

Cough, Expiration and Aspiration Reflexes following Kainic Acid Lesions to the Pontine Respiratory Group in Anesthetized Cats

I. POLIAČEK, J. JAKUŠ, A. STRÁNSKY, H. BARÁNI, E. HALAŠOVÁ¹,
Z. TOMORI²

Department of Biophysics, ¹Department of Biology, Comenius University, Jessenius Faculty of Medicine, Martin and ²Department of Pathophysiology, Faculty of Medicine, Šafárik University, Košice, Slovak Republic

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Summary

The importance of neurons in the pontine respiratory group for the generation of cough, expiration, and aspiration reflexes was studied on non-decerebrate spontaneously breathing cats under pentobarbitone anesthesia. The dysfunction of neurons in the pontine respiratory group produced by bilateral microinjection of kainic acid (neurotoxin) regularly abolished the cough reflexes evoked by mechanical stimulation of both the tracheobronchial and the laryngopharyngeal mucous membranes and the expiration reflex mechanically induced from the glottis. The aspiration reflex elicited by similar stimulation of the nasopharyngeal region persisted in 73 % of tests, however, with a reduced intensity compared to the pre-lesion conditions. The pontine respiratory group seems to be an important source of the facilitatory inputs to the brainstem circuitries that mediate cough, expiration, and aspiration reflexes. Our results indicate the significant role of pons in the multilevel organization of brainstem networks in central integration of the aforementioned reflexes.

Key words

Pontine respiratory group • Cough • Defensive airway reflexes • Kainic acid lesion • Cat

Introduction

The last decade has brought considerable progress in understanding the central mechanisms of cough and other defensive airway reflexes (Shannon *et al.* 1997, 1998, 2000, Tomori *et al.* 1998, Korpáš and Jakuš 2000, Bolser and Davenport 2002, Pantaleo *et al.* 2002). It is obvious that airway reflexes are generated by complex neuronal networks localized within the brainstem. This is in accordance with an older hypothesis

concerning the functional character of the cough center (Korpáš and Tomori 1979). Experiments with recording of neuronal activities within the ventral respiratory group (VRG) of the medulla during mechanically or electrically induced cough (Jakuš *et al.* 1985, Oku *et al.* 1994, Shannon *et al.* 1997, Bongianni *et al.* 1998) revealed substantial involvement of original respiratory neurons in production of the cough motor pattern. Several years ago, the valuable analysis of multiple discharge patterns from respiratory neurons and the interesting model of neuronal

network generating the cough reflex were introduced by Shannon *et al.* (1998, 2000). According to this model neuronal circuitries of the Respiratory Central Pattern Generator can also produce the cough motor pattern. However, the possibility that other brainstem circuits overlapping the main respiratory networks may also participate has not been excluded. Our previous papers (Jakuš *et al.* 1998, 2000) described two brainstem regions, which seemed to be essential for the generation of cough and expiration reflexes. Moreover, some authors (Gestreau *et al.* 1997, Xu *et al.* 1997, Baekey *et al.* 2003) reported the regions outside the medullary respiratory groups containing pools of neurons being activated during the stimulation of cough trigger zones within the airways. In addition, the terminal arborization of neurons from the commissural subnucleus of the nucleus tractus solitarii (the region which receives vagal primary afferents from the lungs, airways and other viscera) was also found in the nucleus parabrachialis medialis and lateralis, and within the Kölliker-Fuse nucleus (Ezure *et al.* 1991, Otake *et al.* 1992). These nuclei mostly correspond to the pontine respiratory group – PRG (Feldman 1986), which was previously referred to as the „pneumotaxic center”. Different functional connections were also established between the neurons of PRG and those in the rostral VRG (Bianchi and St. John 1981, Segers *et al.* 1985, Gang *et al.* 1998), as well as between the neurons in PRG and other medullary regions (King 1980, Smith *et al.* 1989, Gang *et al.* 1993, Gerrits and Holstege 1996), which are proposed as important sites for coughing (e.g. raphe midline, lateral tegmental field). These findings strongly suggest a possible involvement of PRG neurons in integration of the cough and other defensive airway reflexes.

However, only a few publications have studied the role of pons in the generation of cough and defensive airway reflexes (Dubi 1959, Jakuš *et al.* 1987, Baekey *et al.* 1999). Jakuš *et al.* (1987) described the effects of surgical brainstem sections on cough, expiration (ER), and aspiration reflexes (AR) in anesthetized non-decerebrate spontaneously breathing cats. They showed that a transversal section at the level of rostral pons usually led to a strong depression of expiratory efforts during cough and ER, as well as to a smaller depression of inspiratory effort in AR. Such pontine transections probably removed both facilitatory and inhibitory reticular influences between the medulla and the pons (suprapontine areas). However, the role of discrete areas within the rostro-dorsal lateral parts of pons (in PRG) in

the generation of defensive airway reflexes has not yet been established.

Therefore, the aim of this paper was to study the effects of kainic acid lesions of PRG on the elicibility and parameters of both the tracheobronchial (TBC) and laryngopharyngeal coughs (LPhC), as well as ER and AR in experiments on anesthetized cats. The concomitant changes in eupnoic breathing and systemic blood pressure are also described.

Methods

The data were obtained from 12 spontaneously breathing adult cats (mean body weight 2.82 ± 0.22 kg). The animals were anaesthetized with sodium pentobarbitone by an initial intraperitoneal dose of 35–40 mg/kg and a maintenance dose 1–2 mg/kg/h given intravenously. In the course of the experiment, the end-tidal CO₂ was continually monitored by a capnograph (Capnogard, Novametrics). A supplemental dose of pentobarbitone was given if the blood pressure or respiratory rate increased spontaneously or when the end tidal CO₂ dropped below 3.8 % or in response to surgical procedures. Airway reflexes were evoked at least 20 min after the administration of an additional dose of the anesthetic. Arterial blood samples were periodically analyzed for pO₂, pCO₂ and pH using a blood gas analyzer (AVL - Compact 2). Metabolic acidosis, when it occurred, was corrected by an intravenous administration of 8.4 % sodium bicarbonate. Rectal temperature was kept at 37.5 ± 0.5 °C using servo-controlled heating lamps. In order to prevent brain edema and hypersalivation the cats received hydrocortisone (VUAB, Prague) in a dose of 6 mg/kg intramuscularly 15 h before the experiment and 3 mg/kg intravenously with a single intravenous dose of atropine (0.2 mg/kg) during the first stage of anesthesia. The animals were killed by an overdose of pentobarbitone at the end of the experiment. Animal care and use conformed to the guidelines accepted by the European Physiological Society.

The general surgical preparation was described in detail previously (Jakuš *et al.* 1998, 2000, Poliaček *et al.* 2003). Briefly, plastic cannulae were introduced into both ends of the dissected trachea after a tracheostomy, into the right femoral artery and the right femoral vein for measurement of arterial blood pressure (BP) by an electromanometer (Tesla LDP 186), and for further intravenous injections, respectively. Another cannula was introduced into the right pleural cavity for recording of

the pleural pressure (Ppl) by an electromanometer (Tesla LDP 165). Bipolar fine wire electrodes (0.1-0.3 mm in diameter) were introduced into the crural part of the diaphragm (DIA) and into the rectus or external oblique abdominal muscles (ABD) for electromyographic (EMG) recording. The animal was placed in a stereotaxic apparatus and kept in the prone position. Following occipital craniotomy and partial cerebellectomy, the dorsal surface of the pons was exposed.

TBC, LPhC, ER and AR were elicited by mechanical stimulation of the tracheobronchial, laryngopharyngeal, glottal and nasopharyngeal regions, by an elastic nylon fiber (diameter 0.2-0.5 mm).

Chemical lesions of neurons within PRG were induced by bilateral microinjections (150-200 nl) of the excitotoxin kainic acid (Sigma, 2.0 mg/ml) dissolved in phosphate buffered saline (pH 7.4) using a Hamilton syringe (needle tip diameter 0.3 mm). The solution was saturated with Fast Green dye to localize its spreading. The syringe was mounted in a stereotaxic manipulator and the needle tip was inserted into the rostral pons 10.2-10.7 mm rostral to the obex, 4.2-4.5 mm lateral to the midline, and 2.7-3.0 mm below the dorsal pontine surface. Kainic acid produces functional inactivation of the cell bodies within 30 min while sparing neuronal fibers (Coyle *et al.* 1978). Therefore we waited 30-40 min following the injections before testing the effects. At the end of the experiment, the brainstem was removed for histological processing using transversal sections 0.1 mm thick at the level of the pons.

EMG activities of DIA and ABD were amplified (Iso-Dam8 Amplifier, WPI) at low and high pass filters (0.3-3 kHz) and displayed on the screen of oscilloscopes (Tektronix 5223, Tesla OPD 609). EMG signals were recorded online together with Ppl and BP at a sampling frequency of 2 or 4 kHz using PC software (Lab View, National Instruments). The bursts of discharges were integrated "on line" by RC circuits and/or a moving average of raw signals was calculated "off line" by the computer, both with a time constant of 50 ms.

Computer-assisted processing of the recorded signals was performed. The respiratory rate and durations of inspiratory, postinspiratory, and expiratory phases (Richter and Ballantyne 1983) were derived from integrated EMG of DIA during eupnoeic breathing. Similarly, durations of individual bursts of discharges in DIA and ABD were determined from their raw and integrated EMG during the studied airway reflexes. The intensity of breathing and the strength of particular

reflexes were assessed from the peak inspiratory and expiratory pleural pressures. The systolic and diastolic values of the systemic arterial blood pressure were also evaluated during control breathing. The variables of eupnoeic breathing were averaged for each animal separately from five standard respiratory cycles during the control (pre-injection) and the post-injection periods. Similarly, the airway reflexes were elicited both under the control conditions and following kainic acid injection and the variables of evaluated reflexes were determined.

The final results are expressed as means \pm S.E.M. Analysis of variance (ANOVA) and the appropriate parametric and non-parametric alternatives of the t-test were used to determine the statistical significance of the differences.

Results

Histological verification of lesioned sites in rostral pons proved that the injections mostly infiltrated the areas between 9-12 mm rostral to the obex, 3.5-5.5 mm lateral to the midline and 1.5-3.5 mm below the dorsal pontine surface. The lesions (Fig. 1) mainly affected the motor trigeminal nucleus, principal sensory trigeminal nucleus, marginal nucleus of the brachium conjunctivum, Kölliker-Fuse nucleus and parabrachial nuclei (Berman 1968). The area of PRG and adjacent parts of pontine reticular formation were affected in our lesions.

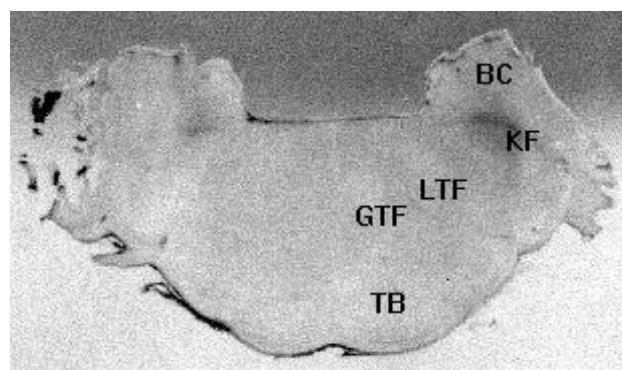


Fig. 1. Transverse pontine section at the level 10.5 mm rostral to the obex showing the bilateral kainic acid lesion to the region of PRG (right is larger). GTF –gigantocellular tegmental field, TB – trapezoid body, LTF - lateral tegmental field, BC – brachium conjunctivum, KF – Kölliker-Fuse nucleus.

The cough reflex was regularly induced by mechanical stimulation of the tracheobronchial or

laryngopharyngeal mucous membrane during control inspiration. This was characterized by a strong burst of discharges in DIA resulting in a negative deep deflection of Ppl, which was immediately followed and partially overlapped by a burst of activity in ABD accompanied by a strong positive swing of Ppl (Figs 2 and 3). Under control conditions we evaluated 72 TBC in 12 cats and 66 LPhC in 11 cats (six cough reflexes with the highest Ppl deflections were evaluated in each animal). The data of both types of cough reflexes were statistically processed and summarized in Table 1. Kainic acid injections abolished any signs of TBC in all 114 tests of tracheobronchial stimulation as well as signs of LPhC in all 79 tests of laryngopharyngeal stimulation. During the post-injection period there were no residual electrical activity in DIA or ABD and no Ppl alterations resembling the cough reflex (Figs 2 and 3).

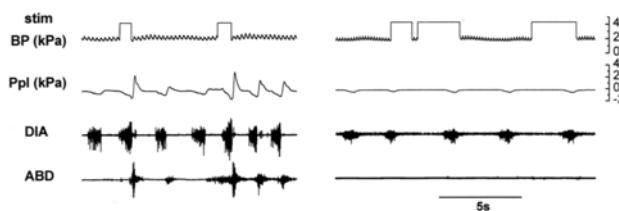


Fig. 2. Tracheobronchial stimulation. Mechanical stimulation of the tracheobronchial tree evoked the tracheobronchial cough (left). No signs of cough reflex were recorded following bilateral lesion to the pontine respiratory group (right). BP – blood pressure, Ppl – pleural pressure, DIA, ABD – electromyographic activities in the diaphragm and abdominal muscles. Periods of repeated stimulations (stim) are superimposed on the BP records.

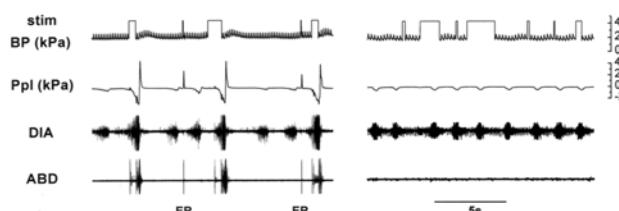


Fig. 3. Laryngopharyngeal and glottal stimulations. Mechanical stimulation of the laryngopharyngeal mucosa evoked the laryngopharyngeal cough (left). Tactile glottal stimulation elicited the expiration reflex (ER, left). Following bilateral kainic acid lesion to the pontine respiratory group no signs of cough and expiration reflex were recorded (right). For other legend see Fig. 2.

Expiration reflex was induced by mechanical stimulation of the vocal folds mostly during control eupnoeic expiration. It was characterized by brief but strong electrical activity in the manifested by a forceful expiratory effort as well as a prompt and steep positive

swing of Ppl without preceding inspiration (Fig. 3). The mean values of determined parameters from 66 ER on 11 animals (six strongest ER were evaluated in each animal) are presented in Table 1. Similarly as in coughing, kainic acid injections given to PRG prevented the appearance of any sign of ER (Fig. 3) in all 213 tests of glottal stimulation.

Aspiration reflex was clearly evoked in nine animals under control conditions by mechanical stimulation of the nasopharynx through a small pharyngostomach opening. AR consisted of a brief but large burst of electrical activity in DIA accompanied by short steep and strong negative deflection of Ppl (Fig. 4). The eight strongest reflexes were taken for evaluation in each animal. Mean values of AR parameters under control (72 AR on 9 cats) and post-injection periods (64 AR on 8 cats) are shown in Table 1. Kainic acid injections abolished the signs of AR in 144 out of 535 tests (27 %) of nasopharyngeal stimulation (reflex responses were detected in 391 tests – 783 %), but AR was completely abolished in one animal only. The “residual” aspiration reflex responses showed a drop in the peak value of Ppl to the 53 % of its control value ($p < 0.001$) and shortening of tDIA activity ($p < 0.01$, Table 1).

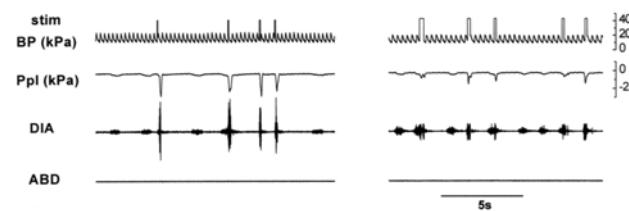


Fig. 4. Nasopharyngeal stimulation. Tactile stimulation in this region evoked the aspiration reflex under both control (left) and post-lesioned conditions (right). The strength of the reflexes following bilateral lesion to PRG was markedly reduced compared to the control reflexes. For other legend see Fig. 2.

The parameters of eupnoeic breathing (Table 2) were only slightly affected by the kainic acid injections, compared to the control. Just the postinspiratory duration was prolonged by about 75 % ($p < 0.001$). Monitoring of the end tidal CO_2 (Table 2) and evaluation of the arterial blood gas tensions and pH revealed no significant changes during the experiments. However, the kainic acid lesions in PRG were accompanied by a drop in both systolic ($p < 0.02$) and diastolic ($p < 0.05$) values of the systemic blood pressure, compared to the pre-injection state (Table 2).

Table 1. Durations of electrical activity in diaphragm (t_{DIA}) and in abdominal muscles (t_{ABD}) and peak inspiratory ($P_{pl\ I}$) and expiratory pleural pressures ($P_{pl\ E}$) during tracheobronchial cough (TBC), laryngopharyngeal cough (LPhC), expiration (ER) and aspiration reflexes (AR) under control conditions and following bilateral kainic acid injection into the pontine respiratory group.

	Control				Kainic Acid			
	n_1	t_{DIA} [ms]	t_{ABD} [ms]	$P_{pl\ I}$ [kPa]	$P_{pl\ E}$ [kPa]	n_2	t_{DIA} [ms]	$P_{pl\ I}$ [kPa]
TBC	72/12	1070±40	450±30	-1.51±0.07	1.69±0.19	114/12		absent
LPhC	66/11	1500±70	400±30	-1.63±0.06	1.8±0.18	79/11		absent
p_1		<0.001	>0.05	>0.05	>0.05			
ER	66/11	-	88±4	-	1.11±0.09	213/11		absent
AR	72/9	78±3	-	-2.7±0.11	-	64*/8	65±3	-1.43±0.08
p_2							<0.01	<0.001
						144/9		absent

The values are means ± S.E.M. n_1 – number of evaluated reflexes / number of animals, n_2 – number of stimulations / number of animals (* evaluated AR out of 391 evoked reflex responses), p_1 – for mean values of TBC compared with LPhC, p_2 – for mean values of AR under control compared with post-lesion conditions.

Table 2. The respiratory rate, inspiratory (t_i), postsinspiratory (t_{E1}) and expiratory time durations (t_{E2}), peak inspiratory ($P_{pl\ I}$) and expiratory pleural pressures ($P_{pl\ E}$), end-tidal CO₂ (ETCO₂) and both the systolic (BP_s) and diastolic blood pressures (BP_d) during eupnoeic breathing under control conditions and following bilateral kainic acid injection into the pontine respiratory group.

	n	rate [min ⁻¹]	Breathing				Blood pressure			
			t_i [s]	t_{E1} [s]	t_{E2} [s]	$P_{pl\ I}$ [kPa]	$P_{pl\ E}$ [kPa]	ETCO ₂ [%]	BP _s [kPa]	BP _d [kPa]
<i>Control</i>	11	23.8	1.28	0.29	1.15	-0.53	-0.15	4.3	23.8	16.7
		±1.8	±0.20	±0.02	±0.11	±0.08	±0.03	±0.3	±1.0	±1.2
<i>Kainic acid</i>	11	24	1.33	0.51	2.02	-0.63	-0.2	5.1	19.7	13
		±4.9	±0.41	±0.05	±0.48	±0.07	±0.03	±0.4	±1.3	±1.3
<i>p</i>		>0.05	>0.05	<0.01	>0.05	>0.05	>0.05	>0.05	<0.02	<0.05

The values are means ± S.E.M. n – number of animals

Discussion

The present paper provides new and important information that kainic acid injections to PRG in both halves of the pons completely abolished the signs of the tracheobronchial and laryngopharyngeal coughs and expiration reflex in anesthetized non-paralyzed cats. In addition, the aspiration reflex elicited from the nasopharynx persisted in 73 % of tests, but it was reduced in its intensity. Our previous findings in cats (Jakuš *et al.* 1987) showed that successive surgical transsections at the level of rostral pons to the pontomedullary junction regularly removed the signs of *cough and ER* under similar experimental conditions. However, it was still not clear if the effects of transsections resulted from

destruction of the cell bodies, the neuronal pathways, or both. Basically, we proposed that such transsections could mainly damage the rostral-caudal interconnections between the neurons within the rostral and caudal pons, the medulla and higher suprapontine regions. Our present findings obtained by focal injections of neurotoxin – kainic acid to the clusters of neurons within PRG have documented the same behavior of the cough and another two estimated reflexes. Because kainic acid functionally mainly destroys neuronal somata in the target region, while sparing nerve fibers (Coyle *et al.* 1978), we speculate that the PRG neurons themselves could play an essential (at least modulatory) role in generation of the cough and ER. Our finding is in accordance with the activation of neurons within the rostro-dorsal lateral pons

during “fictive” laryngeal cough reflex, revealed by c-Fos imunoreactive method (Gestreau *et al.* 1997). In addition, most PRG neurons were found to display tonic discharge patterns during eupnoic respiration and their burst activity markedly changed during fictive cough (Baekey *et al.* 1999).

Along with the idea of Shannon *et al.* (1998) we suppose that the Respiratory Central Pattern Generator may not only produce the cough motor pattern but probably also participates in forming ER motor pattern (Korpáš and Jakuš 2000) or the patterns of other airway reflexes. In support of this, the appropriate changes in neuronal bursting activity during ER (Dyachenko 1990) and the focal cooling experiments within the area of the Bötzinger complex (Jakuš *et al.* 1996) have suggested the significance of the rostral VRG region for production of ER motor pattern.

However, it seems that the medullary VRG region alone is not able to produce the central motor patterns of cough and ER without some functional support from PRG, the medullary raphe midline (Jakuš *et al.* 1998) and the reticular neurons of lateral tegmental field (LTF) (Jakuš *et al.* 2000). Hence, we propose that PRG neurons could be an important source of facilitation for neuronal networks (localized at multiple brainstem levels), which mediate the cough and ER, similarly as was assumed for the role of raphe and LTF neurons (Jakuš *et al.* 1998, 2000). In our study, we did not record electrical activity of pontine neurons or even evaluated their functional connections. Therefore, the mechanism by which these neurons may contribute to integration of the cough and ER could not yet be answered now. Nevertheless, we speculate on the role which may be played by PRG, raphe midline and LTF neurons in the “gating” mechanism of the cough – a recently introduced idea (Bolser *et al.* 1999, Bolser and Davenport 2002). Hence, some neurons in these regions may facilitate the afferent inputs coming to the cough motor pattern generator and/or to the expiratory premotor part of the cough reflex arc. Recently Baekey *et al.* (2003) suggested that PRG neurons are a possible source of both phasic and tonic inputs to the pool of raphe neurons during cough and eupnea. Moreover, PRG neurons have a connection to the pre-motor and motor inspiratory neurons (Yates *et al.* 1999) and together with the medullary neurons are the source for the common pathway to both phrenic and abdominal motor nuclei probably involved in attacks of expulsion during vomiting and defecation (Portillo *et al.* 1994). Taken

together, we assume similar facilitatory effects of PRG neurons to the premotor and motor respiratory neuronal pools during cough and ER.

Furthermore, the present paper has shown that kainic acid microinjection into PRG did not abolish the *aspiration reflex* from the nasopharynx. It is well known that AR is a very stable reflex preserved even under extreme conditions such as hypercapnia, hypoxia, or hypothermia (Korpáš and Tomori 1979, Tomori *et al.* 1998). In our experiments, AR was preserved in 73 % of tests but the strength of the reflex was decreased. Similarly, successive surgical transsections of the pons did not abolish the signs of AR, although AR was weaker (Jakuš *et al.* 1987). In experiments with kainic acid injections into the medullary raphe region and into LTF of the medulla (Jakuš *et al.* 1998, 2000), the elicibility of AR was also preserved, but its intensity decreased to 75 % and 77 %, respectively, while in the case of PRG the strength of the reflex reached 53 % of the control value. Therefore, it cannot be excluded that PRG neurons might be involved in a different manner in the generation of AR compared to the raphe midline and LTF neurons. The area of medullary LTF was established as crucial for the formation of strong inspiratory efforts in gasping (St. John 1990) and gasp-like AR (Fung *et al.* 1994) in decerebrate and artificially ventilated cats. However, Jakuš *et al.* (2000) noticed that following midcollicular decerebration and successive kainic acid lesion of LTF (as referred to by Fung *et al.* 1994) AR definitely disappeared, while in cats without decerebration AR surprisingly persisted even after lesions of LTF. Thus our former and present findings suggest an important role of midbrain and pontine structures, which may play in generation of the aspiration reflex. Moreover, although Fung *et al.* (1994) described LTF of medulla as an indispensable area for both the gasp and gasp-like AR, our findings indicate a multilevel organization of neuronal circuits for this reflex, where LTF and PRG neurons are probably involved.

Numerous projections from the medullary LTF neurons to the parabrachial and Kölliker-Fuse nuclei were established (King 1980). However, the great majority of LTF neurons was silent during breathing under different experimental conditions (both in decerebrate or barbiturate-anesthetized animals). It was thus presumed that these neurons may not participate in basic respiratory control (King and Knox 1982), but they can be involved in production of AR.

Our results have shown that PRG neurons also play a role in shaping of the *eupnoeic respiratory pattern*. Following kainic acid injections into PRG of our cats without vagotomy, the postinspiratory phase of respiratory cycle was prolonged. Similarly, Gautier and Bertrand (1975) and St. John *et al.* (1971) reported a prolongation of the postinspiratory phase and a higher tidal volume in anesthetized vagotomized cats after PRG lesions. Other authors (St. John *et al.* 1972, Denavit-Saubié *et al.* 1980) described a prolongation of the respiratory phase following a functional blockade of neurons within PRG region. In addition, individual subnuclei of PRG region may have different effects on the shape of the final respiratory motor pattern. For example, the kainic acid lesion to the nucleus parabrachialis lateralis potentiated ventilation, whereas similar injections into the nucleus parabrachialis medialis (NPBM) first shortly facilitated and then evoked a long-lasting depression of ventilation or even apnoea in vagotomized paralyzed and anesthetized cats (Hirano *et al.* 1990, 1994). Similarly, xylocaine blockade of NPBM neurons decreased the rate of breathing in both anesthetized vagotomized rabbits (Gromysz 1997) and cats (Pokorski and Gromysz 1997). Nevertheless, the proper localization of the pneumotaxic area within the region of motor nucleus of trigeminal nerve but not in the area of NPBM was referred by Gromysz (1997). On the contrary, Mizusawa *et al.* (1995) found no effect of tPRG

lesion on eupnoeic ventilation in non-vagotomized animals.

Since our kainic acid injections covered all subnuclei of PRG while the vagus nerves were intact, only slight alterations in eupnoeic breathing were revealed in our experiments (Table 1). In this context it is evident that structures localized in each half of the pons are mutually interconnected and may thus function together. For this reason, the differences in the effects following unilateral or bilateral lesions may not be significant, as has been confirmed in our previous papers.

In addition, we suppose that the neurons in the rostro-dorsal lateral pons may also participate in the regulation of systemic arterial blood pressure, because our lesions regularly decreased both the systolic and diastolic blood pressure. By contrast, the electrical stimulations in the region of parabrachial nuclei enhanced the mean arterial blood pressure (Motekaitis *et al.* 1994).

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Reprint requests

I. Poliaček, Department of Biophysics, Jessenius Faculty of Medicine, Comenius University, Malá Hora 4, 037 54 Martin, Slovak Republic. E-mail poliacek@jfmed.uniba.sk