Chemical activation of C₁-C₂ spinal neurons modulates intercostal and phrenic nerve activity in rats

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Chemical activation of C₁-C₂ spinal neurons modulates intercostal and phrenic nerve activity in rats. Am J Physiol Regul Integr Comp Physiol 286: R1069–R1076, 2004. First published February 5, 2004; 10.1152/ajpregu.00427.2003.—Chemical activation of upper cervical spinal neurons modulates activity of thoracic respiratory interneurons in rats. The aim of the present study was to examine the effects of chemical activation of C₁-C₂ spinal neurons on respiratory motor outflows. Electroneurograms of left phrenic (n = 23) and intercostal nerves (ICNs, n = 93) between T₁ and T₉ spinal segments were recorded from 36 decerebrated, vagotomized, paralyzed, and ventilated male rats. To activate upper cervical spinal neurons, glutamate pledgets (1 M, 1 min) were placed on the dorsal surface of the C₁-C₂ spinal cord. Glutamate on C₁-C₂ increased ICN tonic activity in 56/59 (95%) ICNs. The average maximal tonic activity of ICN was increased by 174% (n = 59). After spinal transection at rostral C₁, glutamate on C₁-C₂ still increased ICN tonic activity in 33/35 ICNs. However, the effects of C₁-C₂ glutamate on ICN phasic activity were highly variable, with observations of augmentation or suppression of both inspiratory and expiratory discharge. C₁-C₂ glutamate augmented the average amplitude of phrenic burst by 20%, whereas the increases in amplitude of ICN inspiratory activity, when they occurred, averaged 120%. The burst rate of phrenic nerve discharge was decreased from 34.2 ± 1.6 to 26.3 ± 2.0 (mean ± SE) breaths/min during C₁-C₂ glutamate. These data suggested that upper cervical propriospinal neurons might play a role in descending modulation of thoracic respiratory and nonrespiratory motor activity.

IN WIDELY USED CAT AND RAT MODELS for the study of respiratory control, the number of synapses to activate or suppress respiratory motor neurons by supraspinal neurons appears to differ for phrenic and intercostal motor outflows. Evidence suggests that respiratory activation of intercostal muscles depends to a great extent on spinal interneurons to transmit supraspinal inputs from premotor neurons of the ventral respiratory group (rat) or dorsal and ventral respiratory groups (cat) (4, 20, 24, 35). By contrast, studies in rats show that very strong monosynaptic excitatory and inhibitory inputs to phrenic motor neurons originate from supraspinal cells of the ventral respiratory group (8, 35–37). Studies in cats concerning the strength of monosynaptic input to phrenic motor neurons have yielded conflicting results (3, 4). However, although less pronounced than for rats, substantial projections are likely from both dorsal and ventral respiratory group in cats (4, 11).

The insertion of interneurons between supraspinal premotor neurons and intercostal motor neurons may increase modulation of intercostal respiratory activity (4). For example some thoracic respiratory interneurons (TRINs) driven by supraspinal respiratory activity are assumed to transmit respiratory drive to intercostal muscles in the cat (21). In addition, intersegmental as well as contralateral projections of TRINs have been demonstrated. These projections may be important for modifying supraspinal respiratory drive (21, 22, 32). Studies by our group in the rat likewise demonstrated the presence of TRINs. Their activities could be strongly modulated by noxious visceral and somatic input, whereas effects on phrenic nerve motor outflow were minimal (27). In rats, propriospinal pathways are activated by application of a high concentration of glutamate to the dorsal surface of the C₁-C₂ spinal cord (28, 29, 33). This manipulation could either excite or suppress the activity of dorsal horn and intermediate zone neurons influenced by colorectal distension (29). Similarly, TRINs could be either excited or suppressed by C₁-C₂ glutamate (28). Relevant descending pathways to TRINs from C₁-C₂ could involve neurons in the dorsal and/or ventral horn if distribution of cells is similar to that obtained by fluorogold tracer studies from lumbosacral spinal cord (25).

Because of the strong, albeit variable, effect of C₁-C₂ glutamate on TRINs, we hypothesized that this manipulation would influence motor outflow of intercostal nerves. Results showed that glutamate on C₁-C₂ typically elicited tonic activation of efferent intercostal nerves, whereas phasic respiratory activity could be increased or decreased. Tonic and phasic respiratory responses by intercostal nerves to glutamate were considerably greater than glutamate effects on phrenic motor activity. It is suggested that the upper cervical spinal cord acts as a processor and integrator of respiratory and nonrespiratory information to thoracic motor neurons through its actions on thoracic interneurons. A preliminary report has been published in abstract form (13).

METHODS

Experiments were performed on 36 male Sprague-Dawley rats weighing 320–460 g (Charles River, Boston, MA). The protocol was approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee and followed the guidelines of the American Physiological Society and the International Association for the Study of Pain. Anesthesia was initially induced with a bolus injection of pentobarbital sodium (60 mg/kg ip). Supplemental doses (30–40 mg/kg ip) were given to maintain anesthesia if necessary, until decerebration was completed. A catheter was placed in the left jugular vein for continuous saline infusion (0.5–1.0 ml/kg h⁻¹) throughout experiments. The right carotid artery was cannulated to monitor arterial blood pressure (ABP). Mean ABP was ≥80 mmHg during experiments. Cervical vagi were sectioned bilat-

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erally. After muscle paralysis with pancuronium bromide (0.4 mg/kg iv), animals were artificially ventilated with room air administered by a volume-controlled pump (50–55 strokes/min, 3–5 ml stroke volume). Supplemental doses of pancuronium bromide (0.2 mg·kg\(^{-1}\)·h\(^{-1}\) iv) were administered to maintain muscle relaxation so that no spontaneous respiratory movement was seen after the ventilator pump was stopped. Rectal temperature was kept between 37 and 38°C by using a servocontrolled heating blanket.

Decerebration was performed using a modified technique described in previous studies (35, 39). Briefly, bilateral windows (3×5 mm) through the parietal bones were made with care to avoid damaging the central sagittal and transverse sinuses. Complete transection of the brain stem at the precollicular level was made using a fine wire cautery blade. A wedge of brain tissue was then mechanically removed to avoid damage of the basilar artery on the floor of the skull cavity. Small pieces of gel foam or saline cotton balls were used to stop bleeding.

A dorsal approach was used to expose the left phrenic nerve as it crossed the brachial plexus in the neck and was crushed peripheral to the recording site. A bipolar platinum hook electrode was placed around the nerve to monitor central respiratory drive. Similar electrodes were placed around left external and/or internal intercostal nerves at various thoracic levels (T3–T8) after they were dissected and crushed distal to the recording site. To obtain stable recordings of neural activity and protect nerves from drying, the electrodes were fixed to the nerves by dental impression material. To assure an adequate intercostal nerve signal, CO\(_2\) (up to 3.5%, average 1.7 ± 0.2%) was added to the inspired air in the majority of animals. We used positive end-expiratory pressure (2–4 cm H\(_2\)O) throughout an experiment if the thoracic cavity was opened during surgery (n = 5).

Neural signals were amplified, filtered (300–3,000 Hz), and documented online with the Spike 3 data-acquisition system (CED, Cambridge). Neural discharges were also monitored audibly through speakers during experiments.

Laminectomies were performed to expose the C1-C2 spinal segments. To chemically activate C1-C2 spinal neurons, glutamate (1 M) was absorbed onto filter paper pledgets (2×2 mm) and was placed on the dorsal surface of C1-C2 segments (27, 33). Because 1 M glutamate is hypertonic and hyperosmotic compared with extracellular...
RESULTS

Discharge Patterns of ICN

Electroneurograms for the left phrenic nerve and one to three ICNs at various levels of the spinal cord (T3-T8) were recorded simultaneously in 36 rats. According to phrenic nerve activity, the discharge patterns of ICNs (n = 93) were classified as inspiratory (Insp, n = 39), expiratory (Exp, n = 27), or biphasic (Biph, n = 27). Phasic ICN activity increased in a ramp or step-ramp manner. Inspiratory ICNs exhibited discharges during the expiratory phase (Fig. 1A), and this activity was more frequently recorded from external ICNs than internal ICNs (27/29 vs. 12/64, P < 0.01). Expiratory ICNs discharged during the inspiratory phase (Fig. 1B), and those discharges were obtained much more often from internal ICNs than external ICNs (25/64 vs. 2/29, P < 0.05). Most Exp ICNs (25/27, 93%) commenced discharging in the late period of the expiratory phase and terminated abruptly at inspiratory onset. This type of ICN activity was classified as Exp-late (Fig. 1B). Two Exp ICNs discharged during the whole expiratory phase and were classified as Exp-all (Fig. 1B). Biphasic ICNs discharged in both inspiratory and expiratory phases and were obtained only from internal ICNs, which were further divided into two subgroups: Exp-late and Exp-early according to the onset of expiratory discharges (Fig. 1C).

Distributions of the three discharge patterns at different spinal levels are shown in Fig. 1D. ICNs from T3 to T5 were selected randomly for nerve recording. The probability of obtaining inspiratory activity in external ICNs from rostral (T1-T5) segments was not significantly different from caudal (T6-T8) segments (8/8 vs. 19/21). Analogously, expiratory activity in internal ICNs from rostral segments was similar to caudal segments (11/35 vs. 14/29). However, 70% (19/27) of

![Fig. 2. Effects of C1-C2 glutamate (Glu; 1 M) and hypertonic saline (1 M) on ICN tonic activity. Examples of typical responses are shown: A, effects of C1-C2 hypertonic saline before spinal transection; B, effects of C1-C2 glutamate before spinal transection; C, effects of C1-C2 glutamate after rostral C1 transection. D, summary for maximal tonic responses among different groups of ICNs. *P < 0.05 (compared with baseline); **P < 0.01 (compared with baseline).](http://ajpregu.physiology.org/)

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ICNs with biphasic discharges were obtained from rostral segments compared with eight ICNs from caudal segments.

**Effects of C1-C2 Glutamate on ICN Activity**

To examine the propriospinal descending modulation of ICN activity by the upper cervical spinal cord, neural activities of ICNs in 23 rats were examined after application of glutamate and hypertonic (1 M) saline on the dorsal surface of C1-C2 segments.

**Tonic activity.** Glutamate on C1-C2 increased ICN tonic activity in most ICNs (56/59, 95%, Fig. 2B). For all ICNs tested, the average maximal response of tonic activity increased significantly by $174 \pm 19.9\%$ ($P < 0.01$, $n = 59$). The average latency and duration of tonic responses to glutamate on C1-C2 were $3.0 \pm 0.6$ and $73.0 \pm 5.4$ s, respectively. Hypertonic saline (1 M) on C1-C2 did not significantly change tonic activity of ICNs ($n = 48$, Fig. 2A). After spinal transection at rostral C1 segment, glutamate on C1-C2 still increased ICN tonic activity in 33/35 ICNs (Fig. 2C). The average maximal tonic activity of ICNs increased by $116 \pm 14.9\%$ ($P < 0.01$, $n = 35$). Figure 2D shows responses among ICNs with different types of respiratory activity. Tonic responses to glutamate on C1-C2 were similar among ICN groups of Insp, Exp, and Biph in the intact spinal cord (ANOVA, $P > 0.05$). However, responses of Insp ICNs to C1-C2 glutamate were less than for Exp and Biph ICNs after rostral C1 transection (ANOVA and Tukey’s test, $P < 0.05$). No significant differences were found for net increases in ICN tonic activity by C1-C2 glutamate and for ICN tonic baseline before and after spinal transection.

**Table 1. Response patterns of ICN phasic activities to glutamate on C1-C2**

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<th>Discharge Patterns</th>
<th>Response Patterns to C1-C2 Glutamate</th>
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Values are the number of nerves. Insp, inspiratory activity; Exp, expiratory activity; Aug, augmentation; Sup, suppression.

Fig. 3. Effects of C1-C2 glutamate on ICN phasic activity. A–D: typical responses among ICNs. Aug, augmentation; Sup, suppression. Bimodal responses showed either Aug-Sup or Sup-Aug. E: comparison of effects of C1-C2 glutamate and hypertonic saline on expiratory peak activity of ICNs: ICNs with expiratory peak augmentation during C1-C2 glutamate or hypertonic saline were selected. Exp Peak, expiratory peak; Lat, latency; T-P, time to peak; Dur, duration; $n$, number of nerves; *$P < 0.05$; **$P < 0.01$. **
Phasic activity. Responses of ICN phasic activity with glutamate on C1-C2 were highly variable (Table 1). Of the 38 ICNs with inspiratory discharges, 35 (92%) ICNs exhibited augmentation and/or suppression of inspiratory peak activity with C1-C2 glutamate. In the 34 ICNs with expiratory discharges, 32 (94%) ICNs exhibited changes of expiratory peak activity with C1-C2 glutamate. Inspiratory activity in 3/38 ICNs and expiratory activity in 2/34 ICNs did not change with glutamate on C1-C2. Examples of ICN phasic responses during glutamate on C1-C2 are shown in Fig. 3, A–D.

Augmentation. For ICNs showing inspiratory peak augmentation with C1-C2 glutamate (n/H11005 21, including augmented component of bimodal responses that included augmentation and suppression), the average inspiratory peak activity increased by 120.0 ± 20.3% (P < 0.01) and responses lasted 35.5 ± 8.1 s. For ICNs showing expiratory peak augmentation with C1-C2 glutamate (n/H11005 23, including augmented component of bimodal responses), the average expiratory peak activity increased by 245.4 ± 64.8% (P < 0.01) and responses lasted 88.3 ± 9.8 s. No significant difference was found between augmentations of inspiratory and expiratory peak activity with glutamate on C1-C2.

Effects of C1-C2 Glutamate on Phrenic Nerve Activity

Activities of 23 left phrenic nerves were examined for responses to C1-C2 glutamate and hypertonic saline. Glutamate on C1-C2 decreased the phrenic burst rate in 17/23 nerves and augmented the amplitude of phrenic discharges in 21/23 nerves. The average respiratory rate was significantly reduced from 34.2 ± 1.6 to 26.3 ± 2.0 breaths/min (Fig. 4Aa, n = 23, 4Ba, n = 20, 4C).

Effects of C1-C2 Glutamate and Hypertonic Saline on Phrenic Nerve Activity

Activities of 23 left phrenic nerves were examined for responses to C1-C2 glutamate and hypertonic saline. Glutamate on C1-C2 decreased the phrenic burst rate in 17/23 nerves and augmented the amplitude of phrenic discharges in 21/23 nerves. The average respiratory rate was significantly reduced from 34.2 ± 1.6 to 26.3 ± 2.0 breaths/min (Fig. 4Aa, n = 23, 4Ba, n = 20, 4C).

Effects of C1-C2 Saline on ICN Activity

Among 33 ICNs with inspiratory discharges, 27 ICNs exhibited no change in inspiratory peak activity after 1 M saline on C1-C2, whereas 4 ICNs were augmented and 2 were suppressed. The average inspiratory peak activity did not change significantly. However, in 25 ICNs with expiratory discharges, 16 exhibited expiratory peak augmentation with 1 M saline on C1-C2, whereas 2 were suppressed and 7 did not show significant changes. For ICNs showing expiratory augmentation with saline (n = 16), the average expiratory peak activity increased by 72.4 ± 17.9% (P < 0.01), which was less (P < 0.05) than after glutamate application (245.4 ± 46.8%, n = 23, Fig. 3E).
The effect of C1–C2 glutamate on though small changes in phrenic nerve activity were also involved. Respiratory rate indicates that supraspinal components were much less than the inspiratory peak augmentation of ICNs (21.3 ± 5.1%) was much less than the inspiratory peak augmentation of phrenic nerves (20.3%). After spinal transection at C7–C8 segment, glutamate on C1–C2 still increased phrenic phasic activity and reduced burst rate in 3/3 rats. Unlike the effects of glutamate, hypertonic saline did not significantly affect the amplitude, burst rate, or inspiratory time of phrenic nerve activity (Fig. 4Ba, Bb).

Figure 4C shows the comparison of phasic responses of phrenic and intercostal nerves to glutamate on C1–C2. The inspiratory peak augmentation of phrenic nerves (21.3 ± 5.1%) was much less than the inspiratory peak augmentation of ICNs (120.0 ± 20.3%).

DISCUSSION

The present study demonstrated that phasic and tonic ICN activity changed dramatically with glutamate on C1–C2, although small changes in phrenic nerve activity were also observed simultaneously. The effect of C1–C2 glutamate on respiratory rate indicates that supraspinal components were also involved.

**Firing Patterns of ICNs**

ICN firing patterns in adult rats have been reported previously by Tian and Duffin (34, 35). Neural activity recorded from the rostral (T2–T3) external intercostal nerves was generally confined to the inspiratory phase, with little discharge occurring during expiration, except in nerves from caudal (T6–T8) segments. However, both inspiratory and expiratory activity was observed from internal ICNs and mainly from rostral spinal segments. In spinal segments T6–T8, less activity was observed in either respiratory phase, but when present, it was mostly expiratory. In the present study, the firing patterns of ICN activity are similar to the results observed from rats and cats (1, 14, 17, 23, 34, 35). This includes the distribution of inspiratory and expiratory activity in external and internal intercostal nerves and the appearance of inspiratory activity in both internal and external intercostal nerves in the rostral segments. However, the present study showed that there were no significant differences between rostral (T2–T3) and caudal (T6–T8) spinal segments for recording inspiratory discharges from external ICNs (8/8 vs. 19/21) and for recording expiratory discharges from internal ICNs (11/35 vs. 14/29). Taking into account biphasic ICNs, similar chances of recording inspiratory activities were also obtained from caudal and rostral spinal level (52 vs. 48%). This investigation used artificially ventilated, vagotomized, and decerebrated animals. To assure good intercostal nerve signals, CO2 was added to the inspired air for the majority of animals. This could have resulted in hypercapnia so ICN activity might differ from ICN activity during normocapnia. Differences between this study and other investigations also may be explained in part by position of the electrodes. Distance from the ventral root outlet can influence the composition of fibers, and position of the electrode on the mixed nerve can affect the fibers recorded.

**Responses of ICNs to C1–C2 Glutamate**

Glutamate applied on the surface of spinal cord has been used to activate cell bodies in the cervical spinal cord in previous studies (2, 26, 27, 29, 31, 33) because it does not also affect passing nerve fibers (18). Chemical activation of upper cervical spinal neurons with glutamate primarily inhibits thoracic spinal neurons responsive to splanchnic stimulation, chemical stimulation of cardiac afferents, and noxious esophageal distension (12, 26). It also reduces excitatory responses of lumbar sacral cells to noxious colorectal distension and decreases their spontaneous activity (29). This descending inhibition does not require supraspinal structures, because it still occurs after spinal transection at the rostral C1 segment in rats (29). More recently, it was shown that upper thoracic TRINs, which are located mainly in deeper dorsal horn and intermediate zone, could either be inhibited (45% of TRINs) or excited (31% of TRINs) with glutamate on the C1–C2 segment (28). Because some thoracic interneurons are likely responsible for transmitting respiratory drive to intercostal motor neurons, it appears reasonable that chemical activation of upper cervical spinal neurons in this study resulted in either augmentation or suppression of phasic respiratory ICN activity. In contrast, C1–C2 glutamate increased ICN tonic activity in 95% of cases. This difference might occur because phasic activity of ICNs most likely depended on respiratory motor outflow, whereas tonic activity of ICN could also result from nonrespiratory activation of ventral horn neurons.

In the present study, effects of C1–C2 glutamate on ICN tonic activity still occurred after spinal transection at rostral C1 segment. This demonstrated that descending modulation of C1–C2 spinal neurons on ICN tonic activity did not require supraspinal sites. However, we could not determine the extent...
to which changes in phasic activity of ICNs depended on spinal vs. supraspinal mechanisms because respiratory drive was lost after spinal transection. Nonetheless, phasic increases in inspiratory ICN activity were much greater than for phrenic nerve, and some ICN inspiratory activities were suppressed. These data suggest that significant propriospinal modulation of thoracic respiratory motor activity is possible.

**Responses of Phrenic Nerve to C1–C2 Glutamate**

Results of the present study showed that glutamate on C1–C2 significantly decreased respiratory rate and augmented phrenic nerve peak activity, although the latter changes were much smaller than for inspiratory activity of ICNs. However, glutamate on C1–C2 did not significantly affect central respiratory drive monitored by phrenic nerve activity in a previous study where the animals were anesthetized with pentobarbital sodium and not vagotomized (28). It is possible that lack of anesthesia and/or vagotomy in the present study resulted in this difference.

It is unclear how chemical activation of C1–C2 spinal neurons may have influenced phrenic amplitude and respiratory rate. Phrenic motoneurons within C1–C6 levels of the cervical spinal cord in rats are connected monosynaptically with bulbo-spinal respiratory neuronal groups that generate and integrate central respiratory drive (6, 9, 19, 34). Therefore, synapses from C1–C2 neurons onto phrenic motoneurons are not required. Another possibility is that descending propriospinal pathways from C1–C2 might influence the activity of thoracic propriospinal neurons, which in turn project back to phrenic motor nuclei in the mediventral spinal cord. However, after spinal transection at C7–C8 segments, C1–C2 glutamate still changed phrenic activity (rate and amplitude) in 3/3 rats. Most likely, C1–C2 spinal neurons activated by glutamate could project to medullary respiratory groups and influence central respiratory activity. C1–C2 spinal neurons with projection to dorsal or ventral groups of respiratory neurons in the medulla have not been directly examined. However, anatomical studies have shown that large numbers of neurons in the upper cervical spinal cord have ascending projections to respiratory-related areas of the brain stem (10, 15, 16). These pathways are possible candidates for effect of C1–C2 glutamate on central respiratory activity in the present study.

**Summary Model and Possible Implications**

Figure 5 represents a model for C1–C2 descending modulation of thoracic motor activity suggested by the present results and relevant literature. Stimulation of C1–C2 neurons strongly influences thoracic respiratory and nonrespiratory (tonic) activities of afferent intercostal nerves T3–T8. Because of the diversity of respiratory responses, we speculate that observed effects arise through changes in activity of respiratory and possibly nonrespiratory thoracic interneurons (thoracic spinal integration). Because the tonic response persists after C1 transection, at least part of the pathway is due to propriospinal descending modulation. The small increase in phasic activity of the phrenic nerve was most likely mediated through ascending pathways from C1–C2 to medullary regions that can modify phrenic output. The lack of tonic activation of the phrenic nerve with C1–C2 glutamate may be due to the generally monosynaptic activation of this motor pool from the medulla; this finding also argues against significant modulation by C1–C2 inspiratory neurons located in laminae V and VII. Compared with the effects of C1–C2 glutamate on phrenic nerve outflows, effects on ICNs were stronger and more diverse. This finding is again consistent with propriospinal descending modulation of the latter.

The present results suggest that the upper cervical spinal cord serves not only as a site for visceral afferent convergence and integration as shown previously (5, 7, 30, 38), but might also be the origin of projections to respiratory-related pools of neurons in thoracic spinal cord and brain stem. Such projections may represent potential pathways through which central respiratory activity is influenced by nociceptive and nonnociceptive information from the spinal cord. These integrative mechanisms could help shape respiratory motor responses to noxious and innocuous visceral stimulation.

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