity and the appearance of tonic phrenic discharges (39). In agreement with the interpretation provided for these latter findings (39), we can also advance the hypothesis of a prominent role of pontine influences on the BötC and the pre-BötC in the genesis of tonic apnea that recalls very closely apneusis induced by the ablation of the pneumotaxic center (for a review, see Ref. 57). Pontine influences on respiration seem to be rather complex and involve both excitatory and inhibitory pathways (5, 11, 31, 39, 62); these influences can be mediated at least in part by afferent projections from parabrachial and Kölliker-Fuse nuclei to these two subregions of the VRG (see, e.g., Refs. 5, 11, 17, 19, 49, 57). Furthermore, we propose that pontine inhibitory effects on inspiratory activity can be exerted via synaptic actions on neurons located in the BötC and the pre-BötC (possibly involved in the inspiratory off switch) or in the inhibition of inspiratory neurons during the expiratory phase (see, e.g., Ref. 5). Removal of the target neurons may partly correspond to the ablation of the pontine pneumotaxic mechanisms and lead to tonic apnea or apneusis.

An important problem concerning the generation of eupnea (56, 57) is whether apneic regions are critical for rhythm generation or provide a tonic input necessary for eupnea to be expressed. First of all, the neuronal populations encountered in the injection sites did not display tonic discharge patterns, but rhythmic respiratory activities. In addition, KA-induced cessation of rhythmic respiratory activity was not reverted by increasing the tonic chemical drive by hypercapnia or hypoxia (see Fig. 4). These considerations suggest that we provoked lesions of rhythmic populations of respiratory neurons, which may constitute important elements of the respiratory CPG. Apnea or respiratory depression insensitive to increasing levels of arterial PCO₂ has already been reported to occur with cold blocks or lesions in the BötC and pre-BötC regions of cats and rats (10, 56, 58). However, a eupneic rhythm recovered in response to the activation of peripheral chemoreceptors during apnea induced by KA in the pre-BötC of the rat (56). We cannot rule out that apnea ensued because KA-lesioned areas are involved in CO₂ and O₂ chemoreception or in the transmission and integration of chemical drive inputs (see, e.g., Refs. 10, 34, 38, 51, 52, 59; for a review see Ref. 33). However, because lesioned areas were relatively small, whereas multiple sites for central chemoreception are widely distributed in the brain stem (33), a resumption of rhythmic activity could be expected in response to both hypoxic and hypercapnic stimulation. In the absence of such recovery, a disruption of important connections in the neural network subserving respiratory-rhythm generation seems plausible. The question of the neurogenesis of gasping (see, e.g., Refs. 25, 40, 56, 57) was not addressed in this research; moreover, our hypoxic stimulations probably were not sufficiently long to induce gasping in the rabbit.

Apneic effects, although dramatic, were reversible; an interesting finding was the increase in respiratory frequency and the decrease of peak phrenic amplitude observed during the recovery process (see Fig. 3 and Table 2). These changes in respiratory activity are consistent with the results of unilateral KA microinjections and with those of some previous studies performed on other animal species. Increases in respiratory frequency and reductions in peak phrenic amplitude have been reported to occur during unilateral focal cold blocks in the rostral ventrolateral medulla (10), as well as after unilateral or bilateral KA or electrolytic lesions of the BötC or the rostral VRG (29, 58, 64) and after unilateral tetrodotoxin microinjections into the pre-BötC (40). Recent results obtained in decerebrate cats using excitatory amino acid receptor antagonists (1) suggest that the VRG contains various subregions that differentially affect timing and intensity components of the breathing pattern. Although excitatory amino acid receptor blockades do not appear to be directly comparable with KA lesions, it seems relevant to the present discussion that microinjections of a specific non-N-methyl-D-aspartate (NMDA) receptor antagonist into the iVRG induced transient apnea, followed by increases in respiratory frequency associated with reductions in peak phrenic amplitude. Furthermore, NMDA-receptor antagonism within the same area caused only increases in respiratory frequency. This subregion may correspond at least partially to the portion of the iVRG lesioned by means of KA microinjections in the present study.

The recovery of a eupneic pattern of breathing could suggest that the neural structures affected by KA injections do not have a crucial role in the respiratory rhythmogenesis. However, it could be worth recalling that KA lesions could have affected only partially each investigated region. We did not perform injections of larger volumes to avoid confounding effects due to the involvement of neighboring areas, especially those located in adjacent subregions of the VRG. Nevertheless, combined KA lesions in two adjacent subregions produced apneic responses of much longer duration that were possibly irreversible (see Fig. 5). On the other hand, it does not seem surprising that the extent of the lesioned VRG area may be of importance in determining the arrest of the respiratory rhythm and its duration. Present findings could also suggest the possibility that multiple brain stem regions and, in particular, pontine structures play a role in the generation of the eupnic pattern of breathing (56, 57). Independent respiratory rhythms can be recorded on each side of the brain stem after complete mid sagittal transections (e.g., Refs. 5, 62). In addition, caudal-to-rostral brain stem transections have demonstrated that trigeminal motoneurons continue to discharge rhythmic bursts after a separation of the medulla from the pons; these results suggest that pontine mechanisms might have an important role in the genesis of the respiratory
rhythm (for a review, see Ref. 57). However, the relationships of pontine rhythms to eupnea are unknown. Present results more strictly suggest that the different subregions of the rostral VRG are part of the respiratory CPG. In fact, the rostral VRG subregions appear to control not only the amplitude but also the frequency of inspiratory bursts (12, 39, 55, 62). Respiratory responses to unilateral KA microinjections are in agreement with this interpretation (see Fig. 2 and Table 1). It is also worth noting that VRG subregions more rostrally located have a more prominent role in this control (Table 2).

An attempt can be made to explain the genesis of the rather similar respiratory changes in response to KA lesions in the BötC, pre-BötC, and more rostral portion of the iVRG on the basis of current models of respiratory rhythmogenesis (see, e.g., Refs. 3, 5, 36, 42–44, 47). These models are based on reciprocal inhibitory interactions between different types of propriobulbar neurons and, as more recently suggested (see, e.g., Refs. 43, 47), on a combination of a network of inhibitory interneurons and excitatory pacemaker-like neurons with intrinsic oscillatory bursting properties (hybrid pacemaker-network model). However, these models do not display a high level of anatomic specificity: in most cases, they concern functionally differing groups of neurons without taking into account an exact localization within VRG subregions. In addition, the models do not include in the respiratory network the pons, which (as already mentioned) has been suggested to have an important role in the genesis of eupnea (57). Thus it is rather hard to explain present results in the light of these models. Nevertheless, some general considerations can be of some help for this purpose. Interneurons involved in the respiratory rhythmogenesis are inhibitory (GABAergic or glycineric) and receive inhibitory inputs (see, e.g., Refs. 5, 37, 43, 47); they are distributed prevalently in the BötC and pre-BötC but have been also encountered in the adjacent iVRG (e.g., Refs. 5, 13, 37, 45, 47, 60 also for further references). The importance of inhibitory interactions in the respiratory-rhythm generation is well documented, especially in adult animals (e.g., Refs. 43, 47); network rhythmicity may depend on reciprocal inhibition and postinhibitory rebound that shape the final alternating pattern (e.g., Refs. 27, 47). In addition, as already mentioned, even in the hybrid pacemaker-network model, purely reciprocal inhibitory interactions could generate a respiratory rhythm in the absence of activity in the kernel pacemaker cells (47). Inhibitory interactions and postinhibitory rebound are involved in the synchronization of neuronal systems (e.g., Refs. 9, 61 also for further references); on the other hand, desynchronization of neuronal oscillating networks by blockade of inhibitory synapses leads to reductions in the intensity of output bursts and increases in frequency (e.g., Ref. 9 also for further references). On the basis of these arguments, we can speculate that removal of inhibitory interneurons, which are mainly concentrated in the BötC, pre-BötC, and adjacent iVRG (5, 37, 39, 47), may produce at first the arrest of respiratory rhythm and subsequently after readjustments and compensation phenomena in the respiratory network, a resumption of breathing patterns characterized by decreases in peak phrenic amplitude and increases in respiratory frequency. These patterns of breathing are possibly due to the loss of part of inhibitory interneurons and reduced strength of inhibitory synapses (see also Ref. 44), which cause desynchronization of the respiratory network. We have already provided some comments on the KA-induced apneic responses; in accordance with the above-mentioned considerations, they are probably due to the sudden deficit of inhibitory mechanisms and might represent a level of network desynchronization beyond which the respiratory rhythm cannot be generated.

In conclusion, present findings show that all of the investigated rostral subregions of the VRG exert a potent control on the respiratory frequency and intensity of the inspiratory motor output, thus suggesting that they are important components of the neural network responsible for the generation of the eupneic pattern of breathing. We believe that it is unlikely that a vital function such as respiration would rely on a CPG confined to a small medullary area. Rather, we agree with the view that the respiratory CPG is a distributed neural system characterized by a great deal of redundancy (63).

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REFERENCES


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