GABA receptors in the phrenic nucleus of the rat

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Chitravanshi, V. C., and H. N. Sapru. GABA receptors in the phrenic nucleus of the rat. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R420–R428, 1999.—The phrenic nucleus was identified by microinjections of N-methyl-D-aspartic acid in urethan-anesthetized adult male Wistar rats. Microinjections of GABA_A and GABA_B receptor agonists (muscimol and baclofen, respectively) at the same site decreased the phrenic nerve burst amplitude. Microinjections of GABA_A and GABA_B receptor antagonists (bicuculline and 2-hydroxysaclofen, respectively) blocked as well as reversed the effects of their respective agonists. These results were confirmed by recording extracellular action potentials from single phrenic neurons. Micropressure applications of muscimol and baclofen decreased the activity of single neurons in the phrenic nucleus; this effect was blocked as well as reversed by micropressure applications of bicuculline and 2-hydroxysaclofen, respectively. These results demonstrated the presence of GABA receptors on the neurons in the phrenic nucleus and suggested that their activation results in the decrease of the phrenic nerve burst amplitude. The importance of these results in the identification of neural circuits mediating inhibition of phrenic neurons is discussed.

baclofen; bicuculline; 2-hydroxysaclofen; muscimol; phrenic neurons; transmitters

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Materials and Methods

General Procedures

Male Wistar rats (Charles River Farms) weighing 300–350 g and anesthetized with urethan (1.2 g/kg ip) were used. One of the femoral arteries was cannulated with polyethylene tubing (PE-50) for monitoring blood pressure (BP) via a pressure transducer (Statham P23 Db). One of the femoral veins was cannulated for intravenous administration of various agents. Rectal temperature was monitored by a digital thermometer (model BAT-8; Physitemp Instruments, Clifton, N.J.) and maintained at 37 ± 1°C. The trachea was cannulated with PE-240 tubing, and the animal was artificially ventilated using a rodent respirator (model 683; Harvard Apparatus, South Natick, MA). The animal was paralyzed by an intravenous injection of decamethonium (3 mg/kg iv). The neuromuscular blockade induced by decamethonium lasted for 15–20 min; at this time, administration of decamethonium was repeated. Each time the neuromuscular blocker was administered, the adequacy of the depth of anesthesia was ascertained by lack of limb withdrawal in response to a toe pinch. The heart rate (HR) was monitored by a tachograph (model 7P4; Grass Instruments, Quincy, MA) that was triggered by BP waves; the BP and HR tracings were recorded on a polygraph (model 7D; Grass Instruments). The end-tidal CO_2 was estimated from a continuous measurement of expired gas with an infrared CO_2 analyzer modified for use in small animals (Micro-Capnometer; Columbus Instruments, Columbus, OH). The frequency and tidal volume were adjusted on the ventilator to maintain the end-tidal CO_2 at 4.5–5%. The activity of the PN above the noise level, at an end-tidal CO_2 of 4.5–5%, was considered to be control PN activity; a plateau at the peak of the end-tidal CO_2 tracing represented the correct end-tidal CO_2 value (7–9).

Recording and Analysis of PN Activity

The procedure was generally similar to that published in the literature (8–9, 15). The PN was exposed via a dorsolateral approach under an operating microscope (OPMI-1H; Zeiss, Thornwood, NY) and sectioned distally as it entered the chest cavity, and a small segment was desheathed and placed on a bipolar platinum-iridium electrode. The nerve was immersed in a pool of warm (37°C) paraffin oil. The electrode was connected to a probe head stage (model super-Z, CWE systems), and the whole nerve discharge was amplified (model BMA-830 amplifier, filters set at 5–10,000 Hz; CWE systems). Amplified signals were stored together with the arterial pressure on an FM tape (HP 8868A) and visualized on an oscilloscope (Tektronix R5103N).

After the surgery was completed, sufficient time (usually 30–45 min) was allowed for stable PN recordings. The whole PN activity was full-wave rectified, and a moving average signal was obtained (model MA-821 moving averager, time constant set at 10 ms; CWE systems). At the end of the experiment, the PN was sectioned rostrally and the remain-
ing activity recorded was considered to be the noise level. In the PN recordings, the amplitude of bursts of activity (µV) was assessed from the size of the signal on the oscilloscope screen and the settings on the amplifiers, whereas the frequency of bursts was calculated from the time scale on the oscilloscope screen. The tracings of the moving average of the PN activity were recorded on a polygraph. In the moving average tracings, PN burst frequency was calculated with reference to the time scale. The amplitude of tracings was expressed in arbitrary units that were based on the height (in mm) of the recordings with reference to the baseline obtained at the noise level of the PN activity (7–9).

Functional Identification of Phrenic Nucleus

The rats were placed in a prone position in a stereotaxic instrument (David Kopf Instruments), with the head flexed 11 mm below the level of the interaural line. The skin and musculature overlaying cervical vertebra were removed, a laminectomy was performed, and the dura was removed to expose the spinal cord from C1 to T1 vertebral levels. The dorsal and ventral roots of C3, C5, and C6 spinal nerves, ipsilateral to the PN from which electrical activity was recorded, were sectioned; the dorsal and ventral roots of C4 spinal nerve were left intact. Thus the PN bursts recorded in this preparation represented output from a portion of the phrenic nucleus located in the ipsilateral C4 spinal segment. The phrenic nucleus located in the contralateral C4 spinal segment did not contribute to the PN activity recorded in this preparation (8, 9).

Microinjection Technique

Multibarreled micropipettes, prepared from glass capillaries (S0341005 capillaries, Interscience) were used. The micropipette was mounted on a micromanipulator (David Kopf Instruments). Each barrel of the micropipette was connected through tubing to one of the channels on a picospritzer (General Valve, Fairfield, NJ). The volume of injection, indicated by the displacement of the fluid meniscus in the barrel, was visually confirmed under a custom-made binocular microscope equipped with a graduated reticule in one of the eyepieces. The volume of all microinjections, regardless of the nature of the solution injected, was 50 nl, and the duration was 5–10 s. When multiple injections were made at the same site, the interval between injections was at least 5 min.

Neuronal Recording and Micropressure Ejection

The procedure for single-unit recording published in the literature (7) was followed. Multibarrel micropipettes, prepared from single glass capillaries (TW150F-4; World Precision Instruments, New Haven, CT), were pulled in a vertical pipette puller (model PE-2, Narishige). The central barrel was filled with 0.5 M sodium acetate containing 2% Chicago sky blue and was used for recording, the second barrel contained 0.9% sodium chloride solution that was used to eject normal saline as a control, and other barrels were filled with agonists and antagonists depending on the concerned experiment. The resistance of the barrel used for extracellular recording was checked at 1,000 Hz in saline (microelectrode puller, model BL-1000; Winston Electronics), was 4–8 MΩ. The micropipettes were mounted on a micromanipulator (David Kopf Instruments), and the phrenic nucleus was explored. The recording barrel was connected to a probe head stage, and the spontaneous action potentials were amplified (model DAM 80, amplifier with filters set at 300–10,000 Hz; World Precision Instruments), fed into a window discriminator (model 2503, Frederick Haer), visualized on an oscilloscope (model R5103N, Tektronix), and recorded on an FM tape (model 8868A, Hewlett Packard). The transistor-transistor-logic pulses generated from the window discriminator were fed into a rate/interval counter (model RIC-830, CWE), and the tracing of the firing of the neuron (spikes/burst) was recorded on a polygraph (model 7D; Grass Instruments). The amplitude of neuronal activity (µV) was assessed from the signal on the oscilloscope screen and the settings on the amplifiers. The unitary nature of the recordings was tested by the constancy of spike contour and amplitude, which was ascertained by superimposing 10–20 successive action potentials on the waveform (time-base 0.5 ms/div). Increase in discharge in response to a micropressure ejection of L-glutamate (2–5 mM) indicated that the recordings were made from the neuronal cell bodies rather than fibers of passage. Micropressure ejection of contents in different barrels was accomplished by application of one pressure pulse (15 ms duration, 40 psi); the volume of ejected solution was estimated to be 1–2 nl.

Histology

In the microinjection studies, the sites were marked by injecting diluted India ink (50 nl). In the single neuron recording experiments, the site was marked by the application of micropressure pulses to the barrel containing Chicago sky blue (2% in 0.5 M sodium acetate) to eject 10–20 nl of the dye. The animals were then killed with a high dose of pentobarbital sodium, and heparinized normal saline was perfused through one of the femoral arteries until the fluid exiting from the rostral cut end of the contralateral external jugular vein was clear. The spinal cord was then removed and fixed overnight in 10% formaldehyde solution. Sections were cut (30 µm) in a cryostat (model OTF/AS/MR, Hacker Instruments), mounted on a glass slide, stained with neutral red or cresyl violet, and verified histologically (8, 9).

Statistical Analysis

A paired t-test was used when the animals served as their own controls. Differences between more than two means from different groups of rats were determined by ANOVA followed by Duncan’s multiple-range test. Differences were considered significant at P < 0.05. All values were expressed as means ± SE.

Drugs and Chemicals

- Baclofen HCl (GABA<sub>B</sub> receptor agonist), bicuculline methiodide (GABA<sub>A</sub> receptor antagonist), decamethonium bromide (neuromuscular blocker), L-glutamate monosodium, 2-hydroxyacids (2-OH-sacoden; GABA<sub>B</sub> receptor antagonist), muscimol (GABA<sub>A</sub> receptor agonist), and 2-n-methyl-D-aspartic acid (NMDA) were used. All solutions were freshly prepared in normal physiological saline. The concentrations of drug solutions refer to their salts. The pH of injected solutions was 7.4. Control injections (50 nl) into the phrenic nucleus consisted of normal saline (pH 7.4). Decamethonium and L-glutamate were obtained from Sigma (St. Louis, MO); all other chemicals were obtained from Research Biochemicals International (Natick, MA).

RESULTS

Effects of Microinjections of GABA Receptor Agonists Into Phrenic Nucleus

GABA<sub>A</sub> receptors: Concentration response of muscimol microinjections. To identify the phrenic nucleus, microinjections of NMDA were used. First, concentration response of microinjections of NMDA (0.01–5 mM)
into the phrenic nucleus was studied (n = 5). The volume of microinjections, regardless of the substance injected, was 50 nl in this and all other groups of rats. In this model of phrenic nucleus, maximum responses were obtained by a 5 mM concentration of NMDA; at this concentration, the increase in the PN background discharge was 31.7 ± 2.2 arbitrary units and the duration of this effect was 83.8 ± 15.7 s (Fig. 1A). Therefore, this concentration was selected for identification of phrenic nucleus in all experiments.

The concentration response of muscimol (0.5–3 mM) microinjections into the phrenic nucleus was studied as follows. The phrenic nucleus at the C4 segment of the spinal cord was identified by microinjections of NMDA (5 mM) through one barrel of the micropipette. Microinjections of muscimol (0.5–3 mM) were made at the same site through another barrel of the same micropipette. Muscimol induced a dose-dependent decrease in the PN burst amplitude (Fig. 1B); the decreases in PN burst amplitude were 6.7 ± 0.7 units (from a control value of 27.3 ± 1.5 to 20.7 ± 1.5 units; n = 6) in response to a 0.5 mM solution, 19.3 ± 1.2 units (from a control value of 27.4 ± 1.8 to 8.1 ± 1.2 units; n = 12) in response to a 1 mM solution, and 15.7 ± 1.5 units (from a control value of 23.3 ± 2.1 to 7.7 ± 1.9 units; n = 6) in response to a 3 mM solution. Typical tracings showing the responses to a microinjection of muscimol (1 mM) into the phrenic nucleus are presented in Fig. 2, A and B. The duration of muscimol-induced decrease in PN burst amplitude was 8–10 min.

Reversal of muscimol-induced responses. Microinjection of muscimol (1 mM) into the phrenic nucleus previously identified by a microinjection of NMDA (5 mM) elicited a decrease in the PN burst amplitude (P < 0.05; n = 8) from a control value of 26.5 ± 1.9 arbitrary units (Fig. 1C) to 7.5 ± 0.6 units (Fig. 1C). A microinjection of bicuculline (1 mM) at the same site, 2–3 min after the microinjection of muscimol, reversed the muscimol-induced decrease in PN burst amplitude; the PN burst amplitude increased from 7.5 ± 0.6 to 31.9 ± 2.8 arbitrary units (P < 0.05). The value of PN burst amplitude after microinjections of bicuculline (Fig. 1C) was significantly (P < 0.05) greater compared with the control value. A smaller concentration of bicuculline (0.5 mM) did not completely reverse the muscimol-induced inhibition of PN burst amplitude. In these experiments, a microinjection of muscimol (1 mM) into the phrenic nucleus decreased the PN burst amplitude (P < 0.05; n = 6) to 7.5 ± 0.6 units (from a control value of 26.5 ± 1.9 arbitrary units (Fig. 1C) to 7.5 ± 0.6 units (Fig. 1C). A microinjection of bicuculline (1 mM) at the same site, 2–3 min after the microinjection of muscimol, reversed the muscimol-induced decrease in PN burst amplitude; the PN burst amplitude increased from 7.5 ± 0.6 to 31.9 ± 2.8 arbitrary units (P < 0.05). The value of PN burst amplitude after microinjections of bicuculline (Fig. 1C) was significantly (P < 0.05) greater compared with the control value. A smaller concentration of bicuculline (0.5 mM) did not completely reverse the muscimol-induced inhibition of PN burst amplitude. In these experiments, a microinjection of muscimol (1 mM) into the phrenic nucleus decreased the PN burst amplitude (P < 0.05; n = 8) from a control value of 26.5 ± 1.9 arbitrary units (Fig. 1C) to 7.5 ± 0.6 units (Fig. 1C). A microinjection of bicuculline (1 mM) at the same site, 2–3 min after the microinjection of muscimol, reversed the muscimol-induced decrease in PN burst amplitude; the PN burst amplitude increased from 7.5 ± 0.6 to 31.9 ± 2.8 arbitrary units (P < 0.05). The value of PN burst amplitude after microinjections of bicuculline (Fig. 1C) was significantly (P < 0.05) greater compared with the control value. A smaller concentration of bicuculline (0.5 mM) did not completely reverse the muscimol-induced inhibition of PN burst amplitude. In these experiments, a microinjection of muscimol (1 mM) into the phrenic nucleus decreased the PN burst amplitude (P < 0.05; n = 8) from a control value of 26.5 ± 1.9 arbitrary units (Fig. 1C) to 7.5 ± 0.6 units (Fig. 1C). A microinjection of bicuculline (1 mM) at the same site, 2–3 min after the microinjection of muscimol, reversed the muscimol-induced decrease in PN burst amplitude; the PN burst amplitude increased from 7.5 ± 0.6 to 31.9 ± 2.8 arbitrary units (P < 0.05). The value of PN burst amplitude after microinjections of bicuculline (Fig. 1C) was significantly (P < 0.05) greater compared with the control value. A smaller concentration of bicuculline (0.5 mM) did not completely reverse the muscimol-induced inhibition of PN burst amplitude. In these experiments, a microinjection of muscimol (1 mM) into the phrenic nucleus decreased the PN burst amplitude (P < 0.05;
n = 6) from a control value of 36.0 ± 1.9 to 19.3 ± 2.7 arbitrary units. A microinjection of bicuculline (0.5 mM) at the same site 2–3 min after the microinjection of muscimol reversed the muscimol-induced decrease in PN burst amplitude; the PN burst amplitude increased from 19.3 ± 2.7 to 27.7 ± 2.9 arbitrary units (P < 0.05). Thus the muscimol-induced decrease in PN burst amplitude was reversed by bicuculline, but the value for PN burst amplitude after recovery (27.7 ± 2.9 arbitrary units) was still significantly (P < 0.05) smaller compared with the control value (36.0 ± 1.9 arbitrary units), indicating that the reversal was not complete.

Blockade of muscimol-induced responses. The phrenic nucleus was identified by microinjections of NMDA, as described previously. Microinjections of bicuculline (1 mM) at the same site elicited an increase in the PN burst amplitude (P < 0.05; n = 6) from a control value of 25.2 ± 2.5 (Fig. 1D) to 33.7 ± 2.0 arbitrary units (Fig. 1D). Subsequent microinjections of muscimol (1 mM) into the same site failed to elicit the usual decrease in the PN burst amplitude (Fig. 1D); the PN burst amplitude after the injection of muscimol (30.2 ± 2.9 arbitrary units) was not significantly different (P > 0.05) compared with the PN burst amplitude after the microinjection of bicuculline (Fig. 1D).

A smaller concentration of bicuculline (0.5 mM) did not completely block the muscimol-induced inhibition of PN burst amplitude. In these experiments, microinjections of bicuculline (0.5 mM) into the phrenic nucleus elicited an increase in the PN burst amplitude from a control value of 30 ± 1.5 arbitrary units to 34.8 ± 1.9 units, but the increase was not statistically significant (P > 0.05; n = 6). Subsequent microinjections of muscimol (1 mM) elicited a decrease in the PN burst amplitude; the PN burst amplitude after the injection of muscimol (19.7 ± 2.8 arbitrary units) was significantly smaller (P < 0.05) compared with the value of PN burst amplitude after the microinjection of bicuculline (34.8 ± 1.9 units).

Specificity of effects of bicuculline. In these experiments (n = 6), control value for PN burst amplitude was 32.6 ± 2 arbitrary units (Fig. 3A). Bicuculline (1 mM) was microinjected into the phrenic nucleus; the PN burst amplitude increased to 39.9 ± 2.2 arbitrary units (Fig. 3A) due to blockade of GABA_A receptors. Baclofen (1 mM) was microinjected into the phrenic nucleus 2–3 min after the microinjection of bicuculline; the PN burst amplitude decreased to 24 ± 1.8 arbitrary units due to the activation of GABA_B receptors (Fig. 3A); the PN burst amplitude after the injection of baclofen (24 ± 1.8 arbitrary units) was significantly smaller (P < 0.05) compared with control PN burst amplitude (32.6 ± 2 arbitrary units), indicating that bicuculline did not alter responses to a GABA_B receptor agonist.

GABA_B receptors: Concentration-dependent increase in the PN burst amplitude (Fig. 4A); the decreases in PN burst amplitude were 7 ± 1.1 units (from a control value of 26 ± 1.8 to 19 ± 1.5 units; n = 6) in response to a 0.5 mM injection, 12.6 ± 1.4 units (from a control value of 30.5 ± 1.6 to 17.9 ± 2.2 units; n = 10) in response to a 1 mM injection, and 10.1 ± 1.4 units (from a control value of 28.4 ± 2.1 to 18.3 ± 2.6 units; n = 9) in response to a 3 mM injection. The duration of baclofen-induced decrease in PN burst amplitude was 5–6 min.

Reversal of baclofen-induced responses. A concentration response study of 2-OH-sadofen in the phrenic nucleus was performed (n = 6); maximum increase in the PN burst amplitude was observed in response to microinjections of a 100 mM concentration of 2-OH-sadofen (Fig. 4B). In another group of rats
(n = 12), microinjection of baclofen (1 mM) into the phrenic nucleus previously identified by an injection of NMDA (5 mM) decreased the PN burst amplitude (P < 0.05) from a control value of 29 ± 1.9 units (Fig. 4C) to 20.3 ± 1.7 units (Fig. 4C). A microinjection of 2-OH-saclofen (100 mM) at the same site 2–3 min after the injection of baclofen significantly (P < 0.05) reversed the baclofen-induced decrease in PN burst amplitude from 20.3 ± 1.7 to 31.0 ± 3.2 arbitrary units (Fig. 4C).

Blockade of baclofen-induced responses. The phrenic nucleus was identified by microinjections of NMDA, as described previously (n = 6). Microinjections of 2-OH-saclofen (100 mM) at the same site increased the PN burst amplitude (P < 0.05) from a control value of 27.5 ± 3.4 (Fig. 4D) to 34.3 ± 2.9 units (Fig. 4D). Subsequent microinjections of baclofen (1 mM) within 2–3 min into the same site failed to elicit the usual decrease in the PN burst amplitude; the PN burst amplitude after the injection of baclofen was 34.7 ± 3.5 arbitrary units (Fig. 4D).

Specificity of effects of 2-OH-saclofen. In these experiments (n = 6), control value for PN burst amplitude was 33.5 ± 3.2 arbitrary units (Fig. 3B). 2-OH-saclofen (100 mM) was microinjected into the phrenic nucleus; the PN burst amplitude increased to 40.2 ± 3.4 arbitrary units (Fig. 3B) due to blockade of GABA<sub>B</sub> receptors. Muscimol (1 mM) was microinjected into the phrenic nucleus 2–3 min after the microinjection of 2-OH-saclofen; the PN burst amplitude decreased to 13.5 ± 2.5 arbitrary units due to the activation of GABA<sub>A</sub> receptors (Fig. 3B); the value of PN burst amplitude after the microinjection of muscimol (13.5 ± 2.5 arbitrary units) was significantly smaller (P < 0.05) compared with control PN burst amplitude (33.5 ± 3.2 arbitrary units), indicating that 2-OH-saclofen did not alter the responses to a GABA<sub>A</sub> receptor agonist.

The sites in the phrenic nucleus from which PN responses were elicited were marked by microinjections (50 nl) of diluted India ink; a typical site is shown in Fig. 7A. The sites from which microinjections of NMDA did not elicit a response are depicted in Fig. 7, B–D.

**Effect of GABA Receptor Agonists on Single Phrenic Neurons**

In another group of rats (n = 6), activity was recorded from 47 single phrenic neurons. As stated in MATERIALS AND METHODS, micropressure ejection of L-glutamate increased the firing of the neurons, indicating that the activity was recorded from the neuronal cell bodies and not fibers of passage. The firing of these neurons coincided with the PN bursts, indicating that their activity was related to inspiration (Fig. 5A). Muscimol (1 mM) was micropressure ejected onto 23 out of 47 neurons (48.9%). The activity of these neurons was decreased significantly (P < 0.05) from a control value of 15.2 ± 1.5 (Fig. 6A) to 0.7 ± 0.5 spikes/burst (Fig. 6A). Injection of bicuculline (1 mM) reversed significantly (P < 0.05) the inhibition induced by muscimol (1 mM; Fig. 5B) from 0.7 ± 0.5 to 19 ± 1.8 spikes/burst (Fig. 6A).

In 24 out of 47 neurons (51.1%), bicuculline (1 mM) was micropressure ejected first and muscimol was applied subsequently. Bicuculline transiently increased the firing of such neurons (Fig. 5C) from a control value of 16.0 ± 2.0 to 27.3 ± 2.5 spikes/burst (P < 0.05). The firing of these neurons returned to control (16.0 ± 2.0 spikes/burst) within 2–4 s after the micropressure ejection of bicuculline. Subsequent application of muscimol (1 mM) within 2–3 s by micropressure ejection failed to elicit the usual inhibition of the phrenic neuron (Fig. 5D); the firing remained at 18.5 ± 2.0 spikes/burst, indicating that the effects of muscimol were completely blocked by prior application of bicuculline (1 mM) on the neuron. As expected, changes in the activity of a single phrenic neuron did not alter the frequency (44.2 ± 4.0 bursts/min) or amplitude (33.8 ± 2.2 arbitrary units) of PN bursts.

In another group of rats (n = 8), activity was recorded from 58 phrenic neurons whose activity coincided with PN bursts. Baclofen (1 mM) was micropressure ejected on 33 out of these 58 neurons (i.e., 56.9%); their firing was significantly (P < 0.05) decreased from a control value of 11.9 ± 1.0 (Fig. 6B) to 3.5 ± 0.7 spikes/burst (Fig. 6B). 2-OH-saclofen (100 mM) was micropressure
ejected on these neurons within 2–4 s; a significant \((P < 0.05)\) increase in the firing from a value of \(3.5 \pm 0.7\) to \(8.4 \pm 0.9\) spikes/burst (Fig. 6B) was observed, indicating a reversal of inhibition of muscimol-induced inhibition of neuronal activity by 2-OH-saclofen.

On 25 out of 58 neurons (i.e., 43.1%) whose activity coincided with that of PN bursts, 2-OH-saclofen (100 mM) was micropressure ejected first, causing a significant \((P < 0.05)\) increase in the firing from a control value of \(10.4 \pm 1.7\) to \(13.8 \pm 1.3\) spikes/burst. The firing returned to control values within 2–4 s. Subsequent micropressure application of baclofen (1 mM) within 2–3 s on these neurons failed to elicit the usual inhibition of firing; the firing rate remained at \(10.2 \pm 0.9\) spikes/burst. The control rate (38.6 \pm 2.2 bursts/min) and amplitude (30.0 \pm 2.3 arbitrary units) of PN nerve bursts remained unaltered.

The total number of rats in which neuronal recording in the phrenic nucleus was carried out successfully was 14. As stated earlier, in these experiments also, the phrenic nucleus was first identified by microinjections of NMDA. After completion of the experiment, the neuronal recording site was marked; these sites are depicted in Fig. 7, B–D.

DISCUSSION

To describe our results in proper perspective, we present a discussion of the role of GABA receptors in respiration here. GABA has been implicated as one of the inhibitory transmitters in the neural circuits regulating respiration (3). This conclusion is based on the following reports. 1) Respiratory depression has been observed in different experimental animals after the administration of GABA receptor agonists via different routes (10, 17–19, 25, 26, 30, 31). 2) In cats, intracellular recordings of medullary respiratory neurons exhibited a hyperpolarization, decrease in input resistance, and decrease in spike frequency when GABA or baclofen was applied. 3) In cats, extracellular recordings from dorsal and ventral groups of respiratory neurons showed a decrease in the firing rate when baclofen was applied...
The aforementioned discussion reveals that most of the studies regarding the role of GABA receptors in the phrenic nucleus have been restricted to the brain stem. Phrenic motoneurons represent the final group of respiration-related neurons that integrate the outflow to the diaphragm. Relatively few studies have been reported regarding the role of GABA receptors in the phrenic nucleus (2, 14, 33). The main focus of our study was to investigate the role of GABA receptors in the phrenic nucleus. It was observed that activation of GABA\textsubscript{A} and GABA\textsubscript{B\textsubscript{2}} receptors in this nucleus by microinjections of specific agonists (muscimol and baclofen, respectively) resulted in a decrease of PN burst amplitude. There was no change in the PN burst frequency; this observation is consistent with the established notion that respiratory rhythm-generating mechanisms are located at supraspinal levels (3, 13, 23, 24).

The concentrations of GABA receptor agonists and antagonists used in this study were based on detailed concentration-response studies conducted in the phrenic nucleus. Because there are no reports in which these agents have been microinjected into this nucleus, we compared our drug concentrations with those reported for nucleus of the solitary tract (NTS). In our studies, the maximally effective concentrations of muscimol (1 mM) and baclofen (1 mM) were found to be smaller than the concentrations of muscimol (1.6 mM) and baclofen (2 mM) reported to elicit cardiovascular responses from NTS (4, 29). A concentration of 0.2–0.5 mM of bicuculline has been used by others (4, 11); however, blockade of maximally effective concentrations of muscimol was not attempted in these studies. In our study, a 1 mM concentration of bicuculline blocked the effect of a maximally effective concentration of muscimol (1 mM) in phrenic nucleus. A 20 mM concentration of 2-OH-saclofen has been reported to elicit a 90% reversal of the effect of a 0.1 mM solution of muscimol; complete blockade of a maximally effective concentration of baclofen was not attempted in that study. In our study, a 100 mM concentration of 2-OH-saclofen was necessary to completely reverse or block the response to a maximally effective concentration of baclofen (1 mM). Microinjections of bicuculline and 2-OH-saclofen increased the PN background discharge, suggesting that the phrenic nucleus is under tonic inhibitory control that is mediated via GABA\textsubscript{A} and GABA\textsubscript{B\textsubscript{2}} receptors.

At the maximal concentrations used, the specificity of our antagonists was tested. For example, a 1 mM concentration of bicuculline blocked the effects of a maximally effective concentration (1 mM) of muscimol, but it did not alter the responses to a maximally effective concentration of baclofen (1 mM). Similarly, a 100 mM concentration of 2-OH-saclofen blocked the effects of a maximally effective concentration (1 mM) of baclofen, but it did not alter the responses to a maximally effective concentration of muscimol (1 mM). Changes in PN activity due to distortion at the site of microinjection were also excluded because control microinjections of the same volumes of 0.9% saline did not alter PN activity.

NMDA was used only to identify the phrenic nucleus; investigation of the role of NMDA receptors in the phrenic nucleus was not the focus of our studies. In the...
NTS and hypothalamus, maximal cardiovascular responses have been reported to be elicited by a 0.1 mM solution of NMDA (11, 12, 21). Although microinjections of this concentration of NMDA into the phrenic nucleus elicited an increase in PN background discharge, the responses were not robust. Maximal effects were elicited by a 5 mM concentration of NMDA; therefore, we used this concentration for the identification of the phrenic nucleus. The reasons for the necessity of using higher concentrations of NMDA in the phrenic nucleus are not clear; it may be speculated that the density of NMDA receptors in the phrenic nucleus may be lesser than that in the NTS and/or a difference exists between the subtypes of NMDA receptors in the phrenic nucleus and the NTS.

The effects of microinjections of GABA receptor agonists and antagonists into the phrenic nucleus were confirmed by recording single unit activity in this nucleus. The maximally effective concentrations of the agonists and antagonists were selected from concentration-response data obtained in microinjection experiments. The drug solutions were ejected onto the phrenic neurons using micropressure technique; only one pressure pulse was applied, so that the estimated ejected volume was 1–2 nl. Both GABA\textsubscript{A} and GABA\textsubscript{B} receptor agonists caused a decrease in the firing of these neurons that was reversed as well as blocked by application of their respective antagonists. On the basis of our studies, the location of GABA receptors cannot be assigned exclusively to phrenic motoneurons; one or both types of GABA receptors may be located on the interneurons in the phrenic nucleus. Unlike in the microinjection studies, the PN burst amplitude was not altered, confirming that the volumes of ejection were minute in micropressure studies. Micropressure ejections of both antagonists, bicuculline and 2-OH-baclofen, increased the firing of single phrenic neurons, suggesting that individual phrenic neurons are under tonic inhibition mediated by GABA\textsubscript{A} and GABA\textsubscript{B} receptors.

Perspectives

The sources of inhibitory inputs to the phrenic neurons can only be speculated at this stage and remain to be investigated. Although an earlier report (14) indicated that synaptic inhibition of phrenic neurons during the expiratory phase was weak or nonexistent, subsequent studies (2, 33) indicated that the synaptic inhibition of phrenic motoneurons was significant, especially during late expiration. It has been reported that monosynaptic inhibition of phrenic motoneurons originates from the rostral medullary expiratory neuronal group designated as the Bötzinger complex; these expiratory neurons send long descending inhibitory projections to the phrenic motoneurons (20). Immunohistochemical studies have revealed the presence of GABA-like terminals, with varicosities forming a dense network of synaptic contacts on dendrites and somas of phrenic motoneurons (32). If a robust GABAergic projection from the Bötzinger complex to the phrenic nucleus exists, stimulation of the Bötzinger complex by microinjections of l-glutamate should result in an inhibition of the phrenic nucleus; this inhibition should be abolished by prior injections of GABA receptor blockers in the phrenic nucleus. This may be one of the mechanisms by which cessation of PN activity is mediated in response to the activation of cardiopulmonary C-fiber afferents.

Our results may also prove helpful in explaining the side effects of some clinically used GABAergic agents. For example, baclofen is used intrathecally to treat severe spasticity and muscle spasms due to either spinal cord trauma or multiple sclerosis. Some of the adverse reactions to intrathecal administration of baclofen include respiratory depression, dyspnea, and chest tightness. The mechanism of these respiratory effects is not clearly established. On the basis of our results, it can be hypothesized that intrathecally administered baclofen may reach phrenic motoneurons, depress their activity, and contribute to the respiratory side effects mentioned earlier. These possibilities remain to be investigated.

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