Short-term plasticity of descending synaptic input to phrenic motoneurons in rats

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Submitted 8 July 2002; accepted in final form 7 December 2002

Hayashi, F., C. F. L. Hinrichsen, and D. R. McCrimmon. Short-term plasticity of descending synaptic input to phrenic motoneurons in rats. J Appl Physiol 94: 1421–1430, 2003. First published December 13, 2002; 10.1152/japplphysiol.00599.2002.—Respiratory afferent stimulation can elicit increases in respiratory motor output that outlast the period of stimulation by seconds to minutes [short-term potentiation (STP)]. This study examined the potential contribution of spinal mechanisms to STP in anesthetized, vagotomized, paralyzed rats. After C1 spinal cord transection, stimulus trains (100 Hz, 5–60 s) of the C1–C2 lateral funiculus elicited STP of phrenic nerve activity that peaked several seconds poststimulation. Intracellular recording revealed that individual phrenic motoneurons exhibited one of three different responses to stimulation: 1) depolarization that peaked several seconds poststimulation, 2) depolarization during stimulation and then exponential repolarization after stimulation, and 3) bistable behavior in which motoneurons depolarized to a new, relatively stable level that was maintained after stimulus termination. During the STP, excitatory postsynaptic potentials elicited by single-stimulus pulses were larger and longer. In conclusion, repetitive activation of the descending inputs to phrenic motoneurons causes a short-lasting depolarization of phrenic motoneurons, and augmentation of excitatory postsynaptic potentials, consistent with a contribution to STP.

IN THE NEURAL CONTROL of breathing, there are many examples of persistent responses that outlast the presentation of a stimulus (5, 9, 13). Examples include input from receptors located in the carotid body, the walls of the respiratory tract, and the somatic musculature. This short-term respiratory memory or plasticity appears to integrate and smooth responses to various stimuli and prevent large and rapid fluctuations in ventilation (21).

One of the most commonly observed forms of short-term plasticity is the short-term potentiation (STP) of respiratory motor output that follows activation of carotid chemoreceptor afferent pathways by either chemical or electrical stimulation (5, 9, 24). Mifflin (20) has clearly demonstrated that a component of the STP resulting from carotid sinus nerve stimulation occurs within the nucleus of the tractus solitarius. However, a component of this short-term plasticity may also arise at the spinal cord level. Soma of bulbospinal premotor neurons are located primarily in the ventral respiratory group and provide both excitatory and inhibitory descending projections to spinal respiratory motoneurons (6). High-frequency stimulation of descending pathways in the spinal cord, including axons of bulbospinal ventral respiratory group neurons, reveals a STP of phrenic nerve activity (18). Section of the spinal cord rostral to the stimulation site has no effect on the STP, thereby confirming that spinal circuitry is sufficient for its generation. Short-term plasticity of the descending synaptic input to spinal motoneurons controlling muscles involved in expiratory efforts has also recently been demonstrated in turtles (13), although the response consisted primarily of a depression of synaptic efficacy. Taken together, these findings suggest that plasticity at the spinal level may contribute to the short-term plasticity evident in central respiratory networks.

The work of Mifflin (20) is the only study to date directly examining the subthreshold behavior and synaptic responsiveness of central respiratory-related neurons during the induction of STP. Using intracellular recording, he found that, after a period of high-frequency stimulation of the carotid sinus nerve, the primary response of second-order neurons within the nucleus of the solitary tract was an increase in excitatory postsynaptic potential (EPSP) amplitude. This was accompanied in ~60% of the neurons by a modest (3–5 mV) depolarization of the resting membrane potential (MP).

In earlier work (18), our laboratory identified two distinct categories of phrenic motoneurons that could be distinguished on the basis of their synaptic response to stimulation of descending inputs. One group (type A motoneurons) exhibited a very short-latency action potential after each stimulus pulse in a train of stimuli. A second group (type B motoneurons) displayed a longer latency EPSP that summated over three to five stimuli in a high-frequency (100 Hz) train to give rise to an action potential. Temporal summation caused these neurons to depolarize during the train. On the basis of...
these results and the findings of Mifflin (20) in the nucleus of the solitary tract, we hypothesized that high-frequency stimulation of descending pathways in the spinal cord would result in an augmentation in EPSP amplitude and cause a sustained depolarization in a subset of phrenic motoneurons. These changes would provide an increase in excitability that could contribute to STP elicited by physiological stimuli such as chemoreceptor activation.

In the present study, subthreshold behavior of individual phrenic motoneurons was monitored in response to single-pulse stimulation of descending spinal pathways before and after a period of high-frequency stimulation of these same pathways. We found that phrenic motoneurons exhibit prolonged depolarization consequent to the high-frequency stimulation, with some of these exhibiting bistable behavior. In addition, there is an augmentation of both the amplitude and duration of EPSPs in response to low-frequency test stimuli. These findings are consistent with plasticity at the synapse between bulbospinal respiratory neurons and phrenic motoneurons, contributing to short-term increases in tidal volume after afferent-evoked (e.g., carotid chemoreceptor-induced) increases in breathing.

**MATERIALS AND METHODS**

Experiments were performed on adult male Sprague-Dawley rats (300–600 g; vendor: Charles River). Anesthesia was induced with isoflurane and maintained with inhaled isoflurane or intravenous injection of urethane (1.5 g/kg) that was supplemented as required (0.15–0.3 g/kg iv). Adequacy of anesthesia was assessed at least every 30 min by the absence of changes in arterial blood pressure, heart rate, or respiratory rate in response to a noxious paw pinch. A femoral vein and artery were cannulated, a tracheotomy and bilateral upper airway secretions. Animals were paralyzed with pancuronium bromide (initial dose, 1 mg iv), and additional doses (0.4–1.0 mg iv) were given every hour or as required. End-tidal CO₂ was estimated from a continuous measurement of expired gas with an infrared CO₂ analyzer that was corrected by using a regression equation derived from blood-gas measurements for the ventilator settings used (Datex 223, Puritan-Bennett; see Ref. 9). End-tidal CO₂ was maintained near 5% in a hypoxic condition (inspired O₂ fraction > 0.5).

The vertebral spines in the lumbar and upper thoracic regions were exposed, and the animal was clamped into a stereotaxic holder. The right phrenic nerve was isolated in the neck by a lateral approach, sectioned, and placed on bipolar silver hook electrodes for stimulation and recording. A laminectomy from C₁–C₇ and an occipital craniotomy were performed. The spinal cord was transected at C₁ with the use of forceps in most rats (27 of 37). Completeness of the transection was assessed visually after removal of tissue over a 1- to 2-mm rostrocaudal extent. Care was taken to preserve the anterior spinal artery. The exposed surface of the brain stem and cord was then covered with warmed mineral oil, and at least 1 h elapsed after spinalization before an experiment was begun.

**Stimulation and Recording**

The experimental paradigm is illustrated in Fig. 1. The lateral funiculus was stimulated with a concentric bipolar electrode (SNE-100, David Kopf Instruments) lowered into the C₁–C₂ spinal cord ~3 mm caudal to the transection and at the point of dorsal root entry. The depth of the electrode was adjusted to give the maximum orthodromic monosynaptic response in the phrenic nerve. Single pulses (0.5–1 Hz, 0.1-ms duration, 50–400 μA) or trains of stimuli (100 Hz, 0.1-ms pulse duration, 5–60 s) were delivered through an optically coupled stimulus isolation unit. Stimulus intensity was adjusted to three times the threshold for orthodromic activation of phrenic motoneurons, unless otherwise indicated. In spinal cord-intact animals, stimulation was confined to the inspiratory or expiratory phases with the use of a logic circuit (17) and laboratory computer. Phrenic nerve activity was amplified, filtered (3 Hz to 10 kHz), and integrated (Paynter filter, 15-ms time constant).

Filtered (0–10 kHz) intracellular recordings were made with glass micropipettes (6–40 MΩ) filled with 3 M potas-
sium acetate containing 10 mM potassium chloride. Phrenic motoneurons were identified by antidromic activation of the ipsilateral phrenic nerve. The response of phrenic motoneurons to orthodromic stimulation of bulbospinal pathways was determined. All signals were displayed on a storage oscilloscope and recorded directly on computer as well as on videotape and a chart recorder. MP was measured, and any offset was corrected by subtracting the offset voltage measured after removal of the electrode from the neuron.

Values are expressed as means ± SE. The statistical significance (P < 0.05) of changes was ascertained by Student’s t-test for paired comparisons with the Bonferroni (26) correction for multiple comparisons. The time course of the decay of integrated phrenic nerve (fPhr) activity or the repolarization of MP after stimulation was analyzed by using the following equation: % change in fPhr (or MP) = A · e⁻ⁿᵗ + B, where A is the reciprocal of the time constant and A and B are constants. Least squares regression was used to estimate the relationship between ln(fPhr − B) and time t when B was iterated in increments of 0.5 to maximize r². Logarithm A was the y-intercept, and −λ was the slope of this regression line. Time 0 was chosen at the peak fPhr or at the peak depolarization of individual motoneurons.

RESULTS

Spinalization abolished all respiratory-related phasic activity on the phrenic nerve, even during marked hypercapnia (alveolar PCO₂ > 70 Torr) obtained by increasing inspired CO₂ fraction (0.05) and lowering the ventilator frequency (<60 breaths/min). Mean arterial pressure in spinalized animals averaged 89 ± 6 mmHg, which was lower than that in unspinalized animals (103 ± 4 mmHg; P < 0.05).

Effect of Repetitive Spinal Cord Stimulation on Phrenic Nerve Discharge

Repetitive stimulation (100 Hz; 15–60 s) caused a sustained increase in discharge of the ipsilateral phrenic nerve that outlasted the period of stimulation (Fig. 2). The magnitude of integrated nerve activity increased in the immediate poststimulation period, reaching a peak at 7.8 ± 1.2 s after stimulus termination (n = 12; Fig. 2). Activity then decayed with a time constant of 49.3 ± 8.7 s. A few units continued to fire tonically for 119 ± 14 s. This overall pattern of phrenic response was reproducible within individual animals and was independent of the duration (15–60 s) of stimulation.

Effect of Repetitive Spinal Cord Stimulation on Phrenic Motoneurons

Twenty-four phrenic motoneurons were tested for their response to 100-Hz stimulation. Three qualitatively different response patterns were observed. However, within a given neuron, the response pattern was independent of the duration of stimulation (2–5 trials per neuron, 5- to 30-s duration stimulus trains).

Depolarizing response. As shown in the example in Fig. 3, neurons exhibiting this pattern of response (10 of 24) underwent a progressive membrane depolarization during stimulation to a new plateau, followed by a brief repolarization at the termination of stimulation (note the repolarization following termination of the stimulus train in Fig. 3C) and then a second depolarization (Fig. 3, top, between C and D; n = 10 of 24). As mentioned above, the overall pattern of potentiation did not vary for depolarizing neurons exposed to different stimulus durations. The neuron illustrated with a depolarizing response to a 5-s period of high-frequency stimulation in Fig. 3 also exhibits a depolarizing response to either 15- or 30-s stimulation periods (Fig. 4). The general pattern of the response (i.e., depolarization) remains the same, despite subjecting the neuron to a full range of stimulus periods.

The average response of motoneurons exhibiting the depolarizing response is shown in Fig. 5A (n = 10). During a 15-s stimulus train, these neurons depolarized by 7.9 ± 1.8 mV (n = 7; P < 0.01, compared with no change). On stimulus termination, they partially repolarized (by an average of 4.5 ± 1.4 mV; Fig. 5A) but then spontaneously underwent a secondary depolarization (6.3 ± 0.5 mV; n = 7; P < 0.05) from prestimulus control, attaining a peak depolarization at 5.3 ± 0.6 s (n = 7) poststimulation. These neurons then gradually repolarized to control levels, with an average time constant of 33 s (change in MP = +1.1 ± 0.6 mV at 1 min; n = 8; P > 0.1) (Figs. 3 and 5A). For the calculation of the time constant, time 0 was set at the peak depolarization.

As illustrated in Fig. 3F, high-frequency stimulation also elicited a short-term increase in both the amplitude and duration of the evoked synaptic response (EPSP) to low-frequency (0.5–1 Hz) test pulses.
Repolarizing response. One-third of the tested motoneurons (8 of 24) exhibited the response pattern illustrated in Fig. 6. This consisted of a progressive depolarization to a new MP during stimulation, followed by an exponential return toward the prestimulus MP immediately poststimulation without a secondary depolarization.

Neurons exhibiting the repolarizing response pattern depolarized an average of $13.1 \pm 3.4$ mV ($n = 5$; $P < 0.01$) during the period of stimulation (Fig. 5B), but, after stimulus termination, the membrane repolarized to the prestimulus MP with a time course that could be described by a single exponential with a time constant averaging $14.4 \pm 1.8$ s ($n = 5$). For the calculation of the time constant, time 0 was set at the peak depolarization, i.e., immediately after the final stimulus pulse.

Similar to the effect on EPSP amplitude and duration in neurons exhibiting the depolarizing response, these neurons also exhibited an increase in both the amplitude and duration of the evoked synaptic response (compare EPSPs in Fig. 6A with 6D).

Bistable response. The final response pattern consisted of a bistable behavior that was observed in 25% of neurons tested (6 of 24). As shown in Figs. 7 and 8, these neurons depolarized during stimulation to a new steady level that was maintained for ~1 min after stimulation (bistable behavior).

During stimulation, these neurons moderately depolarized by an average of $4.4 \pm 0.7$ mV ($n = 6$; $P < 0.05$)
that was maintained at a relatively stable level for at least 45 s after stimulation (range = 45–115 s). The depolarization was sufficient in some of these neurons to exceed their firing threshold (Fig. 7). Firing in these neurons had a very regular rhythm. Single-stimulation pulses of the descending pathways delivered during this period could reset the firing rhythm if the resulting EPSP was sufficient to bring the neuron to firing threshold (Fig. 7B). A depolarizing intracellular step could elicit prolonged depolarization, even in the absence of descending pathway stimulation. A bistable response was also obtained in two rats with intact spinal cords (Fig. 8). In these motoneurons, the phasic polarization between bursts of inspiratory activity on the phrenic nerve was not sufficient to reset the membrane to the prestimulus potential. Intracellular injection of a large-amplitude hyperpolarizing pulse (≥5 nA; 2 s; n = 2) restored these neurons to their prestimulus MP (Fig. 8B).

Effect of Repetitive Spinal Cord Stimulation on EPSP Waveform

As indicated above for depolarizing (Fig. 3) and repolarizing (Fig. 6) neurons, a period of 100-Hz spinal cord stimulation led to a short-term increase in the amplitude and duration of EPSPs evoked by low-frequency (0.5–1 Hz) test pulses in neurons exhibiting any of the three response patterns to high-frequency stimulation (depolarizing, n = 7; repolarizing, n = 5; bistable, n = 1). The average response for all neurons is shown in Fig. 9. Five seconds poststimulation, the EPSP amplitude had increased 76.1 ± 10.1% above a control of 4.1 ± 0.3 mV (P < 0.01; n = 10). EPSP slope (measured as the rise time from 20 to 80% of the peak) increased an average of 84.6 ± 30.9% (n = 12; P < 0.01) above control (4.13 ± 0.48 mV/ms), then decayed toward control, but remained elevated (35.1 ± 7.1%; n = 10; P < 0.01) 1 min poststimulation.

Presence of Interneurons Proximal to the Phrenic Nucleus

Probable interneurons in the vicinity of the phrenic nucleus (n = 10 in spinal cord intact, n = 2 in spinalized rats; Fig. 10) were identified by using four criteria: 1) the presence of respiratory modulated activity but differing from that of the whole phrenic nerve in spinal cord-intact animals; 2) neurons not antidromically activated from the ipsilateral phrenic nerve at stimulus intensities supramaximal for eliciting a maximal antidromic field potential; 3) neurons orthodromically activated by phrenic nerve stimulation; and 4) neurons located within the region from which the phrenic nerve antidromic field potential could be recorded (i.e., within or proximal to the phrenic nucleus). Figure 10 shows an extracellular recording of an interneuron exhibiting tonic activity with a superimposed phasic inspiratory pattern that was orthodromically activated during spinal cord stimulation.

DISCUSSION

A STP in phrenic nerve activity followed high-frequency stimulation of the ipsilateral lateral funiculus. As previously shown (18), STP of phrenic nerve activity was unaffected by section of the spinal cord, thereby confirming that supraspinal circuitry is not required. Short-term plasticity of the descending synaptic input to spinal motoneurons controlling muscles involved in expiratory efforts has also been recently shown in turtles (13), although, in the turtle, the response consisted of a depression of synaptic efficacy with little effect on motor output to inspiratory muscles. It is also clear that the spinal cord is not uniquely responsible for STP, and other pathways can make a significant contribution. Mifflin (20), for example, noted that the STP in respiratory motor output resulting from carotid sinus nerve stimulation occurs within the nucleus of the solitary tract. Taken together, these findings suggest that both excitatory and inhibitory short-term plasticity of breathing may occur at several locations within the respiratory control system, including brain stem and spinal cord circuitry.
Comparison of STP Elicited by Spinal Cord vs. Primary Afferent Stimulation

Poststimulation STP of phrenic nerve activity in anesthetized rats or cats has virtually identical time courses, whether it is evoked by carotid sinus nerve stimulation (9, 25) or spinal cord stimulation. The similarity of these response patterns is consistent with a common, spinal mechanism contributing to both responses. However, marked differences in both the magnitude and time course of STP on respiratory motor output have been reported. These differences can arise in response to activation of a number of different afferent inputs or even with stimulation of the same afferent pathway but under different experimental conditions (see Ref. 24). For example, STP of hypoglossal nerve activity elicited by stimulation of the superior laryngeal nerve in anesthetized cats decays with a slower time constant than STP of the same nerve elicited by carotid sinus nerve stimulation (12). Because stimulation of either the superior laryngeal or the carotid sinus nerves elicits similar increases in the hypoglossal motor output, it is difficult to explain the difference in STP solely on the basis of a change in the interaction between premotor and motoneurons. A more attractive explanation is that the differences depend on processing of afferent information upstream from the premotor neurons. Differential processing within the nucleus of the solitary tract is one upstream candidate and is consistent with the demonstration that STP occurs in at least some second-order neurons within the nucleus of the solitary tract in response to stimulation of the carotid sinus nerve (20).

Nevertheless, plasticity in the interaction between premotor and motor circuitry provides a simple mechanism to explain marked differences in the pattern of plasticity on different respiratory motor outputs. This is consistent with the demonstration of Jiang et al. (12) that superior laryngeal nerve stimulation elicits a substantial increase in activity and STP on hypoglossal nerve activity. In contrast, superior laryngeal nerve stimulation inhibits phrenic nerve activity, and there is no poststimulus STP of phrenic nerve activity. A STP mechanism that is activity dependent and located within the premotor and motor circuitry would readily explain this independence of STP observed on the hypoglossal and phrenic nerves. Thus it seems likely that there are multiple nodes of plasticity in the central respiratory control system, with different sites partic-
The poststimulation secondary depolarization in motoneurons exhibiting the depolarizing response could be the result of two parallel processes. For example, temporal summation of fast EPSPs could combine with a slow membrane depolarization. In this instance, termination of the stimulus would result in a rapid decay of the temporally summed fast EPSPs, whereas a slower process mediating membrane depolarization extends beyond this period. Thus a brief repolarization would be produced. The same mechanism that produces the slow response may also modulate the amplitude of the EPSPs (see below). Alternatively, the poststimulus depolarization could represent a balance between changes in both inward and outward currents. After stimulus termination, outward currents could initially dominate, tending to repolarize the membrane, but a more rapid decline in net outward current could result in the secondary depolarization.

To our knowledge, this is the first description of bistable behavior in a subset of phrenic motoneurons and is similar to previous descriptions in which a relatively brief excitatory input caused a sustained increase in the excitability of other spinal motoneurons (1, 3, 15). Because the intracellular injection of a hyperpolarizing current reset the MP to the prestimulus control, it indicates that this behavior was likely to
arise from a change in the motoneuron property rather than as the result of increased activity in their presynaptic neurons. A serotonin-dependent, calcium-mediated plateau potential is one mechanism that can cause bistable behavior (11). A serotonergic contribution to the STP in the present paradigm could be argued, if descending serotonergic pathways were ac-

Fig. 8. Spinal cord stimulation-induced bistable response in spinal cord-intact rat. Top traces in A–C are Phr activity: A and B are integrated; C is raw Phr activity. Bottom traces in A–C are $V_m$ of the same phrenic motoneuron. A: 30-s period of stimulation (100 Hz, 0.1-ms pulse) elicited an ~3-mV depolarization that was maintained for ~40 s beyond the period of stimulation. There was also an increase in the magnitude of the phasic depolarization occurring during neural inspiration. au, Arbitrary units. B: the response shown in A was reproduced by repeat stimulation. The poststimulation depolarization could be reversed by injection of hyperpolarizing pulse (2 s) that transiently hyperpolarized the $V_m$ beyond its prestimulus control value. C: superimposed successive orthodromic responses of Phr and motoneuron during high-frequency stimulation. The spike latency of the motoneuron decreased as the neuron depolarized, coincident with neural inspiration.

Fig. 9. Time course of increase above prestimulus control in EPSP amplitude and slope for phrenic motoneurons ($n = 13$) during and after 100-Hz spinal cord stimulation. Values are means ± SE. * Different from control, $P < 0.05$.

Fig. 10. Interneuron recorded within the phrenic motor nucleus in spinal cord-intact rat. A: top trace, extracellular recording of discharge pattern of interneuron. Bottom trace, integrated phrenic neurogram. Note phasic increase in discharge rate during neural inspiration. B: 7 superimposed traces showing orthodromic response to spinal cord stimulation. Action potential latency varied between 2.1 and 4.0 ms.
tivated by the spinal stimulation. Alternatively, the plateau depolarization could arise from a persistent inward dendritic current (15). In the case of phrenic motoneurons, this would imply that dendrites possess active mechanisms for differential control of their response to, for example, descending respiratory inputs vs. nonbreathing behaviors, such as coughing or emesis.

In the present study, an increase in EPSP amplitude also contributed to the increase in motoneuron excitability. The increased EPSP amplitude and slope could result from either an increase in transmitter release or an increase in the postsynaptic response. An increase in transmitter release could result from an accumulation of calcium in presynaptic terminals (27). It is also conceivable that high-frequency stimulation resulted in recruitment of interneurons interposed between the bulbospinal and the motoneurons. Once recruited, these neurons could act as an amplifier to increase the gain of synaptic input to phrenic motoneurons.

The relative contribution of mono- vs. polysynaptic pathways to the EPSPs observed in phrenic motoneurons is not clear, but the response latencies are consistent with a paucisynaptic pathway. In a previous study (18), the EPSP latency in phrenic motoneurons after stimulation of the descending pathways was used to divide phrenic motoneurons into two distinct groups. In one, the latency to EPSP onset was too short (<1 ms) to be resolvable from the stimulus artifact. This was almost certainly a monosynaptic input. In the second group of motoneurons, the EPSP onset latency was ~1.1 ms, and time to EPSP peak averaged 2.4 ms. This latency is at least consistent with the inclusion of an interposed interneuron (18). Interestingly, paired-pulse potentiation was evident in the form of temporal summation of EPSPs only in the motoneurons with a latency of this IPSP suggests a monosynaptic input, and a monosynaptic inhibitory pathway originating in the Botzinger complex has been confirmed in cats (19). Alternatively, the inhibition may be mediated via interneurons located in the rostral cervical spinal cord. Neurons within the rostral spinal cord could have been activated in the present study either directly by current spread or via synaptic inputs. However, few connections have been described between these neurons and phrenic motoneurons (4, 16, 23).

In summary, a significant finding of this study was that repetitive activation of descending inputs to phrenic motoneurons causes STP, resulting from a short-term increase in their excitability. Each phrenic motoneuron exhibits one of three different patterns of depolarization and an increase in the amplitude of evoked EPSPs. The overall time course of increased excitability is similar to that of the decay of the STP recorded on the whole phrenic nerve. This short-term plasticity of respiratory motor output is likely to contribute to the short-term increases in breathing that follow activation of a variety of respiratory afferent pathways.

We thank Dr. George Alheid for helpful comments and discussion. This work was supported by National Heart, Lung, and Blood Institute/National Institute of Neurological Disorders and Stroke Grants HL/NS-40336 and HL/NS-60997 and the National Sudden Infant Death Research Foundation of Australia.

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