INTRODUCTION

A group of neurons in the medullary raphe nuclei is involved in respiratory control. Raphe neurons are known to be the major source of serotonergic projections to other respiratory areas of the brain stem and spinal cord (1-3). Previous studies have shown that stimulation of the neurons in the raphe pallidus (RP) produces excitatory effects on respiratory activity (3, 4). It is well accepted that some of the responses to medullary raphe stimulation are dependant to serotonergic influences (4, 5). The effect of 5-HT receptor activation on on-going respiratory changes relays on the routes of application, the exact site of stimulation or specific subtypes of 5-HT receptors (5, 6).

Systemic application of specific 5-HT1A receptor agonist 8-OH-DPAT enhances phrenic nerve activity in the spontaneously breathing rats (7). However, iontophoretic application of 8-OH-DPAT on expiratory neurons in the lower brainstem decreases neuronal activity with no change in the phrenic nerve activity of the cat (5). Underlying mechanisms of these respiratory effects are complex and they are not clearly established.

The basic respiratory behavior of anesthetized animals is attributed to phrenic nerve activity. The phrenic motor nucleus (PMN) receives descending serotonergic projections originating from the RP (3). These projections make important contributions to the changes in discharge patterns of the phrenic nerve activity. It has been shown that raphe induced respiratory facilitation of the phrenic nerve activity is attenuated following intravenous administration of the serotonergic receptor antagonist methysergide (3). Therefore, we hypothesized that chemical stimulation of RP would produce excitatory responses that are mediated through 5-HT1A receptors in the PMN. The present study was performed to investigate changes in phrenic nerve activity with chemical stimulation of RP by the synaptic excitant, D,L-homocysteic acid (DLH). Additionally, this study examined the role of 5-HT1A receptors in the PMN on the excitatory response elicited from RP.

METHODS

The protocol for this study was approved by the Ethical Committee for Biomedical Research of the University of Split School of Medicine, Split, Croatia. All experiments were carried out in accordance with the National Research Council's guide for the care and use of laboratory animals.

General procedures

Experiments were performed on adult male Sprague-Dawley rats weighing 280-330 g. Anesthesia was performed with intraperitoneal injection of 20% solution of urethane in 0.9% saline (1.2 g/kg; supplemental dose 0.2 g/kg). The adequacy of anesthesia was assessed by the absence of a withdrawal reflex after noxious paw pinch. The femoral vein and artery were cannulated for intravenous drug delivery, blood pressure monitoring, and sampling of arterial blood. Blood samples were taken at regular intervals, and arterial blood gasses were maintained within physiological limits by infusion of bicarbonate solution. The trachea was cannulated through midline incision. All animals were vagotomized bilaterally. End-
tidal CO₂ concentration was continuously monitored with a GEMINI respiratory gas analyzer (CWE Inc., USA) and maintained within physiological limits. Rectal temperature was monitored by digital thermometer and maintained between 37 and 38.5°C by means of external heating pad (FST, Germany).

The rats were placed in a prone position in a stereotaxic instrument (Lab Standard, Stoeling, USA). The right phrenic nerve was dissected using a dorsal approach at the level of C₃ nerve rootlet, mounted on bipolar silver wire electrodes and covered with silicone gel to prevent it from drying. Phrenic nerve activity was amplified, filtered (band-pass 300 Hz-10 kHz) and rectified; the moving time average of phrenic nerve activity was obtained using MA-1000 Moving Averager, System 1000 Modular Instrumentation (CWE Inc., USA) with a 50-ms time constant.

Functional identification of the raphe nuclei

The micropipette was inserted into the medulla oblongata, by a dorsal approach, for stimulation of the caudal portion of the raphe nucleus (raphé pallidus, RP). First, an occipital craniotomy was performed and the dorsal surface of the brainstem was exposed. The dura was opened and the cerebellum was carefully lifted and pushed rostrally under the microscopic observation. This approach provided clear access to the obex which was used as a referential point for measurements. The rostrocaudal, mediolateral and dorsoventral coordinates of the micropipette tip were determined with respect to the obex, midline and dorsal surface respectively. DL homocysteic acid (DLH; 20±5 nl; 10 mM; Sigma Aldrich, St Louis, USA) was microinjected with fine adjustments of coordinates as necessary to locate a site at which an increase in phrenic nerve activity was evoked.

Functional identification of the phrenic motor nucleus

The skin and cervical muscles overlying the cervical part of the spinal cord were removed. Phrenic motoneurons are concentrated at segments C₃ and C₄ (8). In this study a micropipette was introduced into the phrenic motor nucleus ipsilaterally to the phrenic nerve from which electrical activity was recorded, at the level of C₄ to C₅ spinal segment. A micropipette tip was introduced vertically into the ventral side of the spinal cord. To identify the phrenic nucleus, microinjections of DLH (20±5 nl) were used, with fine adjustments of coordinates as necessary to locate a site at which an increase in phrenic nerve activity was evoked. Increases in phrenic nerve discharge in response to microinjection of DLH indicated that we successfully targeted the PMN.

Microinjection technique

Glass micropipettes with the external tip diameter of 30-50 µm were used for drug delivery. A triple-barrel micropipette was inserted at coordinates targeting the raphe nuclei. The ejected solutions contained the vehicle, selective glutamate receptor agonist DLH, 0.9% saline and diluted India ink solution. The micropipette remained at the site in the raphe nucleus and another multibarrelled micropipette was inserted at coordinates targeting the region of the PMN. This solution contained the vehicle, 0.9% saline, WAY, N-(2-(4,2-methoxyphenyl)-1-piperazinyl)ethyl-N-2-pyridyl-1-cyclohexene-carboxamide maleate (WAY-100635; 1 mM; Sigma Aldrich Chemie GmbH, Germany) selective antagonist of 5-HT₁₄ receptors, and DLH (10 mM; Sigma Aldrich, St Louis, USA) the selective glutamate receptor agonist. An India ink was also used for labeling purposes. All drugs solutions were dissolved in 0.9% saline. For pressure injections, polyethylene tubing was sealed over the pipette and connected to a syringe. Drugs were loaded or ejected using positive pressure. Drug volumes ejected were measured by monitoring the fluid meniscus movement in the pipette barrel by a microscope equipped with a fine eyepiece reticle. Drug volume ejected was 20±5 nl. Microinjection of saline was used as a control injection and had no effect on phrenic nerve activity.

Experimental protocol

As a first step, we mapped the raphe nucleus of the medulla oblongata in responses to microinjections of the excitatory neurotransmitter glutamate agonist DLH. Effective injection sites were found at the following coordinates: 0.2-0.3 mm rostral from the obex, in the midline and 2.5-2.7 mm deep from the dorsal surface of the brainstem. Once an increase in phrenic nerve activity was observed, microinjection of WAY-100635 into the phrenic nucleus was performed to inactivate the restricted neuronal population of 5-HT₁₄ receptors. Therefore, microinjection of selective 5-HT₁₄ receptor antagonist, WAY-100635 was made into the PMN. After a waiting period of 3-5 min, another DLH microinjection in the raphe nucleus was repeated while the effects on phrenic nerve activity and blood pressure were monitored.

Histology

At the end of the experimental microinjections diluted India ink (20±5 nl) was injected to mark the injection sites. Each animal was then perfused transcardially under deep anaesthesia with Zamboni’s fixative (4% formalin and 15% saturated picric acid in 0.1 M phosphate buffer). The brainstem was removed and stored in 4% formaldehyde in 0.1 M phosphate buffer at 4°C until 100 µm coronal sections were cut with vibratome (Vibratome Series 1000, Pelco® 101, USA). Native sections were examined microscopically. The location of injection sites were defined on the basis of the distances of marked sites to known anatomical structures (9).

Statistical analysis

The response to injections of DLH and WAY-100635 were analyzed on the phrenic motor output in terms of inspiratory duration (Tᵢ), expiratory duration (Tₑ), respiratory cycle duration (TTₑᵢ), and peak amplitude of phrenic nerve discharge. Nerve activity and blood pressure were averaged over sequential 10 seconds periods before, during and after the microinjection and were subsequently expressed as percentage changes from the pre-injection control. Baseline values for all of the variables were determined by averaging these values 10 seconds prior to central application of drugs. Amplitude of integrated phrenic nerve activity was normalized to pre-injection baseline activity, which was set at 100% in each animal.

All the values are reported as means±S.E. One-way analysis of variance was used for comparison between the groups followed by Bonferroni correction. Statistical significance was set at p<0.05.

RESULTS

Central administration of glutamate receptor agonist DLH into the raphé pallidus nucleus enhanced phrenic nerve activity in the spontaneously breathing anesthetized rats (Fig. 1A). Microinjections of selective 5-HT₁₄ receptor antagonist, WAY-100635, into the phrenic nucleus did not change phrenic nerve activity.
activity (Fig. 1B), but attenuated stimulatory effect on phrenic nerve activity elicited by DLH microinjections into the raphe nucleus (Fig. 1C).

Onset of the phrenic nerve activity changes began at 28.5±4.8 seconds following the central microinjections of DLH, with the peak excitation occurring at 40 to 60 seconds. Administration of DLH into the raphe nucleus produced an increase in the amplitude of phrenic nerve activity by 55.18±6.8% (n=6, p<0.001) as shown in Fig. 1A and 2. Both TTOT and TE showed no significant changes compared to control values. In contrast, TI increased by 8.49±2.65% (n=6, p<0.05).

Alteration in the mean blood pressure after the administration of DLH into the raphe nuclei was rather inconsistent and not statistically different compared to baseline. There was no significant change in the respiratory frequency compared to control (44.7±1.98 vs. 46.8±0.87 breaths per minute).

Reproducibility of the DLH evoked response was examined by repeating the DLH microinjection after 15 min.

Microinjections of DLH into the PMN were made in order to identify the site at which the tonic increase in phrenic nerve activity (Fig. 1B), but attenuated stimulatory effect on phrenic nerve activity elicited by DLH microinjections into the raphe nucleus (Fig. 1C).

Fig. 1. An original tracing of arterial blood pressure (BP), integrated phrenic nerve activity (IPNA), and averaged (raw) phrenic nerve activity (RPNA) in one representative animal is shown. Panel A presents an increase in IPNA and RPNA in response to microinjection (at the arrow) of the synaptic excitant DLH (10 mM; 20±5 nl) into the raphe pallidus nucleus (RP) of the medulla oblongata during control condition. Microinjection of the WAY (1 mM; 20±5 nl) into the PMN by itself produces no changes in IPNA nor RPNA (panel B). Panel C demonstrates an absence of any response in IPNA and RPNA to microinjection of the same dose of DLH after microinjection of WAY into the phrenic motor nucleus (PMN).

Fig. 2. Relative changes in the phrenic nerve activity induced by DLH microinjections in the RP of the medulla oblongata. Variability is indicated by standard error bars; * denotes significant difference in the phrenic nerve activity before and after microinjections of WAY in to the phrenic motor nucleus (PMN), p<0.05.
activity occurred, which was indicative of phrenic motoneurons activation (Fig. 3). After recovery of baseline activities, WAY-100635 was microinjected from another barrel of the micropipette into the PMN. Microinjection of WAY-100635 into the PMN had no significant effect on baseline phrenic nerve activity or blood pressure (Fig. 1B).

However, the excitation of phrenic nerve activity evoked from the RP was significantly attenuated after the blockade of 5-HT1A receptors in PMN (Fig. 1C and 2). The raphe pallidus-induced increase in the peak amplitude of the phrenic nerve activity was only 4.44±1.42% (n=6, p<0.05) after blockade of 5-HT1A receptors in the PMN compared to increase seen prior to blockade of 5-HT1A receptors in the PMN (Fig. 2). T101 did not significantly change mainly due to changes of T1 and T6 in opposite directions. T1 increased by 8.46±2.5% (p<0.05), whereas T6 decreased by 4.82±1.65% (p<0.05). Disturbances in blood pressure were not significantly different from the baseline values. There was no significant change in respiratory frequency before and after microinjections of DLH in the RP (45±2.05 vs. 47±1.84).

Microinjection sites of WAY-100635 in the RP and PMN were verified histologically as shown in Fig. 4.

**DISCUSSION**

The present study demonstrates that microinjections of the synaptic excitant DLH into the RP leads to an increased amplitude of the phrenic nerve activity and that response can be attenuated by blockade of 5-HT1A receptors in the PMN.

Raphe nuclei have been shown to send serotonergic projections to the respiratory-related brain areas: the ventral respiratory group (VRG), (1, 2); dorsal respiratory group (DRG), (10) and the PMN (3, 11).
The anatomic foundation has been provided by immunohistochemical studies that located serotonergic neurons predominantly in the raphe pallidus (RP), whereas GABA-containing neurons are predominately within the raphe magnus (RM). Neurons in the raphe obscurus (RO) contain both GABAergic and serotonergic neurons (3).

Serotonin agents have been shown to play an important role in the modulation of breathing although with conflicting results yielded in different studies. Different routes of administration, species studied, or various 5-HT receptors might explain such discrepancies. Among the various 5-HT receptor subtypes, 5-HT$_{1A}$ receptors represent the most extensively expressed receptor subtype in the central respiratory network, but also in many brain areas involved in different physiological functions and behaviors (12-16). Activation of 5-HT$_{1A}$ receptors has been shown to inhibit the firing rate of serotonergic neurons (17, 18).

Electrical stimulation of the raphe nuclei can produce either facilitatory or inhibitory effects on respiratory output (19, 20) depending on the exact site of stimulation. Electrophysiological evidence implicates that facilitatory effects are evoked from the caudal RP part and ventral parts of the RO. This facilitatory effect can be attenuated by intravenous administration of broad-spectrum 5-HT$_{1A}$ receptor antagonist methysergide (3). Furthermore, the inhibitory effects can be seen with stimulation of the dorsal parts of the RO and these effects can be attenuated by intravenous administration of a selective 5-HT$_{1A}$ receptor antagonist NAN-190 (19). The increased discharge pattern of the phrenic nerve activity evoked by the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT can be reduced by systemic administration of the selective 5-HT$_{1A}$ receptor antagonist WAY-100635 (7).

Serotonin containing terminals have also been detected in respiratory areas of the reticular formation and PMN (21, 22). Accordingly the site for administration of the selective 5-HT$_{1A}$ receptor antagonist WAY-100635 in this study was PMN. Since there are serotonergic projections from the raphe pallidus to the phrenic nucleus, stimulation of the raphe pallidus by microinjections of synaptic excitant DLH should result in excitation of the neurons in the PMN. Indeed, in the present study this excitation was abolished by prior administration of 5-HT$_{1A}$ receptor antagonist, specifically by selective 5-HT$_{1A}$ receptor antagonist WAY-100635.

Microinjections of WAY-100635 in the PMN did not produce changes in the baseline discharge of the phrenic nerve activity in the given dose suggesting that 5-HT$_{1A}$ receptors do not contribute to the tonic control of respiratory activity, but rather are activated in the modulatory role in response to a specific challenge.

Although electrical and chemical stimulation of the raphe nuclei produce similar effects, the possibility of activation of passing fibers due to electrical stimulation can be avoided by chemical stimulation of the raphe nuclei. Truly, chemical stimulation of the raphe nuclei produces similar effects and thus synaptic excitant DLH was used in this study to stimulate neurons within the RP.

The question remains whether the functional serotonergic pathway from RP to PMN is direct (3) or mediated through brainstem respiratory areas such as VRG (11, 23-25). Immunohistochemical studies provided evidence about serotonin-containing neurons from the raphe pallidus, raphe magnus and raphe obscurus that project to the rostral VRG (11). Serotonin-containing neurons in the RP have been shown to mediate facilitatory effects on respiration (4, 26, 27). However, there is evidence to support the presence of several other types of neurons in raphe nuclei containing GABA, glycine, substance P and thyrotropin releasing hormone (TRH). These neurons could also be involved in transmitting the effects of raphe stimulation (23, 28-31). Given the numerous serotonergic inputs and receptor types within the medullary and spinal cord region future studies should provide evidence of the serotonin circuitry involved in the neuronal control of breathing.

In conclusion, the present study shows that stimulation of the raphe pallidus induces excitation of the phrenic nerve activity. The microinjections of the 5-HT$_{1A}$ receptors antagonist WAY 100635 in the PMN decreased facilitatory effects on respiration induced by RP stimulation.

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