Archives Italiennes de Biologie, 143: 207-214, 2005.

CENTRAL NERVOUS MECHANISMS IN THE GENERATION OF THE PATTERN OF BREATHING

T. PANTALEO, F. BONGIANNI AND D. MUTOLO

Dipartimento di Scienze Fisiologiche, Università di Firenze, Viale G.B. Morgagni 63, I-50134 Firenze

INTRODUCTION

The neuronal network responsible for the generation of the eupneic pattern of breathing is localized in the lower brainstem. The basic mechanisms underlying respiratory rhythmogenesis seem to be located within the medulla oblongata (for review see Refs. 4, 10, 25, 26, 34) where medullary respiratory neurons are concentrated in the dorsal (DRG) and ventral (VRG) respiratory groups. Some lines of evidence have suggested that the DRG is mainly involved in phase-switching mechanisms, whilst the VRG has a prominent role in the gain control of the intensity of the inspiratory motor output (4, 34). Lesion studies led to the proposal that these medullary areas were not crucial for respiratory rhythm generation (30, 31). However, focal cold blocks of structures located in the ventrolateral medulla, encompassing the rostral portions of the VRG, caused strong depression of inspiratory activity or even apnea (9). These findings suggested that these structures may have a pivotal role in the respiratory rhythmogenesis probably by mediating the various drive inputs necessary for the maintenance of the respiratory rhythm.

Recently, the respiratory role of the rostral VRG subregions has been reevaluated. The hypothesis has been advanced that these subregions including the Bötzinger complex (BötC) and the pre-Bötzinger complex (pre-BötC) are essential components of the neural network underlying respiratory rhythm generation (10, 25, 27). The BötC contains a condensed population of expiratory neurons, while the pre-BötC, corresponding to the transition zone between the BötC and the more caudally located inspiratory portion of the VRG, is characterized by the presence of a mix of different types of inspiratory and expiratory neurons which are for the most part propriobulbar neurons; furthermore, pacemaker neurons have been identified in the pre-BötC of *in vitro* preparations of neonatal rodents (for review see Refs. 4, 10, 25-27, 34). The pre-BötC has been suggested to be crucial for respiratory rhythm generation not only in neonatal, but also in adult animals. In addition, recent lines of evidence indicate that in the rostral ventrolateral medulla of the neonatal rat there are two distinct oscillators, the pre-BötC and the Pre-inspiratory (Pre-I) networks, whose coupled activity would be necessary to produce the normal breathing pattern

Correspondence to: Prof. Tito Pantaleo, Dipartimento di Scienze Fisiologiche, Università di Firenze, Viale G.B. Morgagni 63, I-50134 Firenze, Italy; Tel. +39 055 4237319 / 4237316 / 4237311, Fax: +39 055 4379506, E-mail: tito.pantaleo@unifi.it

(16, 19). A novel group of Pre-I neurons with a prominent role in the respiratory rhythmogenesis has been found ventrolateral to the facial nucleus and close to the ventral medullary surface and named para-facial respiratory group (22). However, the presence of the Pre-I respiratory network and of the para-facial respiratory group remains to be determined in adult animals. At present, the role played by the BötC and the pre-BötC in respiratory rhythm generation is far from being definitely settled.

In an attempt to get further insights into the respiratory role of these rostral VRG subregions, we have made use of microinjections of the neurotoxin kainic acid (KA) (see e.g. Ref. 20) in anesthetized, vagotomized, paralysed and artificially ventilated rabbits. Since both *N*-methyl-D-aspartic acid (NMDA) and non-NMDA excitatory amino acid (EAA) receptors appear to mediate most of the excitatory drive input to medullary respiratory neurons during their active periods (4, 13), we reasoned that the removal of such input to the BötC and the pre-BötC could correspond, to some extent, to their ablation. Thus, the respiratory effects induced by microinjections of ionotropic glutamate receptor antagonists into the same subregions were investigated to corroborate the results obtained with KA microinjections.

METHODS

Experiments were performed on male New Zealand white rabbits (2.7-3.2 kg) anesthetized with a mixture of α-chloralose (40 mg/kg i.v.; Sigma) and urethane (800 mg/kg i.v.; Sigma), supplemented when necessary (4 mg/kg and 80 mg/kg, respectively). In this report, we present results derived from 32 experiments in which KA lesions were performed and 36 experiments dealing with EAA receptor antagonism. The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw. All animal care and experimental procedures were conducted in accordance with the Directives of the European Community as well as with the Italian legislation.

Animal preparation and experimental procedures have been fully described in previous reports (7, 8, 21). In particular, the animals were vagotomized, paralysed (gallamine triethiodide 4 mg/kg i.v., supplemented with 2 mg/kg every 30 min, Sigma) and artificially ventilated. Intratracheal and arterial blood pressure as well as end-tidal CO_2 partial pressure (PCO_2) were monitored. In paralysed animals, the depth of anesthesia was assessed by monitoring a stable and regular pattern of phrenic activity and the absence of fluctuations in arterial blood pressure whether spontaneous or in response to somatic nociceptive stimulation. Efferent phrenic nerve activity was recorded with bipolar platinum electrodes from desheathed C_5 phrenic roots, amplified, full-wave rectified and integrated (RC filter, time constant 100 ms). Extracellular recordings were made with tungsten microelectrodes (5-10 MΩ impedance at 1 kHz) to localize the different VRG subregions as already described (21). Integrated phrenic nerve activity along with the signals of all variables studied were recorded on an eight-channel rectilinearly writing chart recorder.

Bilateral microinjections (20-50 nl) were performed via a glass micropipette (tip diameter 10-25 μm) by applying pressure using an air-filled syringe connected to the micropipette by polyethylene tubing. KA was obtained from Sigma. The following EAA receptor antagonists (Tocris Cookson) were used: kynurenic acid (KYN), a broad spectrum EAA antagonist, D(-)-2-amino-5-phosphonopentanoic acid (D-AP5), a selective NMDA receptor antagonist, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a selective non-NMDA receptor antagonist. Only one ionotropic glutamate receptor antagonist was tested in each preparation. Drug concentrations were selected in preliminary trials and were in the same range as those previously reported in *in vivo* studies on medullary respiration-related regions (see e.g. Refs. 1, 3, 6, 7, 20, 28 also for further Refs.). To

evaluate chemical sensitivity during respiratory responses, hypercapnic and hypoxic stimuli were used. Hypercapnia was produced by allowing the animal to inspire appropriate mixtures of CO_2 and O_2 from a large bag (150 l); P_{CO2} was adjusted at approximately 30 mmHg higher than the level of spontaneous breathing (range 60-65 mmHg). After a stable level of P_{CO2} had been achieved, hypercapnic stimulation was maintained for at least 3 min. Hypoxia was induced by allowing the animal to inspire a gas mixture containing 6% O_2 and 94% N_2 for about 2 min. The histological control of injection sites was performed on serial frozen coronal sections (20 μm thick) stained with Cresyl violet.

RESULTS

Bilateral microinjections of 4.7 mM KA into either the BötC or the pre-BötC eliminated respiratory rhythmicity within 30 min in the presence of relatively intense tonic phrenic nerve activity (tonic apnea). Rhythmic activity resumed within 50 min as low-amplitude, high-frequency irregular oscillations superimposed on tonic phrenic nerve activity and then displayed a progressive, although incomplete recovery. Marked increases in respiratory frequency and rate of rise of inspiratory activity, associated with prominent reductions in peak phrenic amplitude were seen 60 min after KA microinjections. Decreases in peak phrenic amplitude associated with increases in frequency were still present 120 min after KA microinjections (Fig. 1). The complete absence of responsiveness to hypercapnia or hypoxia was observed during tonic apnea. Both types of chemical sensitivity were present as soon as some rhythmic respiratory activity resumed. Combined bilateral KA microinjections into the BötC and the pre-BötC caused long lasting (> 3 h) and possibly persistent tonic apnea.

Bilateral microinjections of 50 mM KYN into the BötC produced within 3-5 min a pattern of breathing characterized by low-amplitude, high-frequency irregular oscillations superimposed on tonic phrenic nerve activity. Within 10 min after the completion of the injections, tonic apnea developed. Similar microinjections into the pre-BötC caused progressive increases in respiratory frequency and rate of rise of inspiratory activity, associated with the development of tonic discharges and reductions in peak phrenic amplitude; these changes eventually resulted in irregular patterns of breathing (Fig. 1).

Bilateral microinjections of 1 and 10 mM D-AP5 into the BötC caused dose-dependent respiratory responses characterized by increases in respiratory frequency and rate of rise of inspiratory activity, accompanied by decreases in peak phrenic amplitude. Respiratory responses quite similar to those induced by KYN, including tonic apnea, were observed following microinjections of 20 mM D-AP5. Neither hypoxia nor hypercapnia were able to restore respiratory rhythmicity during tonic apnea. However, during the recovery period, when low-amplitude, high-frequency oscillations superimposed on tonic phrenic activity were present, both types of chemical stimuli reduced the level of tonic activity and restored rhythmic ramp phrenic bursts, albeit at high frequency. Blockades of NMDA receptors by bilateral microinjections of D-AP5 (1, 10 and 20 mM) into the pre-

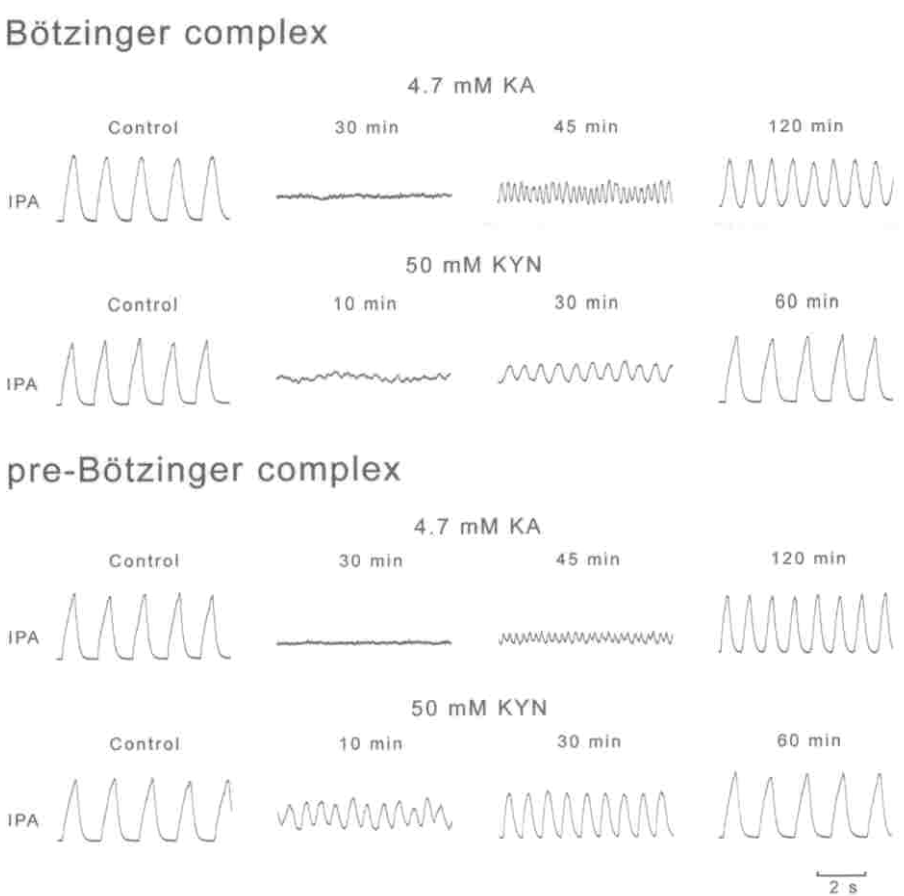

Fig. 1. - *Respiratory responses induced by bilateral microinjections (30 nl) of 4.7 mM kainic acid (KA) and 50 mM kynurenic acid (KYN) into the Bötzinger complex and the pre-Bötzinger complex.* Integrated phrenic nerve activity (IPA) under control conditions and at different times after the completion of the injections.

BötC provoked respiratory responses, to a great extent, similar to those described for the BötC; however, in no case loss of respiratory rhythmicity was observed. Bilateral microinjections of CNQX (1, 10 and 20 mM) into the BötC and the pre-BötC caused much less pronounced effects mainly consisting of increases in respiratory frequency.

Respiratory changes induced by EAA receptor antagonists were reversible over time; the recovery process was characterized by the same phases as those observed during the development of the respiratory responses, but in the reverse order (Fig. 1). All the observed respiratory responses were not accompanied by significant changes in blood pressure.

DISCUSSION

The results of these studies show that the BötC and the pre-BötC exert a potent and rather similar control on both the intensity and frequency of inspiratory activity and that the activation of ionotropic glutamate receptors is involved in the mediation of this control, with a major role played by NMDA receptors. These findings and, in particular, the occurrence of respiratory responses characterized by irregular, high-frequency inspiratory patterns or by tonic apnea insensitive to chemical stimuli support the view that important components of the neural network underlying respiratory rhythmogenesis are located in the rostral VRG subregions.

The reliability of microinjection techniques as well as the localization of the injection sites and the spread of the injectate have been discussed in detail in previous reports (7, 8, 21). Respiratory responses evoked by blockades of ionotropic glutamate receptors and by KA lesions present several common features. In more detail, increases in respiratory frequency associated with reductions in peak phrenic amplitude or irregular oscillations superimposed on tonic phrenic nerve activity were observed both in response to EAA receptor antagonists and during the postapneic period induced by KA microinjections. However, tonic apnea in response to KYN or D-AP5 microinjections was obtained only in the BötC; in this connection, it should be recalled that the removal of a prominent excitatory input to rostral VRG subregions may not correspond completely to their ablation. The finding that not only the amplitude, but also the frequency of respiratory bursts changed in response to KA lesions or EAA receptor blockades suggests that these rostral VRG subregions have an important role in respiratory rhythm generation (see e.g. Refs. 21, 34). Increases in respiratory frequency associated with reductions in peak phrenic amplitude have been reported to occur during unilateral focal cold blocks in rostral ventrolateral medullary regions encompassing the BötC and the pre-BötC (9) as well as following unilateral or bilateral KA or electrolytic lesions of rostral VRG subregions including the BötC (20, 33, 35) and after unilateral microinjections of tetrodotoxin (24) or bilateral microinjections of cobalt chloride (2) into the pre-BötC. Similar breathing patterns have been induced by KYN or NMDA receptor antagonists in rostral ventrolateral medullary sites possibly corresponding, at least in part, to the rostral VRG subregions (1, 18).

The occurrence of apneic responses or irregular and life threatening pattern of breathing further supports the hypothesis that we have operated on important neural substrates subserving respiratory rhythmogenesis. Apneic responses have been obtained in the cat with different types of lesions (29, 33) or focal cold blocks (9) of the BötC. It is worth noting that loss of respiratory rhythmicity combined with the presence of tonic inspiratory activity (tonic apnea) has also been obtained in response to the same experimental manouvres at sites of the ventrolateral medulla within the BötC or close neighbouring regions (9, 20, 29, 33). Persistent or transient apnea has been induced by blockades or ablations of neuronal activities within the pre-BötC or possibly corresponding medullary regions of cats and rats in some previous studies (e.g. Refs. 9, 14, 15, 17, 24, 28, 32; see also Ref. 25), but contrasting results were obtained in other investigations (2, 11). The reasons of these discrepancies are obscure. Present

findings do not support the hypothesis of a crucial role played by the pre-BötC in respiratory rhythm generation of *in vivo* preparations of adult mammals (25); however, they appear to be in keeping with the recent suggestion that a respiratory rhythm can be generated by interactions within the respiratory network even when pre-BötC neurons have been silenced or destroyed (see e.g. Refs. 10, 12, 27).

As already discussed (21), current models of respiratory rhythmogenesis may provide some explanation for the genesis of the observed responses. The importance of inhibitory interactions in respiratory rhythm generation is well documented in adult animals (26, 27); inhibitory interactions and postinhibitory rebound are involved in the synchronization of neuronal systems and may shape the output of the respiratory network. Thus, both neuronal lesions and EAA receptor antagonism may reduce or even cancel the activity of inhibitory interneurons and cause desynchronization of the respiratory network and breathing patterns characterized by increases in respiratory frequency, decreases in peak phrenic amplitude and parallel increases in tonic activity. The observed irregular patterns of breathing and even more tonic apnea may represent the extreme consequence of these neuronal processes. The development of tonic activity either associated with apnea or with irregular patterns of breathing may be due to the lack of potent inhibitory influences on inspiratory activity (see e.g. Ref. 23) such as those arising from rostral expiratory neurons (5, 8; for review see Refs. 4, 34) that are mainly concentrated in the BötC, but are also encountered in the pre-BötC (e.g. Refs. 10, 21, 25, 32). Nevertheless, as suggested by *in vitro* studies on neonatal rodents (10, 25, 27) and in particular by recent studies in adult rats on pre-BötC neurokinin-1 receptor expressing neurons which are glutamatergic and probably excitatory (10, 12), the possibility should be taken into consideration that KA lesions and EAA antagonism have interfered not only with the function of inhibitory interneurons, but also with that of excitatory interneurons located in the rostral VRG subregions.

SUMMARY

The role of the Bötzinger complex (BötC) and the pre-Bötzinger complex (pre-BötC) in the genesis of the breathing pattern was investigated in anesthetized, vagotomized, paralysed and artificially ventilated rabbits making use of bilateral microinjections of kainic acid (KA) and excitatory amino acid (EAA) receptor antagonists. KA microinjections into either the BötC or the pre-BötC transiently eliminated respiratory rhythmicity in the presence of tonic phrenic activity (tonic apnea). Rhythmic activity resumed as low-amplitude, high-frequency irregular oscillations, superimposed on tonic inspiratory activity and displayed a progressive, although incomplete recovery. Microinjections of kynurenic acid (KYN) and D(-)-2-amino-5-phosphonopentanoic acid (D-AP5) into the BötC caused a pattern of breathing characterized by low-amplitude, high-frequency irregular oscillations and subsequently tonic apnea. Responses to KYN and D-AP5 in the pre-BötC were similar, although less pronounced than those elicited by these drugs in the BötC and never characterized by tonic apnea. Microinjections of 6-cyano-7-nitroquinoxaline-2,3-

dione (CNQX) into the BötC and the pre-BötC induced much less intense responses mainly consisting of increases in respiratory frequency. The results show that the investigated medullary regions play a prominent role in the genesis of the normal pattern of breathing through the endogenous activation of EAA receptors.

Acknowledgements. - This study was supported by grants from the Ministero dell'Istruzione, Università e Ricerca of Italy.

REFERENCES

1. Abrahams, T.P., Hornby, P.J., Walton, D.P., Taveira Dasilva, A.M. and Gillis, R.A. An excitatory amino acid(s) in the ventrolateral medulla is (are) required for breathing to occur in the anesthetized cat. *J. Pharmacol. Exp. Ther.*, **259**: 1388-1395, 1991.
2. Anderson, M.K. and Speck, D.F. Evidence for medullary projections of the intercostal nerve-elicited inspiratory termination. *Brain Res.*, **831**: 306-310, 1999.
3. Berger, I., Gillis, R.A., Vitagliano, S., Panico, W.H., Magee, S., Kelly, M., Norman, W.P., Mcmanigle, J.E. and Taveira Dasilva, A.M. NMDA receptors are involved at the ventrolateral nucleus tractus solitarii for termination of inspiration. *Eur. J. Pharmacol.*, **277**: 195-208, 1995.
4. Bianchi, A.L., Denavit-Saubie, M. and Champagnat, J. Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters. *Physiol. Rev.*, **75**: 1-45, 1995.
5. Bongianni, F., Fontana, G. and Pantaleo, T. Effects of electrical and chemical stimulation of the Bötzinger complex on respiratory activity in the cat. *Brain Res.*, **445**: 254-261, 1988.
6. Bongianni, F., Mutolo, D., Carfi, M. and Pantaleo, T. Area postrema glutamate receptors mediate respiratory and gastric responses in the rabbit. *NeuroReport*, **9**: 2057-2062, 1998.
7. Bongianni, F., Mutolo, D., Carfi, M. and Pantaleo, T. Respiratory responses to ionotropic glutamate receptor antagonists in the ventral respiratory group of the rabbit. *Pflugers Arch.*, **444**: 602-609, 2002.
8. Bongianni, F., Mutolo, D. and Pantaleo, T. Depressant effects on inspiratory and expiratory activity produced by chemical activation of Bötzinger complex neurons in the rabbit. *Brain Res.*, **749**: 1-9, 1997.
9. Budzinska, K., Von Euler, C., Kao, F.F., Pantaleo, T. and Yamamoto, Y. Effects of graded focal cold block in the solitary and para-ambigual regions of the medulla in the cat. *Acta Physiol. Scand.*, **124**: 317-328, 1985.
10. Feldman, J.L., Mitchell, G.S. and Nattie, E.E. Breathing: Rhythmicity, Plasticity, Chemosensitivity. *Annu. Rev. Neurosci.*, **26**: 239-266, 2003.
11. Fung, M.L., Wang, W. and St John, W.M. Medullary loci critical for expression of gasping in adult rats. *J. Physiol. (Lond.)*, **480**: 597-611, 1994.
12. Gray, P.A., Janczewski, W.A., Mellen, N., Mccrimmon, D.R. and Feldman, J.L. Normal breathing requires pre-Bötzinger complex neurokinin-1 receptor- expressing neurons. *Nat. Neurosci.*, **4**: 927-930, 2001.
13. Haji, A., Takeda, R. and Okazaki, M. Neuropharmacology of control of respiratory rhythm and pattern in mature mammals. *Pharmacol. Ther.*, **86**: 277-304, 2000.
14. Hsieh, J.H., Chang, Y.C., Su, C.K., Hwang, J.C., Yen, C.T. and Chai, C.Y. A single minute lesion around the ventral respiratory group in medulla produces fatal apnea in cats. *J. Auton. Nerv. Syst.*, **73**: 7-18, 1998.
15. Huang, Q., Zhou, D. and St John, W.M. Lesions of regions for in vitro ventilatory genesis eliminate gasping but not eupnea. *Respir. Physiol.*, **107**: 111-123, 1997.

16. JANCZEWSKI, W.A., ONIMARU, H., HOMMA, I. AND FELDMAN, J.L. Opioid-resistant respiratory pathway from the preinspiratory neurones to abdominal muscles: in vivo and in vitro study in the newborn rat. *J. Physiol. Lond.*, **545**: 1017-1026, 2002.
17. KOSHIYA, N. AND GUYENET, P.G. Tonic sympathetic chemoreflex after blockade of respiratory rhythmogenesis in the rat. *J.Physiol. (Lond.)*, **491**: 859-869, 1996.
18. MCMANIGLE, J.E., PANICO, W.H., DASILVA, A.M. AND GILLIS, R.A. Respiratory effects produced by microinjection of L-glutamate and an uptake inhibitor of L-glutamate into the caudal subretrofacial area of the medulla. *Eur. J. Pharmacol.*, **280**: 257-275, 1995.
19. MELLEN, N.M., JANCZEWSKI, W.A., BOCCHIARO, C.M. AND FELDMAN, J.L. Opioid-induced quantal slowing reveals dual networks for respiratory rhythm generation. *Neuron*, **37**: 821-826, 2003.
20. MORIN-SURUN, M.P., CHAMPAGNAT, J., BOUDINOT, E. AND DENAVIT-SAUBIE, M. Differentiation of two respiratory areas in the cat medulla using kainic acid. *Respir. Physiol.*, **58**: 323-334, 1984.
21. MUTOLO, D., BONGIANNI, F., CARFI, M. AND PANTALEO, T. Respiratory changes induced by kainic acid lesions in rostral ventral respiratory group of rabbits. *Am. J. Physiol Regul. Integr. Comp. Physiol.*, **283**: R227-R242, 2002.
22. ONIMARU, H. AND HOMMA, I. A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J. Neurosci.*, **23**: 1478-1486, 2003.
23. PIERREFICHE, O., FOUTZ, A.S., CHAMPAGNAT, J. AND DENAVIT-SAUBIE, M. NMDA and non-NMDA receptors may play distinct roles in timing mechanisms and transmission in the feline respiratory network. *J. Physiol. Lond.*, **474**: 509-523, 1994.
24. RAMIREZ, J.M., SCHWARZACHER, S.W., PIERREFICHE, O., OLIVERA, B.M. AND RICHTER, D.W. Selective lesioning of the cat pre-Bötzinger complex in vivo eliminates breathing but not gasping. *J. Physiol. Lond.*, **507**: 895-907, 1998.
25. REKLING, J.C. AND FELDMAN, J.L. PreBötzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. *Annu. Rev. Physiol.*, **60**: 385-405, 1998.
26. RICHTER, D.W. AND SPYER, K.M. Studying rhythmogenesis of breathing: comparison of in vivo and in vitro models. *Trends Neurosci.*, **24**: 464-472, 2001.
27. SMITH, J.C., BUTERA, R.J., KOSHIYA, N., DEL NEGRO, C., WILSON, C.G. AND JOHNSON, S.M. Respiratory rhythm generation in neonatal and adult mammals: the hybrid pacemaker-network model. *Respir. Physiol.*, **122**: 131-147, 2000.
28. SOLOMON, I.C. Ionotropic excitatory amino acid receptors in pre-Botzinger complex play a modulatory role in hypoxia-induced gasping in vivo. *J. Appl. Physiol.*, **96**: 1643-1650, 2004.
29. SPECK, D.F. Bötzinger complex region role in phrenic-to-phrenic inhibitory reflex of cat. *J. Appl. Physiol.*, **67**: 1364-1370, 1989.
30. SPECK, D.F. AND BECK, E.R. Respiratory rhythmicity after extensive lesions of the dorsal and ventral respiratory groups in the decerebrate cat. *Brain Res.*, **482**: 387-392, 1989.
31. SPECK, D.F. AND FELDMAN, J.L. The effects of microstimulation and microlesions in the ventral and dorsal respiratory groups in medulla of cat. *J. Neurosci.*, **2**: 744-757, 1982.
32. ST JACQUES, R. AND ST JOHN, W.M. Transient, reversible apnoea following ablation of the pre-Bötzinger complex in rats. *J. Physiol. Lond.*, **520**: 303-314, 1999.
33. ST JOHN, W.M., HWANG, Q., NATTIE, E.E. AND ZHOU, D. Functions of the retrofacial nucleus in chemosensitivity and ventilatory neurogenesis. *Respir. Physiol.*, **76**: 159-171, 1989.
34. VON EULER, C. Brain stem mechanisms for generation and control of breathing pattern. In: CHERNIACK, N.S. AND WIDDICOMBE, J.G. (Eds.). *Handbook of Physiology. The Respiratory System. Control of Breathing.* Bethesda, Maryland: American Physiological Society, 1986, pp. 1-67.
35. ZHANG, Z., XU, F. AND FRAZIER, D.T. Role of the Bötzinger complex in fastigial nucleus-mediated respiratory responses. *Anat. Rec.*, **254**: 542-548, 1999.