GABA$_A$-mediated inhibition in respiratory laryngeal motoneurons of cats

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To elucidate synaptic mechanisms underlying peripherally induced inhibitions in laryngeal motoneurons (LMNs), inhibitory postsynaptic potentials (IPSPs) evoked by electrical stimulation of the superior laryngeal nerve (SLN) were recorded in decerebrate cats during iontophoresis of inhibitory amino acid antagonists. Two types of LMNs displaying respiratory-related activities were classified as inspiratory (IN)- and postinspiratory (PI)-LMNs. Stimulation of SLN during inspiration produced a wave of IPSPs in IN-LMNs but not in PI-LMNs. This wave occurred at the latency of 8.5 ± 0.7 ms (n=25), coincident with a transient inhibition of the inspiratory discharge evoked in phrenic (PN) and recurrent laryngeal nerves (RLN). Stimulation during expiration produced IPSPs in PI-LMNs at the latency of 13.6 ± 1.9 ms (n=15) but not in IN-LMNs. This IPSP wave corresponded to inhibition of the expiratory discharge of RLN and to a transient burst-discharge in PN. The amplitudes of IPSPs evoked in IN- and PI-LMNs were decreased by iontophoresed bicuculline but not by strychnine. The present results suggest that laryngeally evoked IPSPs in IN- and PI-LMNs are mediated polysynaptically through the central respiratory network and $GABA_A$ mechanisms are involved in their final pathway.

Keywords: laryngeal motoneurons, IPSPs, $GABA_A$ receptors, microiontophoresis

Intoduction

Laryngeal motoneurons (LMNs) are located in the ventrolateral parts of the medulla oblongata adjacent to or within the nucleus ambiguus. They send axons through laryngeal motor nerves and govern the movements of upper airway muscles[1, 2]. For instance, cricothyroid muscles are innervated through the superior laryngeal nerve (SLN) and thyroarytenoid muscles through the recurrent laryngeal nerve (RLN). These muscles behave to open or close the upper airway in synchrony with the respiratory rhythm. These LMNs display a respiratory rhythmic activity and, therefore, are designated as respiratory LMNs[3-5].

Chemical or mechanical stimulation of laryngeal mucosa induces laryngeal reflexes, which are associated with a corresponding change in the respiratory rhythm. In experimental animals, electrical stimulation of laryngeal afferents during inspiration elicits a transient inhibition in the phrenic nerve (PN) discharge and stimulation during expiration prolongs the expiratory period[6-10]. Also, these stimuli evoke postsynaptic potentials in various types of bulbar respiratory neurons. Previous studies have focused mainly on the short-latency excitatory postsynaptic potentials (EPSPs) evoked in non-laryngeal inspiratory neurons[11-13]. Although the evoked inhibitory postsynaptic potentials (IPSPs) are assumed to contribute largely to the laryngeal inhibitory reflexes and to the respiratory rhythm changes, they have not been fully evaluated[6,9,14]. To characterize the possible presynaptic sources for such IPSPs and their pathways within the respiratory network is important to understand the electrophysiological and pharmacological profiles of these potentials.

In the present study, the laryngeally evoked IPSPs were recorded from two types of respiratory LMNs and the effects of iontophoretically applied antagonists of putative inhibitory neurotransmitters were examined in decerebrate, vagotomized and artificially ventilated cats. We used a co-

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axial multibarrelled microelectrode, which allowed simultaneous intracellular recording and extracellular iontophoresis of drugs close to the impaled cell *in vivo*[15,16].

MATERIALS AND METHODS

General Surgery

Experiments were performed on adult cats of either sex, weighing 2.3-4.0 kg, in accordance with Guiding Principles for the Care and Use of Laboratory Animals Approved by the Japanese Pharmacological Society. The animals were anesthetized with halothane evaporated in the oxygen-enriched air. The concentration of halothane was set at 2.0-2.5% during induction and at 1.5-1.8% during surgery. The depth of anesthesia was controlled by confirming the absence of any movement response to noxious stimuli and by monitoring systemic blood pressure and heart rate. Tracheal intubation was performed through tracheostomy below the larynx. Polyethylene cannulae were inserted into the femoral artery, femoral vein and urethra. To secure hemostasis during decerebration, the external carotid arteries were ligated distal to the branching of the lingual artery. The head of the animal was placed on a stereotaxic frame, and decerebration was performed by aspirating the brain rostral to the mid-collicular transection. After decerebration, the animals were paralyzed with pancuronium bromide (0.3 mg/kg initially and 0.1 mg/kg hourly). The lungs were artificially ventilated with oxygen-enriched air. The ventilatory stroke volume (10 ml/kg body weight) and frequency (18-30 strokes/min) were adjusted to maintain the fractional concentrations of end-tidal O_2 and CO_2 in the range of 0.29-0.31 and 0.04-0.05, respectively. An expiratory flow resistance of 1-2 cm H_2O was applied to prevent collapse of the lungs. Mean arterial blood pressure was maintained over 100 mmHg by infusing a lactate-glucose Ringer solution as required. Rectal temperature was kept at 37-38℃ by external heating.

PNs were isolated from the C5 spinal root and cut distally. The central cut-ends were desheathed and placed on bipolar silver electrodes immersed in warmed mineral oil. The vagus nerves were cut distally after giving off RLNs. In addition, SLNs were dissected bilaterally. The central ends of RLNs and SLNs were placed on bipolar silver electrodes. The dorsal surface of the medulla oblongata was exposed by occipital craniotomy. Bilateral pneumothoraxes were performed to minimize movements of the brainstem associated with ventilation. After the surgery, halothane anesthesia was discontinued and a minimum of 3 hr elapsed before neuronal activities were recorded.

Recording procedures

Efferent discharges of PN and RLN were amplified (30-3000 Hz band-pass), rectified and integrated by a leaky integrator (0.1 s time constant) for monitoring the central respiratory rhythm. Membrane potentials were recorded from respiratory LMNs penetrated by a single glass micropipette or the center recording pipette of a coaxial multibarrelled microelectrode[16]. The recording electrodes were filled with 2 M potassium citrate, having a resistance of 20-40 MΩ. LMNs were identified by antidromic stimulation of the ipsilateral SLN and RLN using a square wave pulse of the suprathreshold intensity (0.1 ms duration and 0.2-0.3 mA intensity), according to the criteria described previously[17,18]. The location of a recorded neuron was given by the stereotaxic coordinate from the topographic atlas of Berman[19]. To evoke IPSPs in LMNs, square wave pulses of a 0.1-ms duration were delivered to the ipsilateral SLN during inspiration (300-500 ms after the onset of inspiration) and during expiration (300-500 ms after the end of inspiration). The current intensity (0.3-1.0 mA) for this stimulation was selected at 1.5 times the threshold to be sufficient to induce a transient inhibition in PN discharge during inspiration and a temporal discharge during expiration. Since the amplitudes of IPSPs are changeable depending on changes in membrane potential and since the specific antagonists for inhibitory neurotransmitters depolarize the membrane of respiratory neurons[20,21], we measured the amplitudes of evoked IPSPs at the same membrane potential adjusted by current clamping after drug application. Clamping currents were passed intracellularly through a recording pipette using a high-frequency current injection and voltage-sampling method. All recordings were stored on magnetic tape and played back later for computer analysis using signal processing software.

Administration of drugs

The multibarrelled microelectrode consisted of an array of 7 drug-pipettes encircling the center recording pipette, the tip of which protruded 40-50 μm beyond the tip of the drug pipettes[16]. Solutions of physiological saline (165 mM NaCl), bicuculline methiodide (5 mM in saline, pH 3), strychnine sulfate (5 mM in saline, pH 7.8), γ-

aminobutyric acid (GABA; 0.5 M in distilled water, pH 4.5) and glycine (0.5 M in distilled water, pH 4.0) were contained in the drug pipettes. All drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The resistance of the drug pipettes ranged from 10 to 50 MΩ. Drugs were ejected with positive currents (50-100 nA), and a retaining current of -10 nA was applied between test periods. The saline pipette was used as a current sink and a drug control.

Data analysis

To measure the latency, duration and peak amplitude of evoked IPSPs, peri-stimulus averages of membrane potentials were taken at 5 consecutive respiratory cycles. The membrane potential just before the stimulation was used as a reference to measure these variables. Data obtained before and after the administration of each drug were averaged for each group of LMNs and expressed as means ± SEM (n=number of cells). Differences between the mean values were evaluated using a paired *t*-test (two sided). Statistical significance was assumed at $P<0.05$.

RESULTS

Responses of phrenic and reccurent laryngeal nerves induced by stimulation of superior laryngeal nerve

As shown in Fig. 1A, PN and RLN displayed augmenting discharges during inspiration and decrementing discharges during postinspiration (stage 1 expiration).The postinspiratory activity was more prominent in RLN than in PN. Both nerves showed no activity during the later part (stage 2) of expiration. Electrical stimulation of SLN provoked two types of responses in PN, depending on the time of stimulation during the respiratory cycle. When a single pulse was delivered during inspiration, a transient inhibition of inspiratory discharge occurred (see the PN trace in Figs 1B and 3). This inhibition started 10.3 ± 0.9 ms after the stimulus pulse and lasted 30.1 ± 5.1 ms (n=10). In some cases, a small excitation (latency; 4.9 ± 0.1 ms, n=6) was evoked prior to the inhibition. When a stimulus pulse was applied during the expiration, a burst discharge appeared 17.6 ± 1.8 ms (n=10) after stimulation and lasted 16.1 ± 1.4 ms (Figs 1B and 4). In four cases, a short and weak burst-discharge occurred 7-11 ms before the onset of the larger burst-discharge.

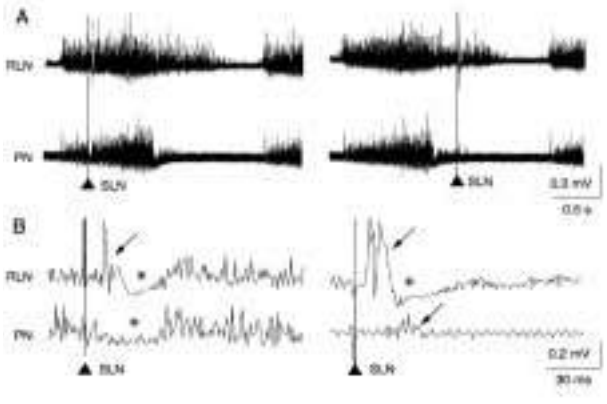

Fig.1. Responses of recurrent laryngeal (RLN) and phrenic nerves (PN) to a single pulse stimulation (0.1 ms duration, 0.5 mA intensity) of the superior laryngeal nerve (SLN) during inspiration (left panel) and during expiration (right panel). Traces in B were taken with a faster sweep speed and averaged at 5 consecutive respiratory cycles. The evoked excitations were indicated by arrows and inhibitions by asterisks.

SLN stimulation evoked a rather uniform response in RLN, irrespective to the time of stimulation (Fig.1). The response consisted of an excitation followed by an inhibition. The early excitation started 5-7 ms after stimulation and the late inhibition occurred 12-20 ms after stimulation. The duration of the late inhibition was 18-25 ms.

Thus, SLN stimulation during inspiration caused inhibition of the inspiratory discharge in both PN and RLN, while stimulation during expiration evoked a transient excitation in PN and inhibition of the postinspiratory discharge in RLN. In addition, SLN stimulation caused a short-latency excitation in both nerves irrespective to the time of stimulation during the respiratory cycle.

Identification of laryngeal motoneurons

Two types of LMNs displaying respiratory-related activities were recorded. Twenty-seven neurons showed augmenting depolarization associated with spike activity during inspiration, which were classified as inspiratory (IN)-LMNs (Fig. 2A1). The membrane potential measured at the expiratory silent phase was -65.7 ± 2.8 mV. The remaining 16 neurons showed a rapid and large depolarization associated with a high frequency discharge at the onset of expiration followed by repolarization during the remainder of expiration. They were classified as postinspiratory (PI)-LMNs, whose membrane potentials measured at the inspiratory silent phase were -64.9 ± 3.1 mV (Fig. 2A2). All neurons recorded were antidromically excited from RLN. Antidromic action potentials were distinguished by their constant and short latency (<3 ms), the absence of synaptic pre-potentials, and the positive collision to spontaneous action potentials (Fig. 2B). Both types

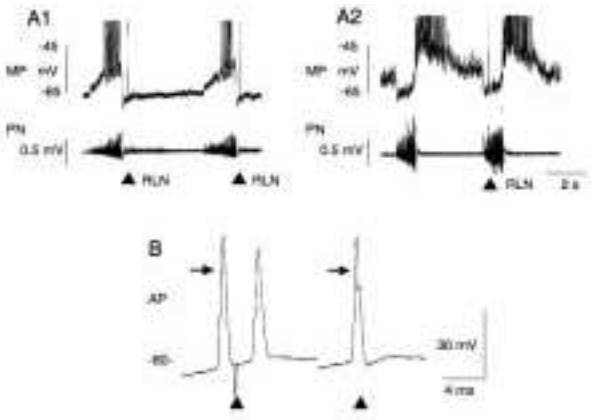

Fig.2. Membrane potentials (MP) of an IN-LMN (A1) and a PI-LMN (A2) combined with phrenic nerve discharges (PN). Electrical stimulation (0.2 mA intensity, 0.1 ms pulse duration) was applied to the ipsilateral recurrent laryngeal nerve (RLN). B: A collision test. RLN stimulation applied 2.5 ms after the onset of spontaneous spike (indicated by an arrow) provoked an antidromic spike. No antidromic spike was induced by RLN stimulation applied 1 ms after the onset of spontaneous spike.

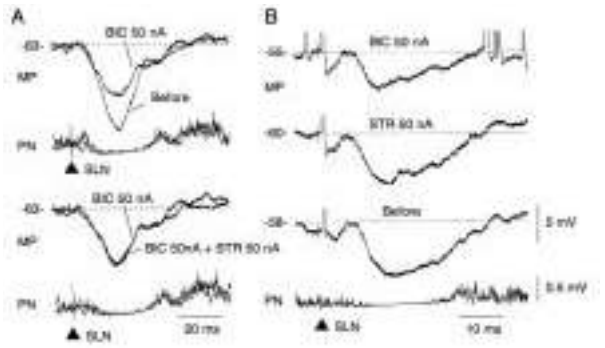

Fig.3. Effects of iontophoresed bicuculline and strychnine on IPSP waves (MP) induced in two IN-LMNs by electrical stimulation of the superior laryngeal nerve (SLN). A: The upper panel shows IPSPs taken before (Before) and during iontophoresis of bicuculline (BIC 50 nA), and the lower panel shows IPSPs taken during iontophoresis of bicuculline and during co-iontophoresis of bicuculline and strychnine (BIC 50 nA + STR 50 nA). B: Traces were obtained before (Before), during iontophoresis of strychnine (STR 50 nA) and during iontophoresis of bicuculline (BIC 50 nA). Traces were cycle-triggered averagings of 5 consecutive respiratory cycles.

of LMNs were found intermingled near and within the nucleus ambiguus.

IPSPs evoked in laryngeal motoneurons

Electrical stimulation of SLN during inspiration provoked a wave of IPSPs in all IN-LMNs (Fig. 3) but not in all PI-LMNs tested. These waves were identified as IPSPs, since they hyperpolarized the membrane, blocked the generation of action potential and decreased in size with hyperpolarizing current injection. In addition, the IPSPs were preceded by a short-lasting wave of EPSPs evoked in all IN-LMNs tested (Fig. 3). The IPSPs evoked in IN-LMNs during inspiration appeared at the latency of 8.5 ± 0.7 ms and lasted 30.5 ± 4.0 ms (n=25). Thus, the time course of SLN-induced postsynaptic response in IN-LMNs correlated to the evoked response in RLN (Fig. 1B). In PI-LMNs, stimulation during inspiration provoked a long-lasting wave of EPSPs, the time course of which corresponded to the period combined early EPSPs and late IPSPs evoked in IN-LMNs and also to the period of inhibition in PN (data not shown).

When SLN was stimulated during expiration, a wave of IPSPs was induced only in PI-LMNs (Fig. 4). This wave appeared at the latency of 13.6 ± 1.9 ms and lasted 27.3 ± 4.1 ms (n=15). The IPSP wave was preceded by a short-lasting wave of EPSPs evoked in all PI-LMNs. The sequential response of PI-LMNs was comparable with that of RLN (Fig.1). In IN-LMNs, stimulation of SLN during expiration induced a series of EPSPs (data not shown). The burst discharge evoked in PN during expiration occurred coincident with the IPSPs in PI-LMNs and with the EPSPs in IN-LMNs.

Since the short-latency EPSPs evoked in both types of LMNs have been well described [11-13], the following study focused on IPSPs evoked in IN-LMNs during inspiration and those provoked in PI-LMNs during expiration.

Effects of iontophoresed bicuculline and strychnine on the IPSPs

Iontophoresis of bicuculline (50-100 nA, 60-120 s) was tested on 17 IN-LMNs and 6 PI-LMNs, and strychnine (50-100 nA, 60-120 s) on 8 IN-LMNs and 4 PI-LMNs. Since either agent itself produced depolarization of the membrane and an increase of action potential firing, the membrane potential was off-set to the pre-iontophoresis potential by current clamping during iontophoresis. Figure 3A illustrates the results of such an experiment, showing an apparent inhibitory effect of bicuculline on the IPSPs evoked in IN-LMNs during inspiration. Additional iontophoresis of strychnine caused no more decrease of the IPSP wave. Strychnine by itself had no significant effect on these IPSPs (Fig. 3B). In PI-LMNs, the IPSPs induced by SLN stimulation during expiration were decreased by bicuculline but not by strychnine (Fig. 4). These figures also show that both antagonists had no effect on the evoked EPSPs. In summary, bicuculline decreased the

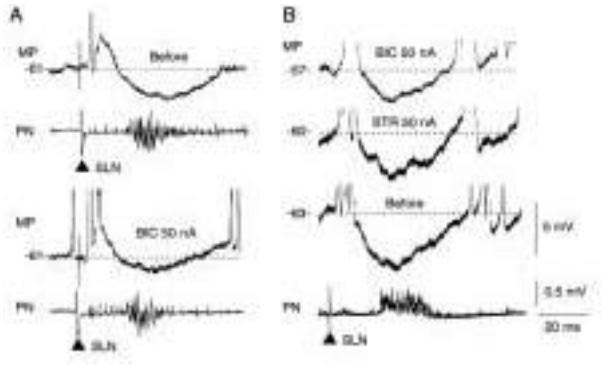

Fig.4. Effects of iontophoresed bicuculline and strychnine on IPSP waves (MP) induced in two PI-LMNs by electrical stimulation of the superior laryngeal nerve (SLN). A: Traces were taken before (Before) and during iontophoresis of bicuculline (BIC 50 nA). Membrane potential during iontophoresis was adjusted to the pre-iontophoresis potential by current clamping. B: Traces were obtained before (Before), during iontophoresis of strychnine (STR 50 nA) and during iontophoresis of bicuculline (BIC 50 nA). Traces were cycle-triggered averagings of 5 consecutive respiratory cycles.

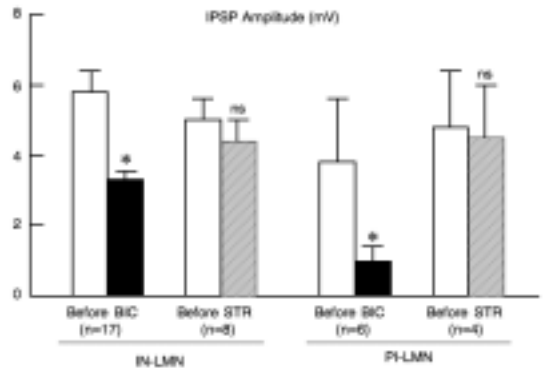

Fig.5. Effects of iontophoresed bicuculline and strychnine on IPSP waves evoked in IN-LMNs during inspiration and in PI-LMNs during expiration by SLN stimulation. Each column shows the mean ± SEM (vertical short bars). n=number of cells examined. *P<0.05, significant difference from the corresponding before value (paired *t*-test). ns: no significant difference.

amplitudes of IPSPs evoked in both types of LMNs and strychnine had no significant effect on them (Fig. 5).

The selectivity of each antagonist was tested with its agonist. As described previously [20,21], iontophoresis of GABA caused hyperpolarization associated with suppression of firing, and this effect of GABA was selectively blocked by co-iontophoresis of bicuculline but not by strychnine. *Vise versa*, the inhibitory effect of glycine was completely blocked by co-iontophoresed strychnine but not by bicuculline (data not shown).

DISCUSSION

Laryngeally evoked IPSPs in laryngeal motoneurons

The present study demonstrated that electrical stimulation of the superior laryngeal afferent nerve produced IPSPs in IN- and PI-LMNs only during their active phase of the respiratory cycle. The IPSPs evoked in one type of LMNs during their active phase corresponded to the wave of EPSPs evoked in the other type of LMNs during their inactive phase. This result suggests the reciprocal interaction between IN- and PI-LMNs. However, it is unlikely that the two types of LMNs are directly coupled with inhibitory synaptic connections via axon collaterals. Since the reciprocal inhibition between non-laryngeal IN and PI neurons have been demonstrated [23-26], LMNs may receive the inhibitory inputs from non-laryngeal presynaptic neurons whose activities are out of phase with either type of LMNs. Since the average latency of the evoked IPSPs was 9 ms in IN-LMNs and 14 ms in PI-LMNs, they are mediated through polysynaptic pathways within the central respiratory network. Furthermore, the conduction times of evoked IPSPs differed remarkably between IN-LMNs and PI-LMNs, suggestive of two different pathways.

Electrophysiological features of the evoked IPSPs were identical to those of IPSPs spontaneously occurring in virtually all respiratory neurons during their inactive phase [5,20-22]. IPSPs were not induced in respiratory LMNs when stimulation was applied during their inactive phase. This may be due to the fact that since membrane potentials of the recorded LMNs during their inactive phase are close to the equilibrium potential for the IPSPs, the evoked IPSPs, if any, are undetectable. Another possibility is that afferent signals are gated in presynaptic neurons by periodic inhibitions occurring at that phase of the respiratory cycle.

The short latency EPSPs are evoked by SLN stimulation irrespective to the type of LMNs and the time of stimulation. This wave is in accordance with the early excitation evoked in RLN and PN. It has been reported that stimuli of laryngeal afferents as well as vagal stretch receptor afferents provoke a short-latency wave of EPSPs in respiratory neurons recorded in the vicinity of the nucleus tractus solitarius [8,11,12,27]. These previous studies suggest that this early excitation can be conducted to the ventral respiratory group neurons through an oligosynaptic pathway. The early EPSPs observed in respiratory LMNs seem to be a similar type of oligosynaptic excitatory response.

Implications of IPSPs on laryngeal reflexes during

inspiration and expiration

Chemical or mechanical stimulation of laryngeal mucosa induces laryngeal defensive responses in synchrony with the occurrence of the respiratory rhythm changes[12,13,28,29]. In the present study, the response of RLN to SLN stimulation during inspiration accorded with that of IN-LMNs and the response of RLN during expiration did with that of PI-LMNs. The results demonstrated that the reflex activity of RLN was derived from either IN- or PI-LMNs, depending on the time of stimulation during the respiratory cycle. Simultaneously, the SLN stimulation during inspiration caused a transient inhibition in PN that corresponded to the occurrence of IPSPs in IN-LMNs and of EPSPs in PI-LMNs. During expiration, SLN stimulation induced a transient burst-discharge in PN which corresponded to IPSPs in PI-LMNs and EPSPs in IN-LMNs. This indicates that, during inspiration, the contraction of laryngeal adductor muscles are inhibited and abductor muscles are led to contract in synchrony with a pause of diaphragm contraction. During expiration, the contraction of abductors is inhibited and adductors are led to contract in synchrony with a transient excitation of diaphragm. Thus, those IPSPs elicited in both types of LMNs play a significant role in laryngeal defensive responses closely related to breathing acts.

Transmitter candidates for evoked IPSPs

The most possible transmitter candidate for spontaneous IPSPs in bulbar respiratory neurons is GABA[15,20,21,25,30]. These spontaneous IPSPs display an electrophysiological feature identical to the hyperpolarization induced by iontophoretically applied GABA, and both the IPSPs and GABA-induced hyperpolarization are similarly blocked by co-iontophoresed bicuculline[15,20,21]. These results indicate that the IPSPs in respiratory neurons are mediated by GABA through GABA$_A$ receptors. Furthermore, GABA-immunoreactive synaptic vesicles were found in the dendrites projecting to the respiratory neurons of the medulla oblongata[31].

The present study clearly shown that bicuculline decreased the IPSPs evoked by electrical stimulation of SLN, but strychnine had no effect on them. This strongly suggests that laryngeally induced IPSPs in LMNs are generated by activation of GABA$_A$ receptors. However, the effect of bicuculline was not complete. This may be due to the insufficiency of bicuculline concentration at active sites to block the effect of endogenously released GABA, because of the location of synaptic contact being remote from the recording site (soma)[15,20,21]. Furthermore, it has been reported that the phrenic inhibitory response induced by SLN stimulation was suppressed by intravenously injected strychnine but not by bicuculline, suggestive of glycinergic mechanisms involved in some sites responsible for this inhibitory response[32]. The present results, however, have clearly documented that GABA-mediated synaptic transmissions contribute to the final pathway.

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