Jpn. J. Pharmacol. 82, 353 – 357 (2000)

Contribution of NMDA Receptors to Activity of Augmenting Expiratory Neurons in Vagotomized Cats

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Received December 15, 1999 *Accepted February 14, 2000*

ABSTRACT—To identify the NMDA receptor-mediated mechanism in augmenting expiratory (E2) neurons, the effects of systemic and local application of dizocilpine on spontaneous and evoked postsynaptic potentials (PSPs) were investigated in decerebrate and vagotomized cats. Intravenously applied dizocilpine reduced the inhibitory PSPs during inspiration and stage 1 expiration, but had little effect on the excitatory PSPs during stage 2 expiration. Iontophoresed dizocilpine caused a continuous hyperpolarization throughout the respiratory cycle. Dizocilpine had no effect on vagally evoked PSPs. These results suggest that the NMDA mechanisms are involved presynaptically in periodic postsynaptic inhibitions and postsynaptically in tonic excitation in E2 neurons.

Keywords: Respiratory neuron, Intracellular recording, NMDA receptor

It is known that blockade of *N*-methyl-D-aspartate (NMDA) receptors by systemic administration of dizocilpine, an antagonist of NMDA receptors, results in apneusis associated with a marked reduction in the active phase depolarization and action potential discharges in augmenting inspiratory (aug-I) and postinspiratory (PI) neurons (1, 2). However, it does not cause such a modification in the active phase of augmenting expiratory (E2) neurons (2, 3). On the other hand, iontophoresis of NMDA antagonists decreases the extracellularly recorded spike discharges in all types of respiratory neurons including E2 neurons (4). Thus, E2 neurons show different responses to systemic and local blockade of NMDA receptors, in contrast to other types of respiratory neurons. However, the previous iontophoresis study combined with extracellular recordings has an obvious disadvantage for clarifying whether the antagonist has any direct effect on postsynaptic potentials (PSPs) in E2 neurons. It is important to elucidate the NMDA receptor-mechanism in each type of respiratory neurons, because this mechanism is essential for respiratory rhythm and pattern generation (5, 6). Therefore, the present study was undertaken to identify the NMDA mechanisms in E2 neurons. We re-evaluated the effects of systemic and local application of dizocilpine on spontaneous and evoked synaptic activities recorded intracellularly using a coaxial multibarrelled microelectrode that allows simultaneous intracellular recording and extracellular iontophoresis of drugs in the vicinity of the recorded neuron (7).

Experiments were performed in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. Adult cats weighing 2.7 – 4.5 kg were used. General surgery was essentially identical to that described previously (1, 7). Under halothane anesthesia, the mid-collicular decerebration, occipital craniotomy, bi-vagotomy and pneumothorax were performed. During surgery, the depth of anesthesia was controlled by monitoring arterial blood pressure and heart rate and by assessing the absence of any movement response to noxious stimuli. The animal was paralyzed with pancuronium bromide (0.3 mg/kg initially and 0.1 mg/kg hourly) and the lungs were mechanically ventilated. The tracheal pressure was kept between 2 and 8 cmH_2O. The end-tidal concentration of CO_2 was continuously monitored and maintained at 4.0 – 4.5%. Mean arterial pressure was kept over 100 mmHg by infusing a glucose-lactate Ringer solution. Rectal temperature was maintained at 37 – 38°C by external heating. At the end of experiments, the animals were killed with an overdose of pentobarbital (>100 mg/kg, i.v.). The central respiratory activity was recorded from the desheathed phrenic nerve. A single micropipette or the center pipette of a coaxial multibarrelled electrode was filled with 2 M potassium citrate (30 – 40 MΩ) and used to record intracellular potentials. The center pipette of the multibarrelled electrode protruded 30 – 50 μm beyond the tips of the drug pipettes. E2 neu-

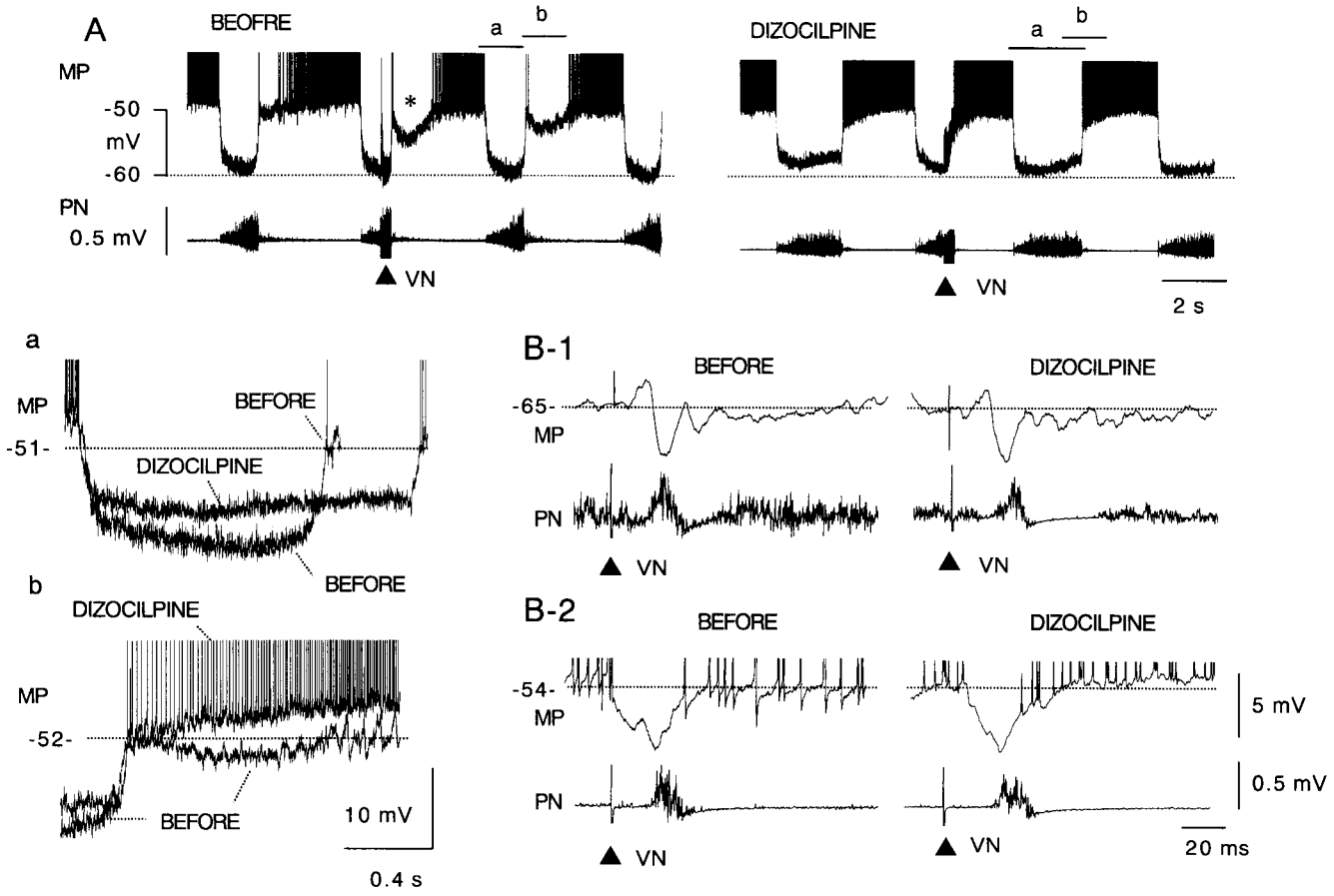

Fig. 1. Effects of dizocilpine (0.3 mg/kg, i.v.) on membrane potential and postsynaptic potentials in two E2 neurons. A: Membrane potential changes (MP) in an E2 neuron before (BEFORE) and 20 min after the intravenous injection of dizocilpine (DIZOCILPINE). Inspiratory off-switching was induced by repetitive stimulation (0.1 ms, 0.2 mA, 20 pulses at 50 Hz) of the vagus nerve (indicated by triangles and VN) at mid-inspiration. An asterisk indicated the induced postinspiratory inhibition. Superimposed traces in a and b correspond, respectively, to traces indicated by bars (a and b) in the top traces. B: Postsynaptic potentials (MP) induced in another E2 neuron by vagal stimulation before and after intravenous dizocilpine. A single pulse stimulation (0.1 ms, 0.7 mA) was applied at mid-inspiration (B-1) and mid-expiration (B-2). PN: phrenic nerve discharge.

rons were sought in the caudal ventral respiratory group, extending 2 – 3-mm lateral to the midline, 2 – 3-mm caudal to the obex, and 1.5 – 2.5-mm below the dorsal surface of the medulla oblongata (8). A single pulse stimulation (0.1-ms duration, 0.5 – 1.0-mA intensity) or a repetitive pulse stimulation (0.1 ms, 0.2 mA, 20 pulses at 50 Hz) was applied to the vagus nerve to evoke excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) in E2 neurons or inspiratory off-switching, respectively. After the completion of surgery, halothane anesthesia was discontinued. Recordings of neuronal activities were started at least 3 h after withdrawal of anesthesia. All recordings were digitized at a sampling rate of 4000 Hz, displayed on a computer display and stored on a hard disk using a signal processing software (Macintosh-MacLab/4s; ADInstruments Pty Ltd, Castle Hill, Australia). Dizocilpine (Research Biochemicals Incorporated, Natick, MA, USA) was dissolved in physiological saline and injected into the femoral vein at a dose of 0.3 mg/kg as previously described (1, 9). For iontophoretic application, dizocilpine (20 mM in saline, pH 8) and NMDA (50 mM in saline, pH 8) were filled in the peripheral pipettes of a multibarrelled electrode (7). Other two peripheral barrels were filled with physiological saline and used for drug control and current sink. Drugs were ejected with negative currents (50 – 100 nA, 10 – 180 s) and retained with a positive current (5 nA) between the test periods. When the current ejection from the saline pipette caused any change in spike discharge of the recorded neuron or when the electrotonically induced changes in the membrane potential were more than 2 mV at the onset and termination of the iontophoretic current, the data were discarded to exclude artifacts associated with iontophoretic currents. Quantitative data were averaged and expressed as the mean ± S.D. (n = the number of cells).

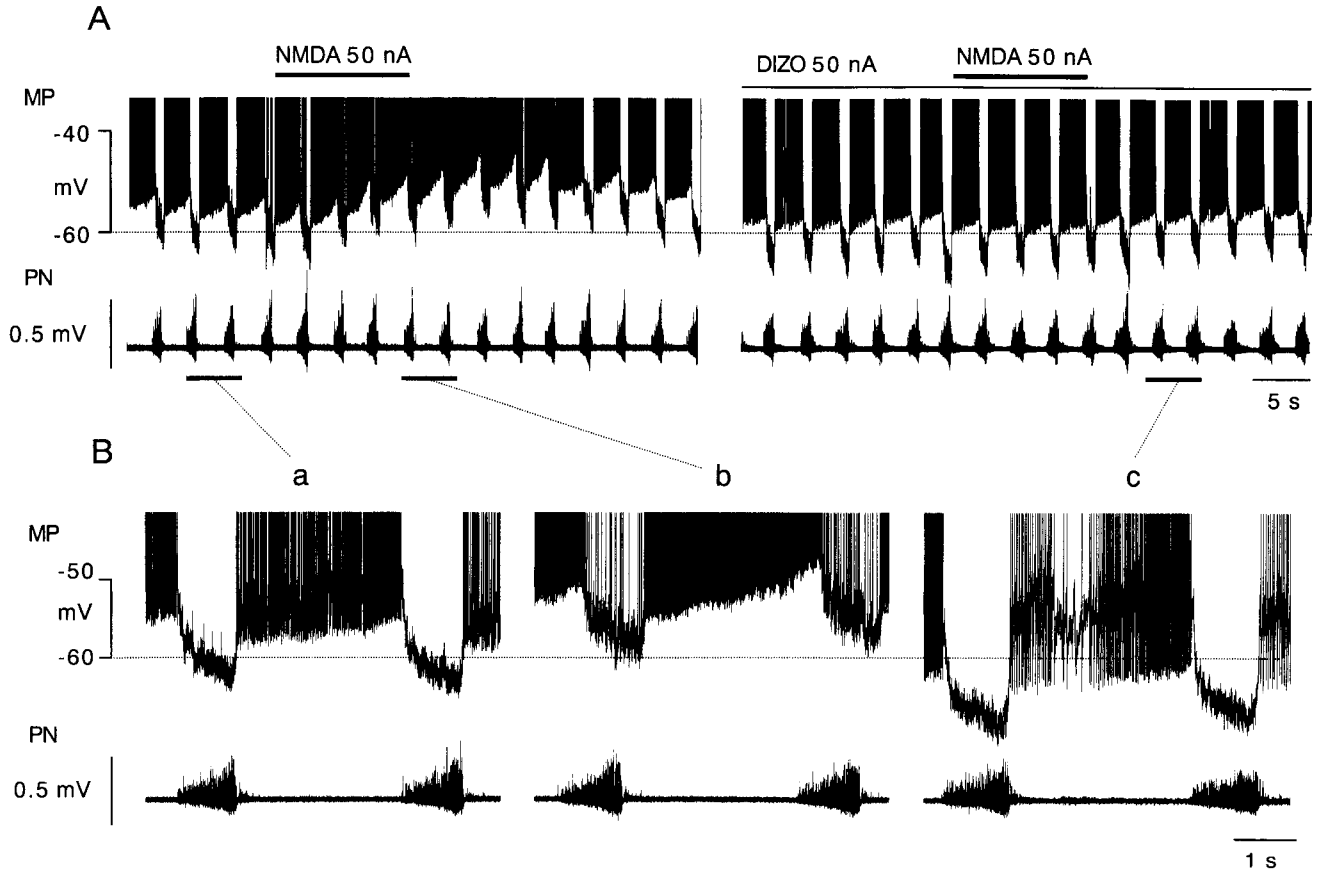

Fig. 2. Effects of extracellular iontophoresis of NMDA (50 nA, 20 s) and of dizocilpine (DIZO; 50 nA, 180 s) on membrane potential (MP) in an E2 neuron. A: Changes in membrane potential and spike discharge during iontophoresis of NMDA and dizocilpine. B: A set of the MP trace and phrenic neurogram (PN) was taken at a faster sweep speed before (a), at the end of iontophoresis of NMDA (b) and during iontophoresis of dizocilpine (c).

Differences between the paired data taken before and after the drug administration were analyzed using Student's paired *t*-test (two-tails), with significance level at $P<0.05$.

The E2 neurons typically show an augmenting depolarization and action potential discharge during stage 2 expiration (Fig. 1A). A large incrementing hyperpolarization appeared during inspiration and a rebound depolarization with a short burst of spiking occurring at the onset of stage 1 expiration (postinspiration), which was followed by postinspiratory hyperpolarization. Intravenous (i.v.) injection of dizocilpine (0.3 mg/kg) caused apneusis (Fig. 1A) as previously reported (1, 2, 9). The duration of inspiration increased from 1.68 ± 0.43 s to 4.15 ± 0.65 s ($n=8$, $P<0.01$) and that of stage 1 expiration decreased from 2.13 ± 0.53 s to 0.70 ± 0.29 s ($P<0.01$). The duration of stage 2 expiration, however, remained unchanged (3.05 ± 0.89 s before and 2.93 ± 0.75 s after dizocilpine, $P>0.05$). In E2 neurons, the stage 2 expiratory depolarization was slightly decreased (hyperpolarized), but this was not statistically significant (-52.7 ± 2.9 mV before and -53.3 ± 3.1 mV after dizocilpine; $n=8$, $P>0.05$). The spike discharge during stage 2 expiration was unaltered or rather increased (Fig. 1A, the upper-right panel). The inspiratory hyperpolarization was changed in the pattern from incrementing to decrementing and decreased in the peak amplitude from -64.2 ± 3.0 mV to -61.5 ± 3.3 mV ($P<0.05$) (Fig. 1A-a). The postinspiratory hyperpolarization became invisible, and the stage 2 expiratory depolarization ensued directly after the end of the inspiratory hyperpolarization (Fig. 1A-b).

When a single pulse stimulation was applied to the vagus nerve at mid-inspiration ($0.5-0.7$ s after the onset of inspiration), a short-latency response was induced in the phrenic nerve, consisting of early inhibition, brief excitation and late inhibition, as was previously reported (7, 10, 11). In E2 neurons, a small EPSP wave and an IPSP wave were consistently evoked in synchrony with the phrenic early inhibition and brief burst, respectively (Fig. 1B-1). The average latency, duration and amplitude of the EPSP wave were 11.8 ± 2.5 ms, 11.4 ± 1.8 ms and 2.2 ± 0.8 mV ($n=6$),

respectively. Those values for the IPSP wave were 23.0 ± 2.2 ms, 17.6 ± 3.1 ms and 4.3 ± 0.8 mV (n = 6), respectively. Vagal stimulation at mid-expiration (1.5 – 2.0 s after the end of inspiration) evoked a brief discharge in the phrenic nerve with the same time course as that of the brief excitation induced at mid-inspiration (Fig. 1B-2). The IPSP wave in the E2 neuron started to arise about 10 ms before the onset of the phrenic brief discharge. This may be due to the presence of a large stage 2 expiratory depolarization, which would mask the small and early EPSPs and unmask the early part of IPSPs. The average latency, duration and amplitude of the IPSP wave were 8.9 ± 1.6 ms, 27.8 ± 9.9 ms and 5.3 ± 0.7 mV (n = 6), respectively. These EPSP and IPSP waves, evoked by vagal stimulation at either mid-inspiration or mid-expiration, occurred in an exactly same fashion before and after the i.v. injection of dizocilpine.

Extracellular iontophoresis of dizocilpine (50 nA, 180 s) combined with intracellular recording was successful in 10 E2 neurons. It caused a continuous hyperpolarization throughout the respiratory cycle (Fig. 2). The average membrane potential was increased from −61.3 ± 4.5 mV to −66.1 ± 4.3 mV ($P<0.05$) during inspiration, from −54.4 ± 4.9 mV to −58.3 ± 4.7 mV ($P<0.05$) during stage 1 expiration and from −51.6 ± 4.0 mV to −54.5 ± 3.9 mV ($P<0.05$) during stage 2 expiration. The magnitudes of hyperpolarization, however, were statistically equivalent among the three phases of the respiratory cycle. In addition, iontophoresed dizocilpine decreased the spike discharge during stage 2 expiration in all E2 neurons (Fig. 2B-c). Finally, dizocilpine completely antagonized the effect of iontophoresed NMDA (50 nA, 20 s) (n = 5). NMDA itself caused a continuous depolarization and increased action potential discharges (Fig. 2B-b).

The present study demonstrated that iontophoresis of dizocilpine depressed the stage 2 expiratory depolarization and associating discharge activity in E2 neurons. This is in accordance with the result reported by Pierrefiche et al. (4), where iontophoresed AP7, another antagonist of NMDA receptors, decreased the extracellularly recorded spike discharges in E2 neurons. Moreover, iontophoresed dizocilpine caused a continuous hyperpolarization throughout the respiratory cycle. Since iontophoresis of NMDA produced a tonic depolarization together with an increase in spike discharge and since iontophoresed dizocilpine exerted a complete antagonism against NMDA, the above results indicate that iontophoresed dizocilpine acted directly on the recorded E2 neurons and blocked the NMDA receptor-mediated excitation. Therefore, it is evident that NMDA receptors are tonically active in E2 neurons and contribute to maintain a steady-state depolarization. It has been suggested that virtually all types of respiratory neurons receive tonic excitatory inputs (4, 14), which likely derive from the pontine respiratory structures (9, 12) and/or the central chemosensitive structures (13, 14).

In contrast to the continuous hyperpolarizing effect of iontophoresed dizocilpine, i.v. dizocilpine did not depress the stage 2 expiratory depolarization in E2 neurons, but it caused a significant decline (depolarization) of inspiratory and postinspiratory inhibitions. In addition, the postinspiratory hyperpolarization became indiscernible and the inspiratory hyperpolarization changed from incrementing to decrementing. These effects are in good agreement with the previous results (2, 3, 9). The changes in the inactive phases of the respiratory cycle may result from depressed activities of presumed presynaptic inhibitory neurons. It has been shown that i.v. dizocilpine depresses the active phase depolarization in aug-I and PI neurons (1, 2, 4) and changes the decrementing discharge pattern of early inspiratory neurons to a more continuous one (2). These neurons are thought to be presynaptic sources for these phasic inhibitions in E2 neurons, and their activity patterns after i.v. dizocilpine are well correlated to the changes in the inactive phase hyperpolarizations in E2 neurons. Since, i.v. dizocilpine does not cause any direct effect on IPSPs and since it did not cause depolarization of membrane, dizocilpine may act preferentially on the presynaptic inhibitory neurons that exert phasic inhibitions in E2 neurons. Although E2 neurons receive tonic excitatory inputs as was shown in the present study, the contribution of NMDA receptors to the active phase excitation in E2 neurons is suggested to be less than in other types of respiratory neurons. This may explain the relative inefficiency of i.v. dizocilpine on the stage 2 expiratory depolarization of E2 neurons.

A single pulse stimulation of the vagus nerve induced a series of EPSP and IPSP waves in E2 neurons. The sequence of PSPs is a mirror image of those induced in aug-I neurons by vagal stimulation (5, 15), which is due to reciprocal interactions between the two neurons (5, 6). That these induced postsynaptic responses were not affected by i.v. dizocilpine is in accordance with our previous results obtained in aug-I and PI neurons (7, 15). Therefore, these evoked PSPs are not mediated by NMDA receptors.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 09680761).

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