Trigeminal reflex regulation of the glottis depends on central glycinergic inhibition in the rat

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Received 17 August 2001; accepted in final form 21 November 2001

Dutschmann, Mathias, and Julian F. R. Paton. Trigeminal reflex regulation of the glottis depends on central glycinergic inhibition in the rat. Am J Physiol Regulatory Integrative Comp Physiol 282: R999–R1005, 2002; 10.1152/ajpregu.00502.2001.—In an unanesthetized decerebrate in situ arterially perfused brain stem preparation of mature rat, strychnine (0.05-0.2 μM) blockade of glycin receptors caused postspiratory glottal constriction to occur earlier, shifting from early expiration to inspiration. This resulted in a paradoxical inspiratory-related narrowing of the upper airway. Stimulation of the trigeminal ethmoidal nerve (EN5; 20 Hz, 100 μs, 0.5–2 V) evoked a diving response, which included a reflex apnea, glottal constriction, and bradycardia. After strychnine administration, this pattern was converted to a maintained phrenic nerve discharge and a reduced glottal constriction that was interrupted intermittently by transient abductions. The onset of firing of postspiratory neurons shifted from early expiration into neural inspiration in the presence of strychnine, but neurons maintained their tonic activation during EN5 stimulation, as observed during control. Inspiratory neurons that were hyperpolarized by EN5 stimulation in control conditions were powerfully excited after loss of glycinergic inhibition. Thus the integrity of glycinergic neurotransmission within the pontomedullary respiratory network is critical for the coordination of cranial and spinal motor outflows during eupnea but also for protective reflex regulation of the upper airway.

Ventral respiratory group; upper airway patency; synaptic inhibition; diving response

The network model for respiratory rhythm generation comprises three phases of respiration: inspiration, postinspiration, and expiration. During eupnea, there is a respiratory modulation of the upper airway such that the vocal fold is dilated (abducted) to decrease airway resistance during inspiration and constricted (adducted) during early expiration or postinspiration. This postspiratory glottal constriction is particularly important for slowing expiratory airflow out of the lungs to allow time for efficient gas exchange and to maintain functional residual capacity, thereby preventing lung collapse (for review, see Ref. 3). Moreover, postspiratory laryngeal adductor motoneurons play a vital role in the mediation of defensive reflexes of the upper airway, such as cough (10–11, 26, 30), sneeze (23, 25), protective apnea (9, 28), and the Hering Breuer reflex (12, 29). Interestingly, recent in situ and in vitro studies reported a shift in the firing of postspiratory neurons from early expiration to inspiration after either blockade of glycinergic neurotransmission (5, 8) or hypoxia (8, 16) in neonatal rat and mature mice. The consequence of such a shift in firing on defensive reflex regulation of the upper airway is unknown but may have serious functional implications.

In the mammal one of the most potent upper airway protective reflexes occurs during diving. Activation of nasotrigeminal afferents evokes a diving response (7, 17) that includes a profound constriction of the glottis (15) to ensure that water cannot enter the bronchi. In the present study, we assessed the role of central glycinergic neurotransmission for the functional integrity of upper airway adduction during the diving response. We show that both the ongoing eupneic modulation of the upper airway and the reflexly evoked glottic closure during a diving response are massively disrupted after blockade of glycinergic neurotransmission.

MATERIALS AND METHODS

Preparation of animals. Experiments were performed on mature rats (Wistar, 70–100 g) of either sex. We employed the intra-arterially perfused working heart-brain stem preparation (WHBP). This preparation allows a spectrum of analyses (intracellular recording to measures of glottal function) in a single preparation. With the near absence of an arterial pulse, there is improved mechanical stability for sharp microelectrode impalements of brain stem neurons. Furthermore, the preparation permits application of toxic drugs systemically without the complications of changes in arterial pressure that could confound interpretation of data. In the perfused rat, arterial pressure is clamped constant despite the presence of a lethal drug. The preparation was originally designed to allow an in vivo-like analysis in an in vitro environment. The work fully conforms with the guiding principles for research involving animals of the American Physiological Society.

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A detailed description of the WHBP was published (18), and only a brief account is given here. Rats were anesthetized deeply in a saturated atmosphere of halothane. Once respiration was depressed and the animal failed to respond to a noxious pinch of the tail or a toe, a phrenic nerve (PN) was sectioned below the diaphragm. In ice-chilled Ringer solution gassed with 95% O2-5% CO2 (carbogen), rats were decerebrated at the precollicular level and cerebellectomized. After the aorta was cannulated and perfused using a peristaltic pump, the preparation was transferred to a recording chamber. The descending pressure. The osmolarity of the artificial cerebrospinal fluid as well as 1.25% Ficoll (Sigma) to maintain colloid osmotic pressure was 7.35/11006 was 298/H11006 was 5 mosmol/l, and on gassing with carbogen the pH was 7.35 ± 0.05. To block glycinergic inhibition, we added strychnine (0.05–0.2 μM; Sigma) to the perfusate. The concentration employed was based on that reported to be specific for glycine receptors (14) and found effective in the WHBP previously (5).

Stimulation of trigeminal afferents. The diving response was evoked by electrical stimulation of the trigeminal ethmoidal nerve (EN5). After removal of the olfactory bulb, the EN5 was identified as being laterally attached to the osnasale. The bare end of a Teflon-insulated silver wire was wrapped around the nerve. The wire and nerve were isolated electrically in low-melting-point paraffin wax applied topically. The indifferent electrode was placed into the orbital cavity. Electrical stimulation was performed with a 10-s train of stimuli at a frequency of 20 Hz (100-μs pulses and 0.5–2 V).

Recording of cardiovascular and respiratory parameters. Perfusion pressure within the aorta was monitored via one port of the double-lumen catheter. This lumen was connected to a pressure transducer, and pressure was set to 70–90 mmHg by adjusting pump flow rate.

To obtain an index of glottal resistance, we perfused the larynx with a constant stream of warmed, humidified oxygen in the expiratory direction via a tracheal cannula with its tip placed below the larynx pointing toward the buccal cavity (19). Subglottal pressure (SGP) was recorded from a side arm of this cannula. Increases and decreases in SGP pressure were indicative of constriction and dilatation, respectively, thereby giving a direct index of the dynamic changes in glottal resistance during the respiratory cycle. The exact airflow for SGP measurements was set for each preparation to obtain a normal respiratory modulation of glottal resistance (i.e., inspiratory dilatation and early-expiratory constriction). Because SGP was measured indirectly via a side arm catheter of relatively high resistance, the pressures measured will be in excess of those within the airway. It was not our aim to determine the absolute SGP, but rather its relative changes during the respiratory cycle. In preparations where SGP was measured, no neuromuscular blocker (see above) was used.

In all experiments, a PN was cut at the level of the diaphragm, and the discharge was recorded from its central end using a glass suction electrode. Rhythmic and ramping PN discharge persisted for 5–6 h. An electrocardiogram was recorded simultaneously with the PN, and instantaneous heart rate was calculated from the R-R interval using a window discriminator and Spike 2 software (CED). Peripheral nerve activity was amplified and filtered (8 Hz to 3 kHz) and integrated (time constant, 100 ms) using the CED Spike 2 program. All signals were displayed on a computer using a 1401 interface (CED).

Neuron recording. Respiratory neurons were recorded intracellularly from the ventrolateral region of the medulla oblongata using fine-tipped glass microelectrodes filled with either 3 M potassium chloride or 3 M potassium methyl sulfate containing 5 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (Sigma). The input resistance of the electrodes varied between 40 and 80 MΩ. The neuronal activity was amplified (Axon Instruments) and via a 1401 interface displayed and stored on a computer running Spike 2 software (CED). In cases where an intracellular impalement failed and the neuron was not damaged, we analyzed the extracellularly recorded action potentials. The microelectrodes were aimed for the ventral respiratory group and placed 1–2 mm rostral to calamus, 1.8–2.5 mm lateral to midline, and 1.5–2.5 mm from the dorsal surface of the brain stem.

Data analysis. We measured PN activity (PNA) cycle length, heart rate, and SGP. The effect of strychnine on the eucapnic modulation of the glottis was analyzed by comparing the peak responses of SGP during inspiration, postinspiration, and late expiration for 30 respiratory cycles both in control and 5 min after administration of strychnine. The strychnine-evoked shift of postsipiratory glottal constriction into inspiration was quantified by comparing the amount of PNA (integrated) that occurred during the onset to peak of the glottal constriction for 30 respiratory cycles both in control and 5 min after administration of strychnine. For EN5 reflex-evoked responses, control data were taken 10 s before the stimulus and compared with the average value recorded during stimulation. Three reflex responses were compared before and during strychnine application in each preparation. Interruptions in the EN5-evoked glottal dilatation or additional contractions superimposed on EN5-evoked constriction before and after strychnine were also analyzed. These were defined by decreases or increases of SGP of <20 mmHg and <0.5 s in duration. Statistical analysis of the effects of strychnine was performed with repeated-measures ANOVA followed by Tukey’s honestly significantly different post hoc test. All data are expressed as means ± SE, and n refers to the number of preparations or neurons recorded. Differences were taken as significant at 95% confidence level.

RESULTS

Influence of strychnine on eucapnic glottal modulation. We analyzed the effect of strychnine administration on the eucapnic modulation of glottal resistance during constant air perfusion of the upper airway in the expiratory direction. The glottis showed a respiratory modulation characterized by a decrease in SGP during inspiration, corresponding to glottal dilatation, and an increase in SGP during postinspiration, indicative of a glottal constriction. During the expiratory interval, SGP decreased steadily to a plateau level (Fig. 1A). Blockade of glycinergic neurotransmission
significantly reduced the postinspiratory-related increase in SGP from 83 ± 2.1 to 59.3 ± 1.1 mmHg (Fig. 1; P < 0.01). Furthermore, strychnine reduced the inspiratory dilatation as revealed by an elevation of SGP levels during this phase from 14.8 ± 1.8 to 22 ± 1.4 mmHg (Fig. 1; n = 5, P < 0.05).

The most prominent effect of strychnine was seen on the timing of respiratory modulation of the glottis. After blockade of glycine receptors, the glottal constriction appeared earlier relative to PNA and coincided with neural inspiration (Fig. 1B). This was quantified by comparing the amount of integrated PNA from onset to peak of postinspiratory glottal constriction (compare dotted lines in Fig. 1, A and B). In the presence of strychnine, integrated PNA coinciding with glottal constriction increased from 23 ± 1.9 to 80.6 ± 2.9 µV/100 s or 250 ± 48% (Fig. 1B; n = 5; P < 0.01). The latter indicated that blockade of glycinergic inhibition caused an earlier onset for the glottic constriction during the respiratory cycle, which, paradoxically, coexisted with neural inspiration.

**Influence of strychnine on the nasotrigeminal reflex.** The nasotrigeminal reflex as evoked by electrical stimulation of the EN5 produced expiratory apnea accompanied by intense glottal constriction and bradycardia (Fig. 2). Strychnine converted the EN5-evoked apnea to a stimulus-locked tonic discharge in the PN. Integrated PNA during the stimulus period increased significantly from 39 ± 8.7 µV/100 ms during control to
activity of postinspiratory neurons from early expir- sence results in a dramatic shift in the onset of spiking postinspiration from inspiration such that their ab- phases. Glycine receptors act functionally to segregate the integrity of glycine receptors within the brain stem is coincident with the end of the inspiratory phase (Fig. 3).

The EN5-evoked bradycardia was increased from −131.5 ± 6 beats/min during control to −173.7 ± 17.6 beats/min after strychnine application in 10 preparations but slightly decreased from −129 ± 11.4 to −114 ± 12.1 beats/min in four preparations.

Intracellular recordings. Consistent with the notion of an earlier onset of glottic constriction coinciding with PNA, there was a change in the timing of discharge of postinspiratory neurons. In eupnea, postinspiratory neurons exhibited a hyperpolarization and absence of action potentials during inspiration and a marked depolarization and decrementing volley of activity coincident with the end of the inspiratory phase (Fig. 3A). However, after strychnine administration, the inspiratory-related hyperpolarization was abolished and neurons started to discharge earlier in the inspiratory phase (Fig. 3B; n = 3). Analyzing the effect of strychnine on the synaptic response of respiratory cells during EN5 stimulation revealed that postinspiratory neurons showed no obvious change in their evoked firing behavior (Fig. 4A; n = 3). In other words, they depolarized and fired tonically after strychnine just as they did under control conditions. However, the EN5-evoked firing rate was increased by 8.3 ± 2.6 Hz (see Fig. 4A). In contrast, inspiratory neurons (n = 3) that were hyperpolarized and ceased discharging during EN5 stimulation in control exhibited a pronounced depolarization with burst discharges after pharmacological blockade of glycine receptors. Their activity correlated with the evoked persistent discharge recorded in the PN (Fig. 4B).

DISCUSSION

Our results indicate that in the mature rat the integrity of glycine receptors within the brain stem is essential for the phase locking of the three respiratory phases. Glycine receptors act functionally to segregate postinspiration from inspiration such that their absence results in a dramatic shift in the onset of spiking activity of postinspiratory neurons from early expiration into midinspiration. This reflects that postinspiratory neurons either receive excitatory synaptic drive during neural inspiration, which is normally effectively shunted by an overwhelming glycine-mediated inhibition, or that glycine receptor antagonism unmasks an excitatory input that is not present in control. Furthermore, because PNA was our measured output, we cannot rule out the possibility of strychnine effects at this level.

The shift in the timing of postinspiratory neuronal discharge has severe physiological consequences on the normal (eupneic) respiratory modulation of the glottis. Laryngeal adductors become active during inspiration, evoking a paradoxical inspiratory glottal constriction. Moreover, reflex regulation of the respiratory system is massively disturbed, such that the expiratory apnea, a trademark component of the diving response, was abolished and converted into a stimulus-locked tonic activation of inspiratory firing. The latter effect was observed in both the phrenic neurogram and single unit recordings from inspiratory neurons. Furthermore, the diving-evoked glottal constriction was reduced, which presumably reflects a simultaneous activation of both abductors and adductors.

Effect of glycine receptor blockade on the respiratory modulation of the glottis. A shift in activity of postinspiratory neurons after blockade of glycine receptors was reported previously in the WHBP of mice (5). Low oxygen was found to produce comparable effects in the WHBP of neonatal rats (8) and also in a rhythmic in vitro thick slice preparation encapsulating parts of the ventral respiratory group (16). It is likely that hypoxia
depresses glycinergic function (24) and hence produces an effect on respiratory network function qualitatively similar to that of strychnine. It was concluded that anoxia or loss of glycinergic inhibition transforms the eupneic respiratory rhythm into gasping (see Ref. 27) or into a two-phase rhythm, which then becomes dependent on GABAergic inhibition and intrinsic membrane properties for phase switching (5). Hitherto, the functional consequence of the loss of the postinspiratory activity in both of these previous reports was not determined, as the appropriate kinesiological experiments were not performed.

The respiratory pattern can be divided into three neuronal phases: inspiration, postinspiration, and expiration (Fig. 1; for reviews, see also Refs. 4, 23, and 24). Our data show that glycine receptor-mediated synaptic inhibition (presumably fast chloride) is essential for the expression of the three-phase eupneic respiratory pattern (see also Refs. 6, 13) that includes modulation of the upper airway. However, as predicted by in vitro studies (1, 20, 25), a respiratory-like rhythm was still generated after blockade of glycine receptors, although this was disturbed compared with control. Furthermore, blockade of glycinergic inhibition caused a paradoxical activation of glottal adductor muscles in the inspiratory phase, which would lead to inspiratory upper airway obstruction. This is explained by the earlier occurrence of activity of postinspiratory neu-

Fig. 4. A: effects of EN5 stimulation on a postinspiratory neuron before and after strychnine. Intracellularly recorded postinspiratory neuron was depolarized and produced tonic activity during EN5 stimulation. After strychnine administration, the neuron still fired tonically; the average discharge frequency during EN5 stimulation was even increased compared with control (29 to 41 Hz). B: strychnine reverses the EN5-induced hyperpolarization in an inspiratory neuron. Electrical stimulation of the EN5 evoked hyperpolarization and a cessation of activity in intracellularly recorded inspiratory neurons during control. After administration of strychnine, EN5 stimulation evoked a depolarization and pronounced spiking activity.
rons into inspiration. Although we did not identify these postinspiratory neurons as laryngeal motoneurons (see Ref. 2), it is suggested that a similar change in the timing of activity must occur in laryngeal adductors based on the reduction in SGP during the early expiratory phase. This is substantiated by comparable findings in WHBP of neonatal rats where the postinspiratory activity recorded from the recurrent laryngeal nerve was decreased (8). All told, the latter neonatal data and the present findings suggest that the integrity of glycinegic inhibition within the respiratory network of mammals is crucial for eupneic modulation of the glottis from birth.

Central glycinegic inhibition is essential for reflex modulation of the upper airway. Loss of glycinegic inhibition powerfully disrupted the respiratory component of the nasotrigeminal reflex. Important features of the nasotrigeminal reflex, such as the inspiratory off switch and complete glottal closure, were both compromised significantly. The disturbance of the postinspiratory phase and the firing of postinspiratory neurons during inspiration can explain this. In the context of respiratory rhythmogenesis in vivo, postinspiratory neurons provide an irreversible off-switch mechanism for inspiration (12, 21, 28, 29). Thus, to explain our diving response data, a group of postinspiratory neurons must be glycinegic and provide the inhibition to laryngeal adductor muscles. Because postinspiratory neurons/laryngeal adductors are activated by many reflexes, including Hering Breuer, cough, aspiration, sneezing, and swallowing (9–12, 28–30), we propose that these behaviors will also fail if glycine receptor function is affected. The latter may occur because of a point mutation, pharmacological blockade, or hypoxia. Our findings may have considerable clinical relevance to our understanding of cot death as well as the snoring that is associated with patients with sleep apnea.

Our study stresses the importance of a kinesiological approach for gaining a fuller appreciation of the physiological significance of respiratory neuronal and motor outflow data. We show that glycine receptors are fundamental for the expression of the eupneic modulation of the airway, as well as its reflex control.

REFERENCES


