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Involvement of nitric oxide in regulation of the medullary respiratory rhythm in neonatal rats

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Abstract. The NADPH-diaphorase (as a neuronal NO-synthase) reactivity in the medullary structures of the respiratory rhythm (RR) generator and the role of NO in the regulation of respiratory activity in the phrenic nerve of artificially superfused semi-isolated medulla-spinal cord preparations were investigated in newborn rats. NADPH-diaphorase positive neurons were found in all nuclei of both dorsal and ventral respiratory groups of neurons. The maximal density of stained cells was present within the rostral part of the ventrolateral medulla (VLM), in the region of the lateral paragigantocellular reticular nucleus. It was found that endogenous NO mediates the mechanism of tonic inhibitory control of the RR frequency located in the rostral VLM under normal and hypoxic conditions, and appears to be involved in generation of the basic RR by the more caudal structures of VLM. It was shown that NO biosynthesis mediates the effect of NMDA receptors activation on the RR.

Key words: NO, NO-synthase, NMDA, NADPH-diaphorase, respiratory rhythm, semi-isolated medulla-spinal cord preparation, ventrolateral medulla, hypoxia

INTRODUCTION

Nitric oxide (NO) is a free radical gas molecule involved as a chemical messenger in different physiological systems (Dawson and Snyder 1994). In the nervous system NO appears to modulate neurotransmission, acting in an intercellular or "retrograde" manner to increase or depress the presynaptic transmitter release or modify the activity of glutamate receptors (Garthwaite 1991, Dawson and Snyder 1994, Garthwaite and Boulton 1995). NO is thought to be released from neurons containing NO synthase (NOS) isozyme I or neuronal NOS. Such neurons were found in different regions of mammalian brain including pons and medulla (Dun et al. 1994). Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase was shown to be a marker enzyme for NOS-containing neurons (Hope et al. 1991, Vincent and Kimura 1992). NOS-immunoreactivity and NADPH-diaphorase reactivity were localized in the structures of the respiratory central pattern generator in adult animals (Vincent and Kimura 1992, Dun et al. 1994), yet not in neonate. NO formation in the brain is stimulated by N-methyl-D-aspartate (NMDA) receptor activation (Garthwaite et al. 1989). In adult cats NO was shown to be involved in the pontine inspiratory off--switch mechanism mediated by NMDA receptor activation (Ling et al. 1992). Similarly, Funk et al. (1993) have demonstrated that NMDA receptors were indirectly involved in regulation of the respiratory rhythm (RR) generated by medullary slices from newborn rats. The existing results of experimental studies on adult animals provide insight into NO as a central excitatory messenger involved in the augmentation of ventilation during hypoxia (Ogawa et al. 1995, Prabhakar et al. 1995). On the other hand, the role of NO in the regulation of RR in newborn animals remains obscure. The aim of the present study was: (1) to examine the distribution of NADPH reactivity in the medullary structures involved in respiratory rhythmogenesis in newborn rats, and (2) to examine the effect of NO-related drugs on RR generated by superfused medullo-spinal preparations from newborn rats under normal and hypoxic conditions.

METHODS

Objects

One hundred and thirty 3- to 4-day-old Wistar rats from the breeding colony of the Bogomolets Institute of Physiology, Kiev, were used in the experiments. All surgical manipulations were performed on animals under deep anesthesia by inhalation of ether. For preparations from the rat pups in situ the method of Marchenko et al. (1995) was used, which may avoid some defects of well--known methods of preparation of completely isolated medullo-spinal preparation (IMSP) *in vitro* by Suzue (1984) and Onimary et al. (1987). The last methods are connected with the traumatic manipulations by isolation and transportation of the preparation into the experimental chamber, and semi-isolated medullo-spinal preparations (SIMSP) practically have no distinctions from IMSP on superfusion quality (Marchenko et al. 1995).

Histochemistry

A modified NADPH-diaphorase histochemistry method (Vincent and Kimura 1992, Petrovicky and Nemcova 1995) was used to localize the distribution of NOS-containing neurons in the medullary nuclei involved in respiratory rhythmogenesis. In anesthetized animals the posterior surface of the skull was opened and the cerebellum was aspirated under sustained superfusion by cool (10°C) Krebs solution. Then the medulla was superfused by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. After 10-min--long superfusion the medulla was removed, postfixed at 4-5° C for 2 h and cryoprotected with 15% sucrose for 24 h. Cryostat sections 50 μm thick were cut and collected in 0.1 M phosphate buffer, pH 7.3. To demonstrate the NADPH-diaphorase reaction, the free-floating sections were incubated in 0.1 phosphate buffer, pH 7.3, containing 0.3% Triton X-100, 0.5 mg/ml nitroblue tetrazolium, 1.0 mg/ml beta-NADPH and 1.2 mg/ml sodium malate (reagents from Sigma, USA) at 37° C for 60 min. Then the sections were rinsed in phosphate buffer, pH 7.3, and mounted onto gelatin-coated slides. After placing the coverslips, the sections were examined under a light microscope.

Electrophysiology

Semi-isolated medullo-spinal preparations were made and left in situ (Fig. 1). Such modification of the preparation allowed us to reduce the trauma of excising a totally isolated preparation and its replacement into a chamber.

In anesthetized animals the posterior surface of the skull was opened and the cerebellum was removed. Then

a dorsal laminectomy down to the lumbar level was performed, the *dura mater* was opened, and the dorsal spinal roots and VI-XI cranial nerves were bilaterally transected. After the wound had been sutured, the animal was turned, ventral surface up. The brain basal surface was opened, and the brainstem was transected at the level of the VI cranial nerve roots (Fig. 1). Above the level of transection tissue was aspirated. Then all body parts below the diaphragm were removed, the right phrenic nerve was prepared, and all thoracic organs were also removed (Marchenko et al. 1995). After ventral laminectomy and opening of the *dura mater*, the spinal cord was transected at the C_6-C_7 level. All ventral spinal roots (excluding the C_3-C_5 roots forming the right phrenic nerve) and the XII cranial nerves were also transected.

In one series of the experiments the VLM was transected at the anterior border of a bundle of the roots of the IX-X cranial nerves, i.e., between the chemosensitive M and S zones (Figs. 1 and 2). In newborn rats transectioning at this level results in an increase of the frequency of RR in the phrenic nerve. This fact demonstrates the mechanism of tonic inhibitory control of RR located in the rostral VLM (Marchenko et al. 1995). Transectioning was performed with a blade fixed in a micromanipulator. To avoid artifacts resulting from mechanical damage, sectioning was made at superfusion of the preparations with the solution cooled to 10° C. Recording of inspiratory activity was begun 10 min after recovery of working solution temperature.

Electrical activity in the phrenic nerve (inspiratory discharges, ID) was recorded with bipolar Ag - AgCl - electrodes (spacing of 5.0 mm) assembled in a plexiglass box. The nerve was covered with paraffin oil to prevent its drying up. Electrical signals recorded from the phrenic nerve were amplified with an AC amplifier (a gain of 10^4-10^5 within the bandpass from 5.0 Hz to 10^4 Hz). Then the signals were discriminated, integrated with an amplitude-frequency integrator (a time constant of 30.0 ms), and through a 12-category AD-convertor addressed to the port of a PC. The software used allowed us to select and statistically process the experimental data and to present the results in a graphical form; it was developed in our laboratory.

Solutions

Preparations were superfused with a modified Krebs solution containing (mM): NaCl, 124.0; KCl, 5.0; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26.0; glucose, 30.0 (pH 7.4). The solution was saturated with carbogene (95% O_2 and 5% CO_2) for 10 min and 1 liter/min rate. Hypoxia was modelled by 3-min-long epi-

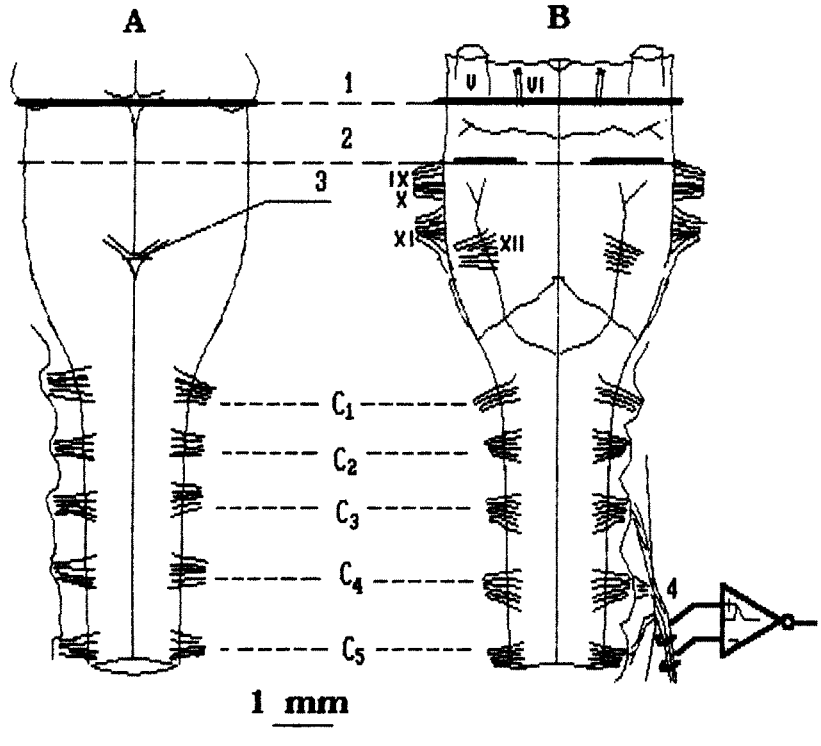

Fig. 1. Scheme of a superfused semi-isolated medullo-spinal preparation of 3- to 4-day-old rats. A and B, dorsal and ventral surfaces, respectively. V-XII are the roots of respective cranial nerves; C_1-C_5 are the cervical spinal roots. 1, the level of transection of the brainstem; 2, that of the ventrolateral medulla; 3, the obex; 4, the phrenic nerve.

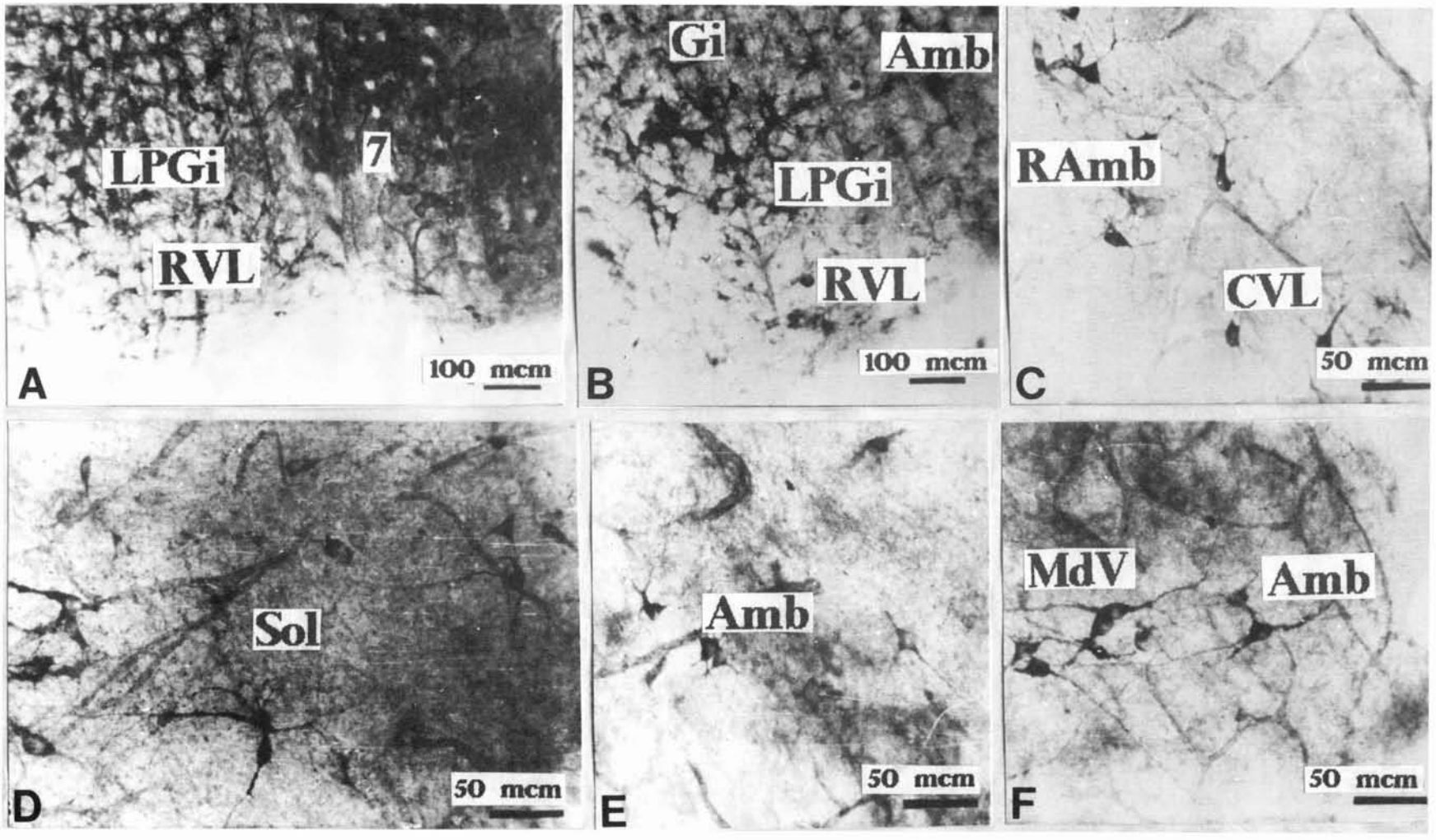

Fig. 2. NADPH-diaphorase-positive neurons within the 4-day-old rat's medulla ($n = 8$). 7, n.facialis; Amb, nucl. amiguus; CVL, nucl. caudoventrolateralis reticularis; Gi, nucl. gigantocellularis reticularis; LPGi, nucl. paragigantocellularis reticularis; MdV, nucl. medullaris reticularis ventralis; Ramb, nucl. retroambigualis; RVL,nucl. rostroventrolateralis reticularis; Sol , nucl. tractus solitarii.

sodes of superfusion of preparations with the solution saturated under the same conditions with a gas mixture containing 95% N_2 and 5% CO_2. The oxygen pressure (pO_2) in the solution on the medullary surface was polarographically controlled during the experiment and maintained within 431 ± 34 mm Hg in control and 36 ± 9 mm Hg in hypoxia. The working temperature of the solutions was controlled with a semi-conductive thermotransducer and maintained at a 26.5° C level. The rate of superfusion was 4.0 ml/min.

Drugs

(1) N^G-nitro-L-arginine methyl ether, a concurrent NO-synthase inhibitor (L-NAME, 10.0 mcM) (Roche et al. 1996, Prickaert et al. 1998, Kalish et al. 1999); (2) hemoglobine, a NO scavenger (Hb, 0.30 mcM); (3) sodium nitroprusside, an exogenous donor (SNP, 10.0 mcM); (4) L-arginine, a substrate of NO biosynthesis (L-Arg, 10.0 mcM); and (5) NMDA (5.0 mcM) were used in the experiments. All drugs were dissolved in the superfusing solution and applied on the medullary surface of SIMSP for 3 min. Applications of the NMDA, hypoxic and acidotic solutions followed 10-min-long superfusions with the solution containing a used drug. In addition, we reversed the preparations by subsequent superfusion with L-arginine solution.

Data analysis

The distribution of NADPH-diaphorase neurons was characterized by their average density in the medullary nuclei (number of cells per 0.1 mm^2). Integrated inspiratory activity was recorded within 9-min-long intervals (3 min of control, 3 min of superfusion with the testing solution, and 3 min of washing out). It was characterized by its frequency (Fi, min^{-1}) and amplitude of the ID (arbitrary units, a. u.). One arbitrary unit corresponds to one point of the PC display (frequency of 5.0 Hz and amplitude of 5.0 mcV of the integrated electrical signal during one AD-convertor cycle). All results were compared using Student's *t*-test ($P<0.05$).

RESULTS

Histochemistry

A number of NADPH-diaphorase-positive neurons were present at all rostrocaudal levels of the medulla. At the rostral level the most pronounced density of stained multipolar, medium-sized (15-25 mcm) cells was found within the ventral reticular nuclei, i.e., n. paragigantocellularis lateralis (27.9 ± 2 .6 cells per 0.1 mm^2), n. rostroventrolateralis reticularis (14.2 ± 2.1 cells per 0.1 mm^2) and n. gigantocellularis (12.7 ± 1.2 cells per 0.1 mm^2) (Figs . 2A, B and 3). This group of cells was displayed down to the caudal border of the IV ventricle with the maximal density of stained cells at the level of the caudal part of n. facialis. At the more caudal levels stained neurons were present within the ventral parts of paramedian reticular nucleus (10.1 ± 1.3 cells per 0.1 mm^2) and ventral medullary reticular nucleus (4.3 ± 0.7 cells per 0.1 mm^2) (Figs. 2C and 3). Single, small-to-medium sized, stained cells were scattered at all rostrocaudal levels of n. ambiguus (2.9 ± 0.3 cells per 0.1 mm^2) (Figs. 2D, E and 3). Groups of multipolar, medium-sized neurons were observed within the ventrolateral parts of n. tractus solitarii (6.2 ± 0.4 cells per 0.1 mm^2) (Figs. 2F and 3).

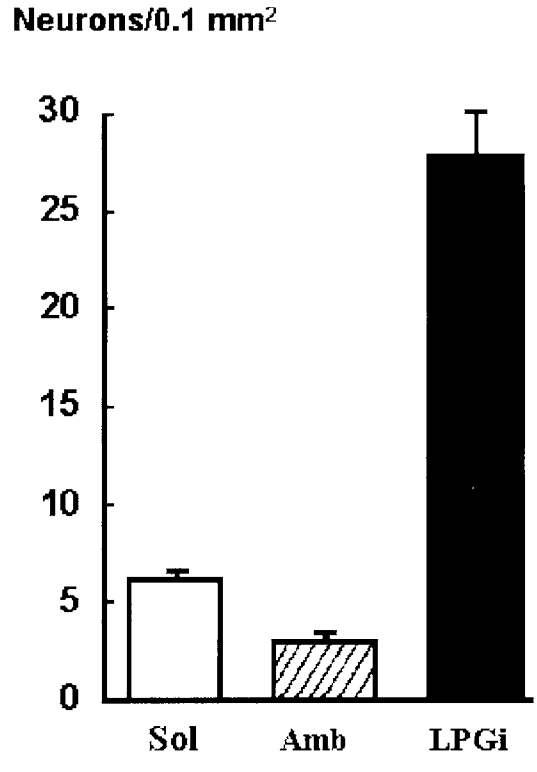

Fig. 3. Veraged density of the NADPH-diaphorase-positive neurons (cell per 0.1 mm^2) within the main nuclei involved in respiratory rhythmogenesis. Designations are the same as in Fig. 2 ($n = 8$).

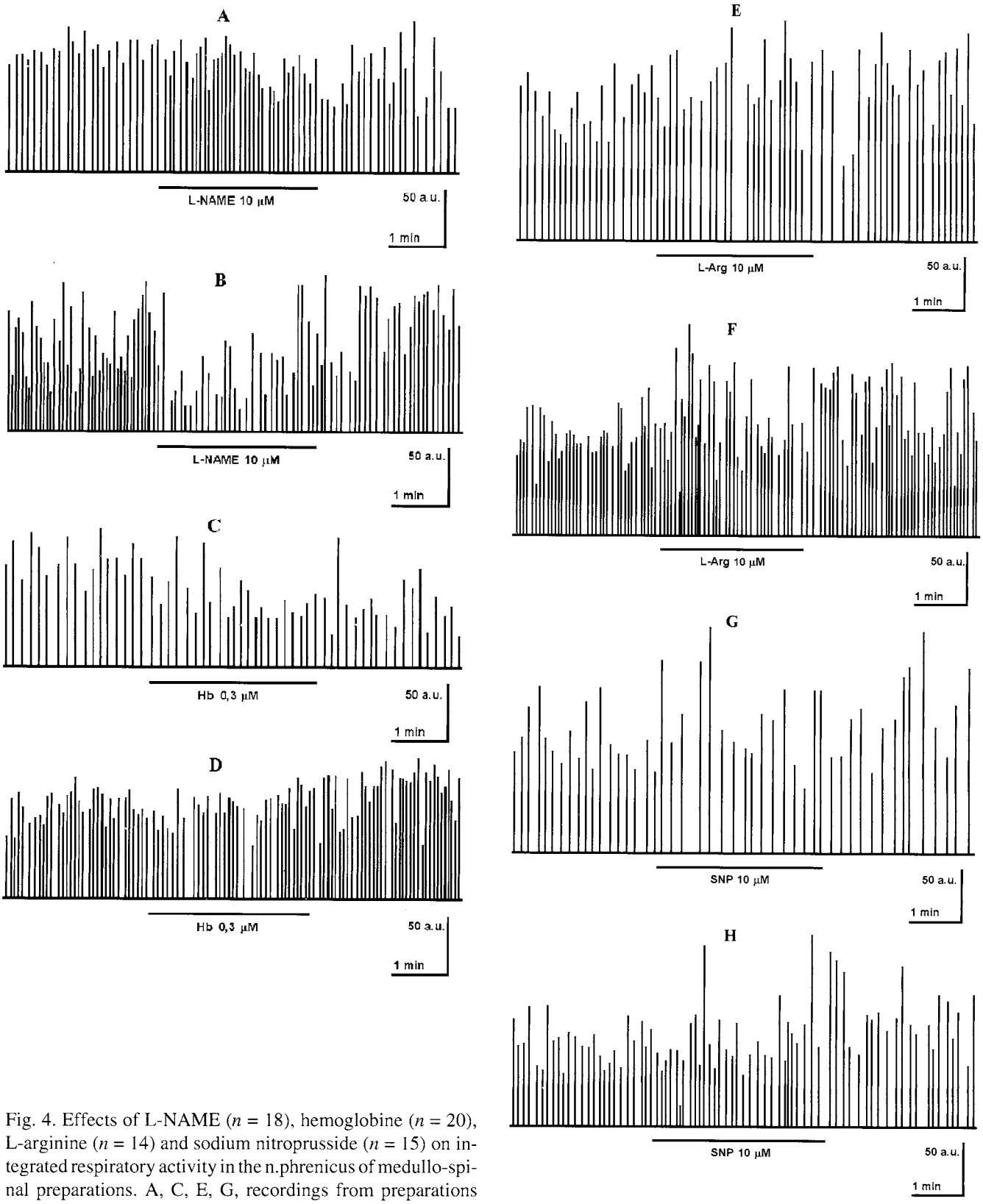

Fig. 4. Effects of L-NAME (n = 18), hemoglobine (n = 20), L-arginine (n = 14) and sodium nitroprusside (n = 15) on integrated respiratory activity in the n.phrenicus of medullo-spinal preparations. A, C, E, G, recordings from preparations with intact ventrolateral medulla; B, D, F, H, from those with a transection through level 2 (see Fig, 1).

Electrophysiology

THE EFFECT OF NO-RELATED DRUGS ON SIMSP'S RESPIRATORY RHYTHM IN CONTROL

In initial SIMSP configuration, superfusion of the preparations with L-NAME significantly increased the ID frequency (by 34.6%); simultaneously their amplitude dropped by 14.0% (Figs. 4 A and 5). After the rostral VLM (zone M) had been separated, application of L-NAME began to evoke opposite changes in the ID frequency: it dropped by 41.7% (Figs. 4B and 5). The same changes were evoked after Hb application (Figs. 4C, D and 5). Three-min-long application of L-Arg on intact SIMSP's resulted in a trend toward the decrease in the frequency (by 6.2%) and a significant increase in the amplitude of ID by 18.1% (Figs. 4E and 5). After separation of the rostral VLM the L-Arg application resulted in a significant increase in ID frequency (Figs. 4F and 5). At SNP applications the respiratory pattern generated by SIMSP significantly changed in the same fashion (the ID frequency dropped by 21.1% and the rise of ID amplitude by 14.9%). After rostral VLM separation the same SNP application significantly increased the ID frequency (Figs. 4G, H and 5).

THE EFFECT OF NO ON REALIZATION OF NMDAS INFLUENCE ON SIMSP'S RESPIRATORY RHYTHM

Three-min-long application of NMDA resulted in a significant (by 49.1%) rise in the ID frequency; a trend toward an increase in the ID amplitude was also noticed (Figs. 6A and 7). Preliminary 10-min-long superfusion with a L-NAME-containing solution resulted in an absence of significant changes in the ID parameters when NMDA was applied (Figs. 6B and 7). The same results were obtained when Hb was earlier administered (Figs. 6 C and 7). NMDA applied against the background of 10--min-long L-Arg application evoked a more prominent increase in the ID frequency (2.25 times), as compared with the control reaction (Figs. 6D and 7).

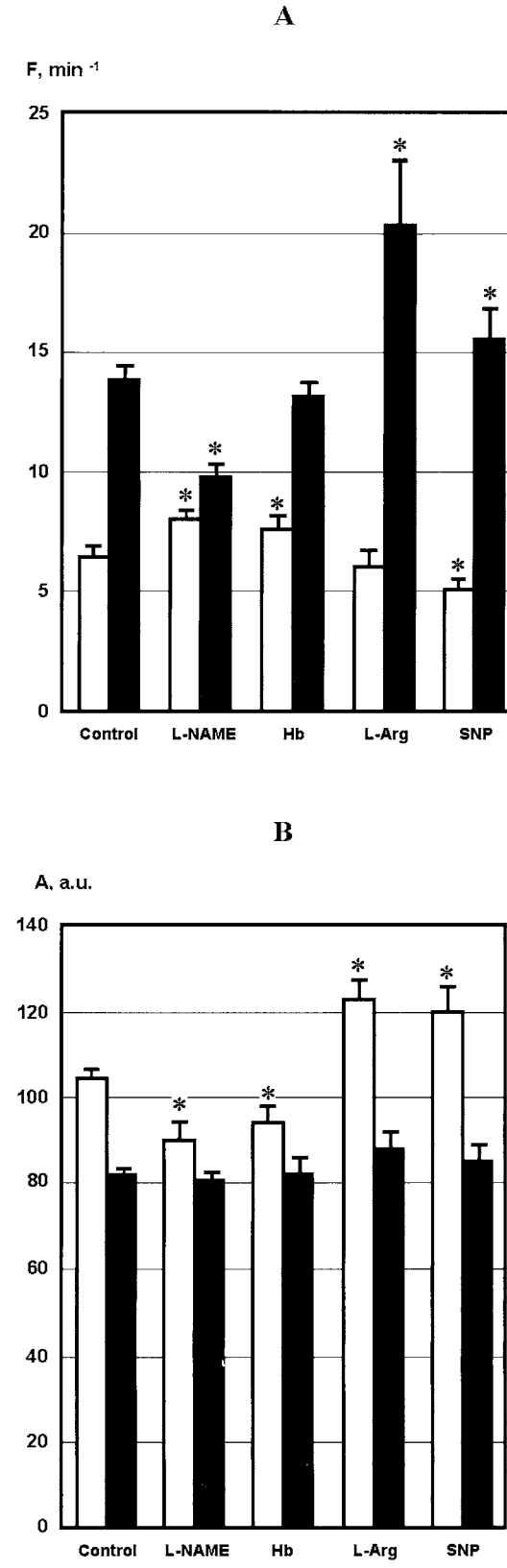

Fig. 5. Effects of L-NAME (n = 18), hemoglobine (n = 20), L-arginine (n = 14) and sodium nitroprusside (n = 15) on frequency (A) and amplitude (B) of integrated inspiratory discharges in the n. phrenicus of medullo-spinal preparations. Data averaged from intact preparations (open bars) and from those with a transection through level 2, Fig. 1 (filled bars). Significant differences (P<0.05) from the control values are shown by asterisks.

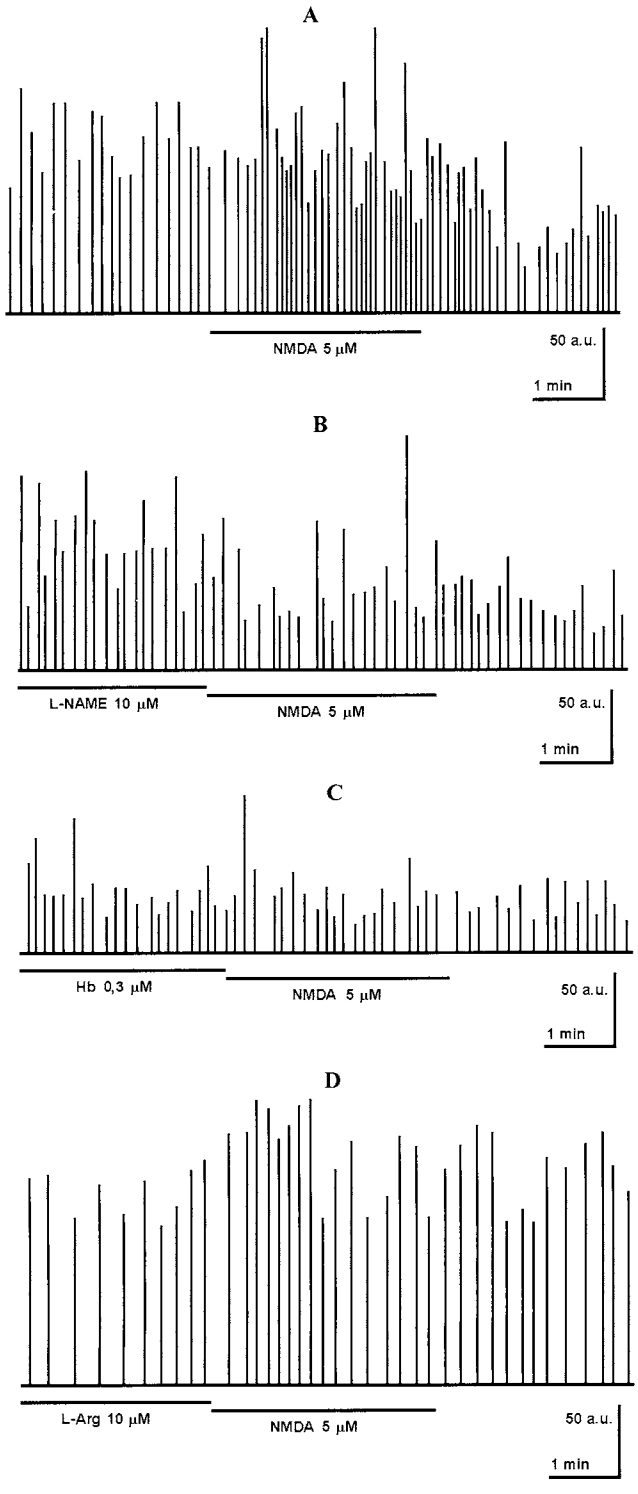

Fig. 6. Effects of NMDA on integrated respiratory activity in the n. phrenicus of medullo-spinal preparations. In B-D, L--NAME, hemoglobine and L-arginine, respectively, were preliminarily applied.

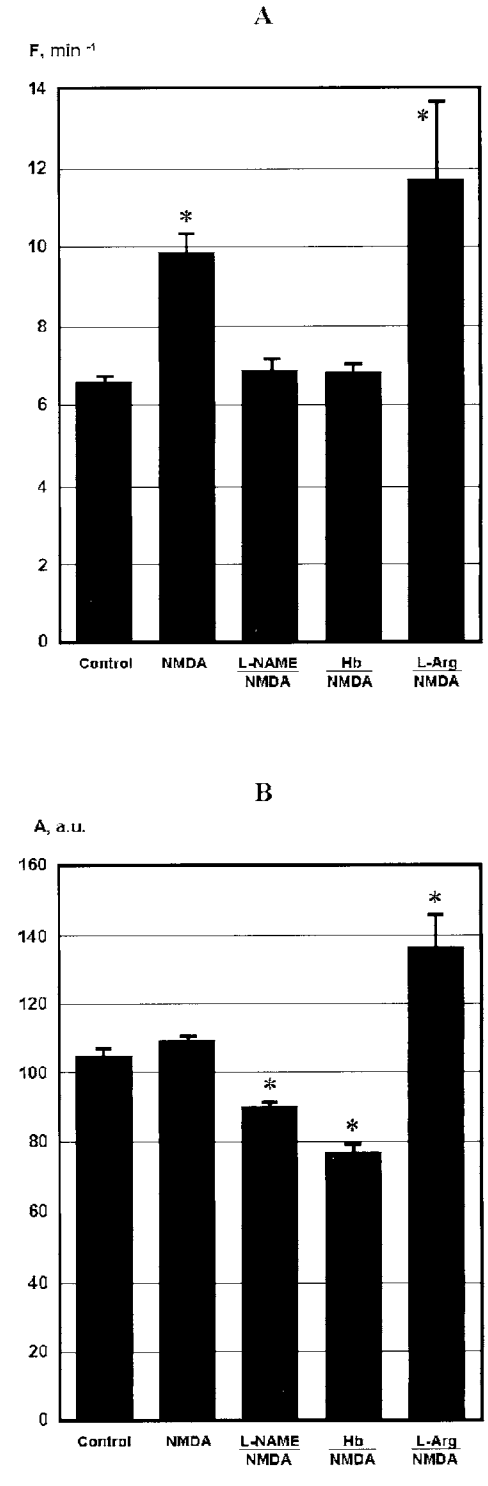

Fig. 7. Effects of L-NAME ($n = 18$), hemoglobine ($n = 20$) and L-arginine ($n = 14$) on frequency (A) and amplitude (B) of integrated inspiratory discharges in the n. phrenicus of medullo-spinal preparations, measured under NMDA application. Significant differences ($P<0.05$) from the control values are shown by asterisks.

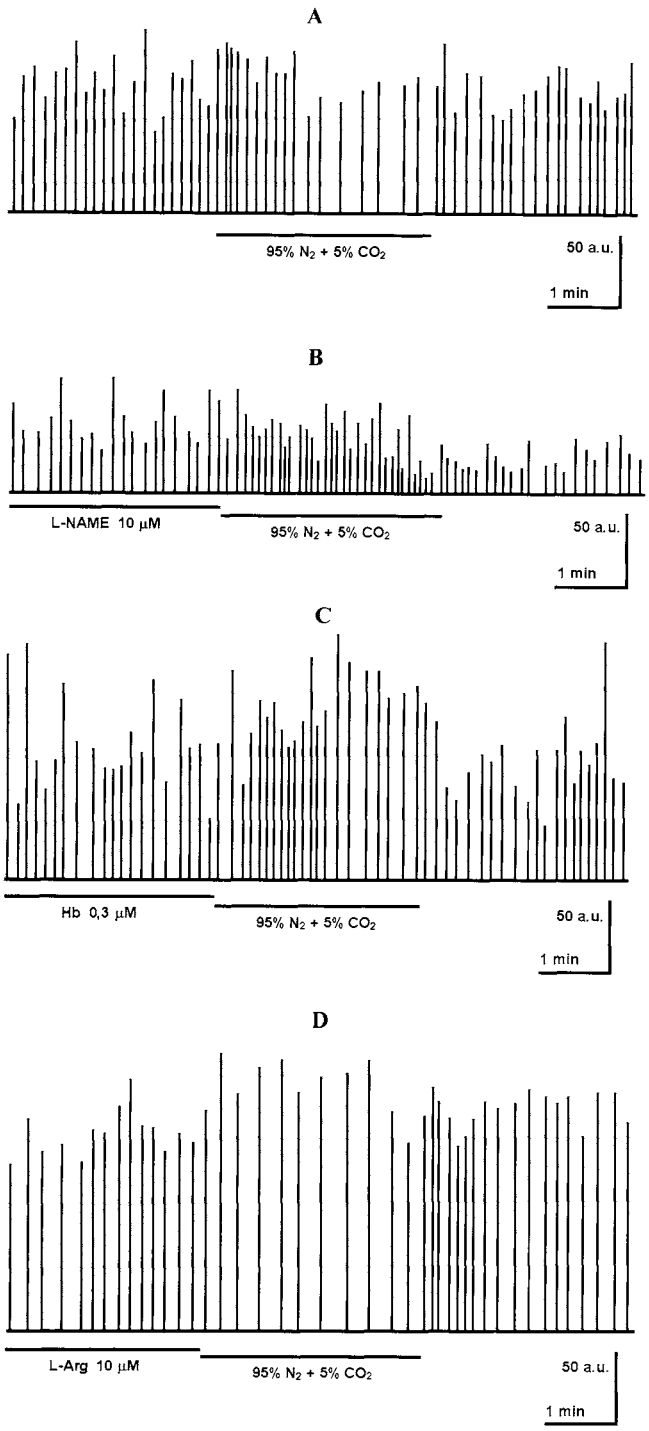

Fig. 8. Effects of hypoxia on integrated respiratory activity in the phrenic of medullo-spinal preparations (n = 27). In B-D, L-NAME, hemoglobine and L-arginine, respectively, were preliminarily applied.

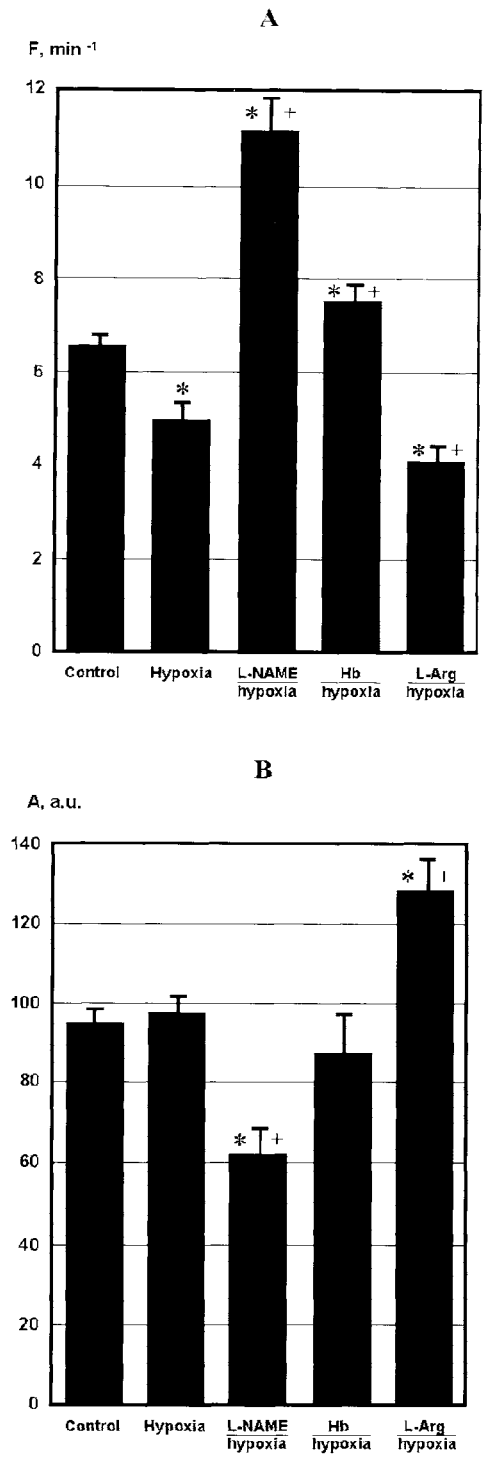

Fig. 9. Effects of L-NAME, hemoglobine and L-arginine on frequency (A) and amplitude (B) of integrated inspiratory discharges in the n. phrenicus of medullo-spinal preparations, measured under hypoxia (n = 27). Significant differences (P<0.05) from the control values are shown by asterisks, and those between the "normal" and "treated" preparations under hypoxia - crosses.

THE EFFECT OF NO ON SIMSP'S RESPIRATORY RHYTHM UNDER HYPOXIA

In intact preparations three-min-long superfusion by the anoxic isocapnic solution evoked a significant drop of averaged ID frequency (by 23.2%) (Figs. 8A and 9). After 10-min-long L-NAME application the ID frequency was significantly increased (by 66.8%) and the amplitude was significantly dropped (by 10.4%) during the subsequent hypoxic test (Figs. 8B and 9). The preliminary Hb application resulted in a similar effect under hypoxia (Figs. 8C and 9). Indices of ID under hypoxic condition after preliminary 10-min-long L-Arg application were changed in an opposite fashion: a significant drop in the frequency (by 31.4%) and rise of amplitude (by 7.0%) were observed (Figs. 8D and 9).

DISCUSSION

In our experiments the maximal density of NADPH--diaphorase positive neurons were observed in the reticular formation within the lateral paragigantocellular nucleus (LPGi). It is shown that in adult rats a group of stained cells is present within the same region (Vincent and Kimura 1992). Von Euler (1986) suggested that the LPGi may be involved in RR generation. The proposal was based on neuroanatomic evidence (retrograde transport of horseradish peroxidase from the n. ambiguus), and the reversible apnea induced by focal cooling of the LPGi (Andrezik et al. 1981, Budzinska et al. 1985). It cannot be ruled out that the LPGi region is the most essential source of endogenous NO modulating respiratory rhythmogenesis in newborn rats. Our data is compatible with the results obtained by Dun et al. (1994), who demonstrated the presence of NOS-positive cells within the LPGi in adult rats.

We have found NADPH-diaphorase-containing neurons within the main parts of ventral (n. ambiguus) and dorsal (n. tractus solitarii) respiratory groups. These results demonstrate that in the newborn rats neuronal substrates related to respiratory rhythmogenesis are capable of generating NO.

Results of our electrophysiological experiments allow us to conclude that in newborn rats endogenous NO mediates the influence of NMDA receptor activation on medullary RR. It was shown that the levels of NMDA receptor mRNA in NOS-containing neurons were higher than in non-NOS units (Price et al. 1993). In our previous experiments NMDA receptor blockade evoked significant changes in RR generated by SIMSP's with intact medulla from newborn rats (Volgin et al. 1998). However, NMDA receptors are not essential in generation of respiratory activity in the 400-mcm-thick medullary slices at the level of the pre-Botzinger complex (Funk et al. 1993). In this respect the LPGi as a more rostral medullary structure does not appear to be involved in the respiratory rhythmogenesis. NO is shown to increase glutamate release within the n. tractus solitarii in adult rats (Ogawa et al. 1995). It seems possible that the glutamatergic neurotransmission and NO formation, related to NMDA receptor activation, within the intermediate and caudal VLM excite respiratory neurons responsible for generation of RR. It should be taken into account that NMDA receptors are not directly involved in the functioning of the rhythm-generation network (Funk et al. 1993), but they are probably responsible for transmission of tonically active inputs to this network from the reticular formation neurons. It also cannot be ruled out that NO produced with NMDA receptor activation is able to amplify such activating signals due to intensification of the glutamate release and activation of non--NMDA receptors directly involved in the generation of RR (Funk et al. 1993).

In early postnatal rats the most rostral VLM portions, which projectionally correspond to the M chemosensitive zone, are known to tonically inhibit the RR-generating network (Marchenko et al. 1995), yet neurotransmitter mechanisms of this phenomenon remain obscure. In this respect we should mention that glutamatergic transmission is involved in the tonic inhibitory mechanism of the rostral VLM which supresses the respiratory hypoxic reflex (Dillon et al. 1991). Our data on the influence of the NO synthase blocking effect on RR generated by SIMSP before and after separation of the rostral VLM suggest the involvement of NO in this tonic inhibitory mechanism in newborn rats.

Perinatal hypoxia evokes a cascade of modifications in the brain tissue, which includes increased release of glutamate, activation of the corresponding receptors and an elevation in the intracellular Ca^{2+}. These changes result in the development of hypoxic-ischemic encephalopathy manifested by cramps, hypotonia and depression of respiration (Johnston 1997). We believe that, in addition to other mechanisms, the inhibitory NO-mediated mechanism of the rostral VLM is involved in hypoxic depression of respiration in neonates. It is known that NO, which is synthetized in carotid chemoreceptors, inhibits this activity in normoxia and hypoxia (Prabhakar

et al. 1995, Trzebski et al. 1995). It should be added that NO plays a considerable role of inhibitory neuromodulation in the rostral VLM regions involved in cardiovascular control (Moibenko et al. 1997). Recent data suggest that the hypoxic respiratory responses were augmented in mutant mice deficient in the neuronal NOS (Kline et al. 1998).

These results and our findings allow us to conclude that NO is an important central neuromodulator of the respiratory rhythm in newborn rats.

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