Midbrain and medullary control of postinspiratory activity of the crural and costal diaphragm in vivo

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Subramanian HH, Holstege G. Midbrain and medullary control of postinspiratory activity of the crural and costal diaphragm in vivo. J Neurophysiol 105: 2852–2862, 2011. First published March 30, 2011; doi:10.1152/jn.00168.2011.—Studies on brain stem respiratory neurons suggest that eupnea consists of three phases: inspiration, postinspiration, and expiration. However, it is not well understood how postinspiration is organized in the diaphragm, i.e., whether postinspiration differs in the crural and costal segments of the diaphragm and what is the influence of postinspiratory neurons on diaphragm function during eupnea. In this in vivo study we investigated the postinspiratory activity of the two diaphragm segments during eupnea and the changes in diaphragm function following modulation of eupnea. Postinspiratory neurons in the medulla were stereotaxically localized extracellularly and neurochemically stimulated. We used three types of preparations: precociously decerebrated unanesthetized cats and rats and anesthetized rats. In all preparations, during eupnea, postinspiratory activity was found in the crural but not in the costal diaphragm. When eupnea was discontinued in decerebrate cats in which stimulation in the nucleus retroambiguus induced activation of laryngeal or abdominal muscles, all postinspiratory activity in the crural diaphragm was abolished. In decerebrate rats, stimulation of the midbrain periaqueductal gray abolished postinspiration in the crural diaphragm but induced activation in the costal diaphragm. In anesthetized rats, stimulation of medullary postinspiratory neurons abolished the postinspiratory activity of the crural diaphragm. Vagal nerve stimulation in these rats increased the intensity of postinspiratory neuronal discharge in the solitary nucleus, leading to decreased activity of the crural diaphragm. These data demonstrate that three-phase breathing in the diaphragm during eupnea exists in vivo and that postinspiratory neurons have an inhibitory effect on crural diaphragm function.

THE DIAPHRAGM MUSCLE is the prime mover of tidal air and consists of a crural and a costal segment. Both segments are innervated by the phrenic nerve, with separate branches to the crural and costal segments (Gordon and Richmond 1990; Hammond et al. 1989; Pickering and Jones 2007; Sant’Ambrogio et al. 1963). With regard to its mechanical action on the ribcage, the diaphragm functions as a single muscle (Goldman and Mead 1963). With regard to its mechanical action on the ribcage, the diaphragm functions as a single muscle (Goldman and Mead 1963). However, during quiet breathing (eupnea) or during expulsive acts involving laryngeal or abdominal excitation (such as vocalization, swallowing, vomiting, parturition, or retching), it is not known whether the crural and costal segments function as a single entity or as two different muscles. According to gastrointestinal physiologists, activation of the diaphragm outside the inspiratory cycle prevents the gastric contents from refluxing into the esophagus (Pickering and Jones 2002). Separate examination of the crural and costal diaphragm function during eupnea and subsequent changes of eupnea is required to understand their motor control. An important investigative approach is to examine the postinspiratory function in both diaphragm segments. Studies on brain stem respiration-related neurons suggest that eupnea consists of three phases: inspiration, stage-1 expiration, and stage-2 expiration (Dutschmann and Herbert 2006; Dutschmann and Paton 2002; Paton 1996; Remmers et al. 1986; Richter 1982; Richter and Ballantyne 1983; Richter and Spyer 2001; Richter et al. 1987; von Euler 1893). The stage-1 expiration is commonly referred to as postinspiration and is viewed as a critical phase for both maintenance of eupneic rhythm (Richter 1982; Richter and Spyer 2001; Richter et al. 1987) and modulation of eupnea (Dutschmann and Herbert 2006; Richter et al. 1987; see also reviews by Mörschel and Dutschmann 2009; Rybak et al. 2004, 2007; Smith et al. 2007) during expulsive behavioral acts. Neurons classified as postinspiratory neurons, located in three brain stem regions, the dorsolateral pons (Dutschmann and Herbert 2006; Dick et al. 1994; Kobayashi et al. 2005), the rostral ventrolateral medulla (VLM) (Parkes et al. 1994; Richter et al. 1987), and the nucleus of the solitary tract (NTS) (Subramanian et al. 2007) are hypothesized to mediate the postinspiratory phase (Dutschmann and Herbert 2006; Mon- teau et al. 1985; Richter 1982; Richter and Spyer 2001; Richter et al. 1987; see also reviews by Mörschel and Dutschmann 2009; Rybak et al. 2004, 2007; Smith et al. 2007). It is not well understood how postinspiration is organized in the crural and costal diaphragm during eupnea in vivo and during changes to eupnea. Postinspiratory activity of the diaphragm has indeed been reported in rabbits (D’Angelo et al. 2010), cats (Orem and Anderson 1996), and dogs (Easton et al. 1993, 1995, 1999) as well as in the external intercostals, as shown by Berdah and De Troyer (2001), and in humans (Citterio and Agostoni 1986; Hodges and Gandevia 2000; Hodges et al. 1997); however, these observations were during sleep-wake states, hypoxia, hypercapnea, mechanical loading, lung volume changes, and postural maintenance, but not during eupnea. These findings and their interpretations are compounded by species-specific differences in the muscle-fiber architecture and histochemistry of the diaphragm (Gordon et al. 1989; Keith 1905). Moreover, the electromyograms (EMGs) in rabbits, cats, and humans were obtained mainly from the costal and not from the crural...
diaphragm. For these reasons, we investigated in vivo, in cats and rats, whether both crural and costal segments of the diaphragm exhibit activity during the postinspiratory period. Furthermore, we studied whether interference of eupnea has any effect on the postinspiratory component of the diaphragm. This was undertaken via stimulation of the midbrain periaqueductal gray (PAG), a known behavioral respiratory modulator (Subramanian and Holstege 2010; Subramanian et al. 2008), and the nucleus retroambiguus (NRA), a critical relay of the PAG (Holstege 1989; Subramanian and Holstege 2009) concerning laryngeal and abdominal motor pathways. Lastly, we also examined the influence of medullary postinspiratory neurons on diaphragm function during eupnea.

METHODS

Experimental Protocol

Both cats and rats were used for this study. Data were collected from the same unanesthetized spontaneously breathing precocically decerebrated adult cats (n = 6) as used in previous investigations (Subramanian and Holstege 2009; Subramanian et al. 2008). Two types of rat preparations (Sprague-Dawley rats of either sex weighing 350 – 450 g) were used: unanesthetized spontaneously breathing predecerebrated rats (n = 6) and anesthetized spontaneously breathing rats (n = 12). In all animals, the crural diaphragm EMG was recorded. In decerebrate cats, the effect of laryngeal and abdominal muscle activation (via microstimulation of the NRA) on the postinspiratory function of the crural diaphragm was investigated. In decerebrate rats, the effect of microstimulation in the midbrain PAG on the postinspiratory function of the crural diaphragm was investigated. In anesthetized rats, neurons that showed activation during postsirpiration (designated as postinspiratory neurons) were stereotaxically localized in the area rostral to the NRA in the VLM and in the NTS. In anesthetized rats, the effect of stimulation of these postinspiratory neurons on the crural diaphragm was investigated. In another set of anesthetized rats, the effect of stimulation of the vagal nerve on these postinspiratory cells and on the crural diaphragm was examined. Experiments were undertaken at the University of Sydney, and approval was obtained from the institutional Animal Care Ethics Committee. At the end of the experiments, the animals were euthanized by intravenous administration of bolus of sodium pentobarbital via the femoral vein.

Anesthesia

Cats were anesthetized in a box filled with a mixture of isoflurane and oxygen delivered by a ventilation pump. After induction, the anesthesia was maintained through a facemask, and femoral arterial and venous catheters were inserted. With the use of the intravenous catheter, sodium thiopental (10–20 mg/kg) was injected to enable the insertion of an endotracheal catheter, which was then used for the administration of isoflurane. Rats were initially anesthetized with sodium pentobarbitone (Nembutal; 70 mg/kg ip), and catheters were placed in the femoral artery and vein for monitoring blood pressure and administration of fluids, respectively. Supplementary doses of anesthesia (5 mg·kg⁻¹·h⁻¹ iv) for rats were given during the experimental period as required. Throughout the period of experimentation, the level of anesthesia was monitored by measuring blood pressure and nociceptive and corneal reflexes.

Decerebration

The method of precocicular decerebration was the same for both cats and rats. After induction of anesthesia and cannulation of the femoral artery and vein, the animal’s head was secured in a stereotaxic frame with the body suspended from the frame by straps. To allow access to the midbrain, we drilled two burr holes in the skull on either side of the sagittal sinus. The dura was then incised, and the medial part of the cortex was suctioned. After ligation and removal of a portion of the sagittal sinus, precocicular decerebration was carried out using suction diathermia, a surgical technique that uses pulsations of electrical energy to generate heat and cauterize blood vessels to prevent excessive bleeding while suctioning brain tissue. Bleeding during decerebration was reduced by lowering the mean arterial pressure from 100–130 to 65–70 mmHg by raising the level of anesthesia. All brain tissue rostral to the superior colliculus, including the entire diencephalon, was removed. After completion of decerebration, anesthesia was discontinued, because decerebration rendered both cats and rats insensitive to pain (Silverman et al. 2005). After decerebration, the animals started breathing spontaneously and the mean arterial pressure returned to 100–130 mmHg within 30–60 min. Fluid supplements were administered via the femoral intravenous catheter. Blood pressure was continuously monitored via the catheter placed in the femoral artery in all animals. Tracheal pressure was recorded through a 19-gauge needle inserted in the trachea. Fluid supplements were administered via the femoral venous catheter. Arterial PCO₂ and P₂O₅ were intermittently monitored. A thermostatic infrared lamp was employed to maintain the animal’s body temperature at 37.5–38.5°C.

EMG Recording From Diaphragm and Other Muscles

Fine wire electrodes (bipolar Teflon-coated, 7-strand, 110-μm wires; A-M Systems, Everett, WA) were used for EMG recordings. In all cats and rats, the wire electrodes were surgically implanted into the same crural region of the diaphragm in the same geometric pattern, perpendicular to the long axes of the diaphragm muscle (Fig. 1). This means that the postinspiratory activity seen in the diaphragm EMG signal in this study always originated from the same crural portion of the diaphragm. EMGs of the costal diaphragm and internal intercostal muscle were also recorded in all animals. EMG recordings were also made from the cricohyloid and the external oblique abdominal muscles in the decerebrate cats.

Power and Amplitude Spectral Analyses of the Crural Diaphragm

The power and amplitude spectra for the crural diaphragm were computed on 0.5-s time windows positioned at 0.7 s before
beginning of inspiration. For each signal, the power and amplitude
density were computed for each of 10 breaths and averaged. The
spectra were normalized to the largest individual breath within 10
breaths of control (Konrad 2005; Merletti and Parker 2004).

Technique of Chemical Microstimulation of the Midbrain and Brain Stem Areas

Excitatory amino acid [D,L-homocysteic acid (DLH), 50 mM in 20
mM phosphate buffer (pH 7.4)] microinjections (Fries and Zieg-
gansberger 1974; Goodchild et al. 1982) of very small volumes
(10–30 nl) were used for stimulation of various brain regions in both
cats and rats. A pressure microdelivery system (Picopipette II; Parker
Instrumentation) was used. The volume injected was determined using
the movement of the meniscus on a precalibrated scale. Injections
were made with intervals of 25 min to eliminate any residual effect of
the previous injection. Control microinjections with saline (10–30 nl)
were made to determine any volume-related effect of the microinjec-
tions. Stereotaxic microinjections in cats were based on Berman’s cat
stereotaxic atlas (1968) and in rats, on Paxinos and Watson’s rat brain
atlas (1997).

Chemical Stimulation in the NRA in the Decerebrate Cat

In six cats, microinjections (10–30 nl) were made within the NRA
along its rostrocaudal axis. The injections were delivered via single-
barrel micropipettes (tip diameters 10–30 μm; filled with DLH) that
were inserted into the caudal VLM. Rhodamine beads were added to
the DLH solution to later determine the precise location of the
injection sites.

Chemical Stimulation in the PAG in the Decerebrate Rat

Single-barrel micropipettes filled with DLH were inserted into the
various regions of midbrain PAG. A solution of wheat-germ aggluti-
nin conjugated with horseradish peroxidase was mixed with the DLH
solution for later histological identification of the stimulation sites.

Chemical Stimulation of the Ventrolateral and Dorsal Medulla in the Anesthetized Rat

In eight rats, double-barrel micropipettes (tip diameters 60–70 μm;
one filled with DLH and the other filled with 3 M NaCl) were inserted
in the rostral VLM and in the NTS, where postsynaptic neurons
were stereotaxically localized. Chemical stimulation was undertaken
through the DLH pipette. Rhodamine beads were added to the DLH
solution to later determine the precise location of the injection sites.

Extracellular Recording of Postinspiratory Neurons in the Anesthetized Rat

Micropipettes (in the double-barrel cluster) filled with 3 M NaCl
(direct-current impedance ~8–10 MΩ) were used to localize postins-
spiratory neurons in the rostral VLM and in the NTS region. The
double-barrel electrode technique allowed for both extracellular re-
cording of the cell (via the NaCl barrel) and verification that the
recordings were obtained from the cell bodies and not from fibers of
passage. This verification was done by a standard electrophysiological
testing for extracellular recordings (Fries and Ziegelsnanger 1974;
Goodchild et al. 1982), i.e., by microstimulation via injection of 3–6
nl of DLH through the DLH barrel while simultaneously recording via
the NaCl barrel from the same cell and checking whether this cell
showed excitation as a result of DLH administration. The same
method was used for stimulation with simultaneous recording of
postinspiratory neurons in the rostral VLM. The recording sites were
marked with rhodamine beads through the DLH pipette.

Vagus Nerve Stimulation in the Rat

The right vagus nerve was dissected at the cervical level and cut.
The proximal end was stimulated using a pair of Teflon-coated
stainless steel wire electrodes. The Teflon was cleared for ~2 mm
from the end, and the wires were wrapped around the nerve to form
a cuff. Electodes were appropriately placed to prevent anodal block.
Stimulus parameters were 3–5 V and 7–10 Hz with a duration of 1 ms.

Data Analysis

The band-pass filtered (0.1–5 kHz) EMG activity of the crural and
costal diaphragm, cricothyroid, internal intercostal, and external
oblique abdominal muscles, as well as the tracheal pressure and mean
arterial pressure, were recorded on the pulse code modulator (PCM;
A. R. Vetter) and played back for analysis using the PCM/MacLab/
Apple system. Chart software (AD Instruments) running on an Apple
computer was used for recording and playing the signals. The Chart
software was also used for selection of the sampling rate of 20,000/s.
The recording started at least 60 s before the administration of DLH.
After the injection, the recording continued for at least 5 min. All the
muscle activities were rectified and averaged within and across ani-
mals. The raw diaphragm EMG signal was used for measurement of
inspiratory (Ti) and expiratory (Te) durations and respiratory fre-
quency (RF). The duration from the onset of the diaphragm signal to
its offset was defined as Ti. The duration between the offset of the
diaphragm and its subsequent onset was defined as Te. The exact
points of onset and offset of inspiration were determined via compari-
son of the diaphragm EMG signal with internal intercostal EMG or tracheal
pressure signal. Statview software was used for computation of Ti, Te,
and RF. Statistical comparisons were carried out using analysis of
variance (ANOVA). Significant differences between the mean values
were detected using Scheffé’s least different test, and a probability of
P < 0.05 was considered significant. Chart records have been
transformed into MacDraw Pro software for data representation.

RESULTS

Normal respiratory and cardiovascular values for unanesthe-
etsically decerebrated cats and rats and for anes-
ethetized rats are shown in Table 1. All animals had intact vagal
nerves and were spontaneously breathing and not tracheo-
tomized.

Decerebrate Cats

Postinspiratory activity of the diaphragm during eupnea.
All cats had postinspiratory activity in the crural diaphragm (Fig. 2).

Table 1. Normal respiratory and metabolic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Decerebrate Cats</th>
<th>Decerebrate Rats</th>
<th>Anesthetized Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti, s</td>
<td>0.65 ± 0.05</td>
<td>0.30 ± 0.05</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>Te, s</td>
<td>1.05 ± 0.10</td>
<td>0.45 ± 0.10</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>TPl, %</td>
<td>10 ± 5</td>
<td>5 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>RF, breaths/min</td>
<td>35 ± 5</td>
<td>80 ± 5</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>BP, mmHg</td>
<td>100 ± 5</td>
<td>105 ± 5</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>195 ± 5</td>
<td>400 ± 5</td>
<td>380 ± 5</td>
</tr>
<tr>
<td>Arterial blood pH</td>
<td>7.37 ± 0.05</td>
<td>7.39 ± 0.05</td>
<td>7.40 ± 0.01</td>
</tr>
<tr>
<td>PO2, Torr</td>
<td>24.9 ± 5.0</td>
<td>25.2 ± 5.0</td>
<td>35.2 ± 3.0</td>
</tr>
<tr>
<td>PO2, Torr</td>
<td>108.5 ± 10.0</td>
<td>110.5 ± 10.0</td>
<td>120.5 ± 5.0</td>
</tr>
</tbody>
</table>

Eupneic respiratory and metabolic parameters for precollicularly decere-
bated unanesthetized cats and rats and pentobarbiturate-anesthetized rats. All
animals were nontracheoanestomized and spontaneously breathing. Measurements
were from 6 decerebrate cats and rats and 12 pentobarbiturate-anesthetized
spontaneously breathing rats. Values are means ± SE. Ti, inspiratory duration;
Te, expiratory duration; TPl, postinspiratory duration; RF, respiratory fre-
quency; BP, blood pressure; HR, heart rate.
This postinspiratory activity was even more discernable when the diaphragm EMG was compared with the internal intercostal muscle EMG. The postinspiratory activity lasted for 10–100 ms of the total expiratory duration. Between the cessation of inspiration and onset of postinspiration, a pause in activity of the crural diaphragm was evident. The pause was in the order of 10–40 ms, variable from breath to breath. The onset of the postinspiratory activity correlated with the onset of the increase in tracheal pressure. The costal diaphragm showed activation similar to that of the crural diaphragm but did not show any postinspiratory activity during eupnea (Fig. 2). Figure 3 illustrates the power and amplitude density function of the crural diaphragm during its inspiratory and postinspiratory periods. For the crural diaphragm, the major peak in the power and amplitude density spectra occurred within the range of 200–600 Hz during eupneic inspiration and in the range of 800–850 Hz during postinspiration.

Postinspiratory activity during NRA stimulation. NRA stimulation 3–5 mm and 5–6 mm caudal to the obex led not only to cricothyroid muscle excitation (n = 6) and abdominal muscle excitation (n = 6), respectively, but also to the abolishment of the postinspiratory activity of the crural diaphragm. After cricothyroid and abdominal muscle activation, a postinspiratory pause of 50–100 ms occurred during 3–5 breaths, before prestimulus breathing with normal postinspiratory activity (with a 10- to 40-ms pause) resumed. NRA stimulation 3–5 mm caudal to the obex led to cricothyroid muscle excitation (Fig. 4) and an increase of the RF from 38 ± 50 to 65 ± 5 breaths/min (P < 0.05). It led to a decrease in both Ti, from 650 ± 50 to 400 ± 50 ms (P < 0.05), and Te, from 900 ± 50 to 450 ± 50 ms (P < 0.05). NRA stimulation 5–6 mm caudal to the obex led to activation of abdominal muscles (Fig. 5) and a decrease in RF from 40 ± 3 to 32 ± 3 breaths/min (P < 0.05). This decrease was caused by a decrease in Ti, from 600 ± 50 to 200 ± 50 ms (P < 0.05), and an increase in Te, from 850 ± 50 to 1,500 ± 500 ms (P < 0.05), respectively.

Decerebrate Rats

Postinspiratory activity of the diaphragm during eupnea. Postinspiratory activity was also observed during eupnea in the crural diaphragm in decerebrated rats (n = 6), although it was not always present breath to breath. The postinspiratory activity lasted for 5 ± 2% of the expiration time. A postinspiration pause was seen in 3 of the 6 animals and had a range in the order of 50–100 ms. The tracheal pressure correlation with the postinspiratory function, as seen in the cat, was not observed in rats. Similarly to that in cats, the costal diaphragm did not exhibit any postinspiratory activity.

Postinspiratory activity during PAG stimulation. Stimulation in the dorsal PAG in rats (n = 6) led to the abolishment of...
postinspiratory activity in the crural diaphragm but induced strong postinspiratory activity in the costal diaphragm, which continued throughout half of the expiratory phase (Fig. 6). The RF increased from 85 ± 4 to 115 ± 10 breaths/min (P < 0.05) because there was a decrease in both Ti, from 250 ± 50 to 100 ± 50 ms (P < 0.05), and Te, from 500 ± 50 to 400 ± 50 ms (P < 0.05).

**Anesthetized Rats**

**Postinspiratory activity of the diaphragm during eupnea.** In the anesthetized rat, the postinspiratory activity in the crural diaphragm EMG was only discernable when it was compared with that in an expiratory muscle, in this case, the internal intercostal muscle EMG, because postinspiratory pauses as seen in the decerebrate preparations were not observed (Fig. 7).

**Localizing postinspiratory neurons in VLM and NTS.** Postinspiratory neurons (n = 14) were stereotaxically localized and recorded from the rostral VLM (at 0.25–1.0 mm rostral to the obex, 1.5–2.0 mm lateral to the midline, and 1.5 mm below the dorsal surface) (Fig. 8B) and from the medial part of the NTS (at 0–1 mm rostral to the obex, 0.2 mm lateral to the midline, and 1 mm below the dorsal surface) (Fig. 8A). During eupnea in both the VLM and NTS, these postinspiratory cells showed abrupt onset at the end of inspiration, just before the offset of the crural diaphragm EMG, and continued to discharge 120–200 ms with a decrementing frequency (Fig. 8C).

**Effect of stimulation of VLM postinspiratory neurons on the crural diaphragm.** After excitatory amino acid stimulation (n = 8) of the postinspiratory neuronal recording site in the VLM (0.5 mm rostral to the obex, 2.0 mm lateral to the midline, and 1.5 mm below the dorsal surface), the cell firing increased from 22 ± 1 to 39 ± 1 spikes/s (P < 0.01) and continued to discharge throughout the expiratory phase. Stimulation attenuated the crural diaphragm EMG (Fig. 9), shortening the inspiration time from 400 ± 50 to 200 ± 50 ms (P < 0.05) and extending the expiration time from 500 ± 50 to 700 ± 50 ms (P < 0.05), leading to a virtually unchanged respiratory frequency (70 ± 5 breaths/min; P < 0.05).
Effect of vagal nerve stimulation on NTS postinspiratory neurons and the crural diaphragm. Electrical stimulation of the central end of the cut left vagal nerve (7 Hz, 5V) elicited the characteristic Breuer-Hering reflex, shortening inspiration and extending expiration. Vagal stimulation also extended the activation period of the postinspiratory neurons \((n = 6)\) (Fig. 10), with the cells continuing to discharge with a decrementing frequency discharge pattern. There was a complete suppression of the crural diaphragm EMG immediately following vagal stimulation and a phasic activation of the postinspiratory neuron. After the end of the stimulation, the diaphragm activity resumed at a reduced level, whereas the extent of the postinspiratory neuronal discharge still spanned almost the entire expiratory phase. Discontinuation of the vagal stimulation resulted in an immediate return of activation of the crural diaphragm and the postinspiratory cell as before vagal stimulation.

DISCUSSION

In cats and rats, postinspiration is evident only in the crural and not in the costal diaphragm during eupnea. Costal diaphragm activity during expiration has been demonstrated in

**Fig. 6.** Effect of injection of 20 nl of DLH in the lateral periaqueductal gray (PAG) in a decerebrate unanesthetized, nontracheotomized, spontaneously breathing rat. The stimulation resulted in costal diaphragm discharging into the expiratory phase but inhibited the postinspiratory component of the crural diaphragm. The modulation of eupnea was characterized by increases in the amplitude of both the crural and costal diaphragm. The photomicrograph shows a coronal section of the PAG, illustrating the micropipette tract marked by the tetramethyl benzidine reaction of the wheat-germ agglutinin-horseradish peroxidase deposit.

**Fig. 7.** Simultaneous recording of crural diaphragm and internal intercostal muscle EMG during eupnea in a anesthetized spontaneously breathing rat. The crural diaphragm EMG activity is compared with internal intercostal EMG activity while identifying its postinspiratory discharge. The breaths showing postinspiratory activity are marked to make a comparison with the decerebrate cat wherein postinspiratory activity in the crural diaphragm was seen in all breaths during eupnea (as shown in Fig. 2).
humans, but possibly only during postural maintenance, because Citterio and Agostoni (1986), Hodges et al. (1997), and Hodges and Gandevia (2000) observed this postinspiratory activity in the costal diaphragm particularly in humans in a sitting position and during postural adjustments. In chronically implanted dogs, during eupnea, postinspiration was seen in the crural and not in the costal diaphragm (Easton et al. 1999). However, this crural postinspiratory activity was mainly present when the dogs were lying in the right lateral decubitus position and was absent when the dogs were standing, signifying the involvement of posture. Our experiments exclusively represent the state of eupnea, because the effect of posture is eliminated. The data on the crural diaphragm confirm that three-phase breathing is not only a neurorespiratory event (Richter 1982; Richter and Spyer 2001) but also a respiratory motor act during eupnea. Our findings complement previous postinspiratory observations made in various respiration-related nerves such as the phrenic, intercostal, laryngeal (Oku et al. 1993; St John and Zhou 1989), and vagus nerves (Paton 1996).

**Differences in the Diaphragm Construction and Function Across Species**

Although the diaphragm is one of the most intensively examined muscles in mammals, there is little agreement on its construction and function. There exist contradictory anatomic descriptions (muscle-fiber architecture, histochemistry, and end-plate distribution) of the diaphragm across mammalian species (Gordon et al. 1989; Keith 1905). For example, multiple discontinuous end-plate fiber banding is seen in cat and dog diaphragms, whereas rat and rabbit diaphragms have only a single continuous end-plate band. Although the proportion of fiber types differs in the crural and costal segments in the dog, these proportions are similar in the two segments in cats and rats. These differences could account for differences in the synchronicity of activation. For example, in our study in cats and rats, during eupnea, the onset of crural and costal diaphragm activity occurred at the same time. In the dog, on the other hand, the crural diaphragm was activated earlier than the costal diaphragm (see Easton et al. 1993, 1995, 1999), suggesting that the dog’s diaphragm consists of two separate muscles (De Troyer et al. 1981). The construction of the diaphragm could also reflect on its postinspiratory activation during eupnea. Postinspiration was evident in the crural, but not in the costal, diaphragm in cats and rats. In the anesthetized rabbit, however, postinspiration was found in both the crural and costal diaphragm (D’Angelo et al. 2010). Another interesting finding is that in the decerebrate cat, postinspiratory pause was seen breath to breath, which was not the case in the decerebrate rat. This could be due to the smaller fiber length of the rat diaphragm.

**Postinspiratory Function of the Crural Diaphragm Does Not Correspond With Esophageal Reflux**

The crural diaphragm is an extrinsic component of the gastroesophageal barrier. This pinchcock action of the diaphragm protects against reflux induced by sudden increases in intra-abdominal pressure (Patti et al. 1997). In our study, during eupnea, postinspiration was neither preceded nor accompanied by sudden changes to intra-abdominal pressure. Hence, the postinspiratory discharge observed in our study in the
crural diaphragm does not correspond with the gastroesophageal reflux, as suggested by Pickering and Jones (2002, 2007).

Stimulation of the NRA Abolishes the Postinspiratory Component of the Crural Diaphragm

Subramanian and Holstege (2009) showed that selective stimulation of the nucleus retroambiguus along its rostrocaudal axis produced laryngeal or abdominal muscle activation. According to the present study, this NRA stimulation abolishes the postinspiratory component of the crural diaphragm, because the respiratory motor output changes. Cricothyroid muscle activation by way of rostral NRA stimulation goes together with increases in RF and diaphragm amplitude or tachypnea. On the other hand, caudal NRA stimulation inducing abdom-
inal activation goes together with forced expiration and a decrease in RF. Poststimulus recovery is marked by a breath-to-breath changing postsynaptic pause. These data suggest that multiple gating mechanisms are involved in the conversion of three-phase into two-phase breathing in the crural diaphragm, although such a conclusion on network reconfiguration would require the investigation of cranial postsynaptic motor output in parallel. Apart from elimination of postsynaptic, NRA stimulation does not induce differential activation of the crural and costal diaphragm.

**Midbrain Stimulation Differently Affects Crural and Costal Diaphragm**

The PAG is a powerful respiratory modulator (Hayward et al. 2003; Huang et al. 2000; Iigaya et al. 2010; Subramanian et al. 2008; Zhang et al. 2007) and organizes breathing in the context of various behaviors, such as vocalization, parturition, vomiting, retching, micturition, blood pressure control, nociception, and many other components of emotional behavior (Holstege et al. 2004; Subramanian and Holstege 2010; Subramanian et al. 2008). The present data show that the PAG not only converts the three-phase respiratory patterning into a two-phase rhythm of the crural diaphragm but also induces postsynaptic activity in the costal diaphragm. It also modulates the timing, pattern of discharge frequency, motor unit recruitment, and the type of motor units recruited in both the crural and costal compartments. The PAG does not maintain direct connections with any somatic motoneuronal cell groups in brainstem and spinal cord, but with premotor interneurons involved in motor output (Holstege 1991b). To differentially regulate crural and costal diaphragmatic motor output, the PAG uses its connections with the parabrachial complex, Kölliker-Fuse nuclei, the medullary ventrolateral tegmental field, and the solitary nucleus (Holstege 1991b). In the rat, apart from the roles played by the drosomedial hypothalamus (Holruichi et al. 2009), parabrachial (Hayward et al. 2004), and solitary nuclei (Huang et al. 2000; Subramanian et al. 2007) in mediating PAG-induced changes to eupnea, the precise influence of PAG on the brain stem respiratory-related neurons are yet to be determined. The induction of postsynaptic in the costal diaphragm by the PAG could play a role in expulsive efforts, when the costal diaphragm acts synergistically with abdominal muscles to raise abdominal pressure in events such as vocalization, defecation, vomiting, and parturition. In this regard, the PAG projection to the NRA (Holstege 1989, 1991b) is important, because the NRA is the only cell group that projects to all motoneurons that control abdominal pressure (Holstege 1989, 1991a). Examples are abdominal, internal intercostal, pelvic floor, and several other motoneuronal cell groups (Holstege et al. 1987). In short, the PAG uses the NRA to convert passive breathing into active breathing in the context of survival in general and motor activities that require changes in abdominal pressure in particular.

**Postinspiratory Neurons in the Rat Medulla**

Postinspiratory inhibitory cells are thought to play a key role in the three-phase respiratory patterning. These cells have been recorded exclusively intracellularly (Dutschmann and Herbert 2006; Richter 1982, Richter and Ballantyne 1983, Richter et al. 1987; Swarzhacer et al. 1990) in the VLM. Neurons exhibiting discharge pattern similar to that of these postsynaptic inhibitory cells have also been recorded in the VLM. Classified as expiratory decrementing (E-Dec) neurons, these cells have been found predominantly in the Bötvinger (BötC) complex (Bianchi and Barillot 1982; Ezure and Manabe 1988; Feldman and Cohen 1978; Hayashi et al. 1996). The axonal projections of the BötC E-Dec cells have only been studied antidromically and seem to be propriobulbar, because they are almost never activated from the C4–C5 spinal cord (Manabe and Ezure 1988; see Ezure 1990). The E-Dec cell discharge is facilitated by lung inflation (Bianchi and Barillot 1982; Ezure and Manabe 1988; Manabe and Ezure 1988; Hayashi et al. 1996), suggesting that they receive excitatory inputs from lung stretch receptors. Certain premotor neurons in the NRA involved in upper airway muscle control (see review by Morris et al. 2003) and some laryngeal constrictor motoneurons located in the nucleus ambiguous (Sun et al. 2008) are also activated during postinspiration. Although BötC and NRA expiratory neurons and laryngeal constrictor motoneurons have decrementing firing patterns, they differ from postsynaptic inhibitory cells because these cells only fire during the stage-1 expiratory period and not during the stage-2 expiratory period. The postsynaptic neurons suggested by Richter and Ballantyne (1983) and Richter et al. (1987) orchestrate inspiratory-expiratory phase switching and are hypothesized to be inhibitory premotor cells in the VLM. The present study demonstrates that during eupneic breathing in vivo, the postsynaptic neurons are inhibitory, because stimulation of these cells attenuates the crural diaphragm function. The neurons that excite these postsynaptic cells are not known. It has been hypothesized (Dutschmann and Herbert 2006; see Mörschel and Dutschmann 2009) that the late-inspiratory neurons (see Long and Duffin 1980; Ezure 1990; von Euler 1983) activate postsynaptic cells. The present study shows that neurochemical stimulation of these ventrolateral medullary postsynaptic cells converts them into expiratory phase-spanning. This suggests that they receive sustained excitatory inputs from other respiration-related neurons in the vicinity, which also may include the late-inspiratory cells. Since the microinjections in this study were made in the actual vicinity of the postsynaptic neurons, and since the effect of stimulation was directly measured on the cells, in contrast to only stereotaxic stimulation, they might have affected other network elements in the vicinity. This means that they might have excited not only postsynaptic neurons but also other neurons. This probably accounts for the changes in respiratory rhythm and timing (see recent models of respiratory rhythm-generating networks in this context presented by Dutschmann et al. 2009 and Rybak et al. 2008). The present study also shows that these postsynaptic cells are excited by vagal stimulation, implying that they might play a role in the Breuer-Hering reflex.

**Conclusion**

During eupnea, postinspiration is seen only in the crural diaphragm. Disruption of eupnea by stimulation of both the VLM and the NRA inhibits postinspiration in the crural diaphragm. PAG stimulation differentially modulates the activity pattern of the crural and costal diaphragm; i.e., it abolishes postinspiration in the crural diaphragm but induces postsynaptic inhibition in the costal diaphragm. Medullary postsynaptic neu-
rons seem to have an inhibitory effect on the postinspiratory component of the crural diaphragm. The power and amplitude density spectrum of the diaphragm during eupnea indicates that significant differences exist in the recruitment of respiratory motor output during inspiratory and postinspiratory periods.

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DISCLOSURES

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