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Effects of riluzole and flufenamic acid on eupnea and gasping of neonatal mice *in vivo*

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Abstract

The pre-Bötzinger complex (PBC), part of the ventral respiratory group that is responsible for inspiratory rhythm generation, contains at least two types of pacemaker neurons. *In vitro* studies have shown that bursting properties of one type of pacemaker relies on a riluzole-sensitive persistent sodium current, whereas bursting of a second type is sensitive to flufenamic acid (FFA), a calcium-dependent nonspecific cationic current blocker. *In vitro*, under control conditions, the PBC generates fictive eupneic activity that depends on both riluzole-sensitive and FFA-sensitive pacemaker neurons. During hypoxia the PBC generates fictive gasping activity and only riluzole-sensitive pacemaker neurons appear to be necessary for this rhythm. We carried out pharmacological experiments to test the role of respiratory pacemaker neurons *in vivo* by performing plethysmographic recordings on neonate mice. As reported *in vitro*, eupnea activity *in vivo* is abolished only if both FFA and riluzole are coadministered intracisternally, but not when either of them is administered independently. On the other hand riluzole, but not FFA, drastically reduced gasping generation and compromised the ability of mice to autoresucitate. Neither substance P nor forskolin was able to reestablish respiratory activity after riluzole and FFA coapplication. Our results confirm *in vitro* reports and suggest that eupnea generation in neonates requires a complex neuronal network that includes riluzole- and FFA-sensitive elements and that gasping activity depends mostly on a riluzole-sensitive mechanism.

Keywords: Respiratory rhythm; Pre-Bötzinger complex; Pacemaker neurons; Eupnea; Gasping; Autoresucitation

A key role for respiratory rhythm generation has been assigned to a group of neurons in the ventrolateral medulla, called the ventral respiratory group (VRG). The VRG includes the pre-Bötzinger complex (PBC) and the parafacial respiratory group (pFRG), that contain a heterogeneous population of respiratory neurons some of which have pacemaker properties that are considered important for rhythmogenesis [6,8,9,13,12,20,21]. Under normoxic conditions rhythmic eupneic and sigh activity are produced by the PBC [7,15,13]. During continuous hypoxic conditions, these rhythms are supplanted by another rhythm called fictive gasping [7,18]. Today the evidence points towards the hypothesis that the respiratory rhythm emerges from the coupling of synaptic and intrinsic membrane properties that may require pacemaker neurons [12–15,20,26,27].

The PBC contains at least two types of pacemaker neurons. The bursting mechanism of one group of pacemakers (Type I), relies on a persistent Na^+ current since their bursting activity is abolished by the persistent Na^+ current blocker riluzole [2,3,13,27]. In contrast, bursting by the other group of pacemaker neurons (Type II), seems to rely on a Ca^{2+}-activated nonspecific cationic current (ICAN) [3,13,27]. This is supported by the fact that their bursting activity is blocked by the ICAN blocker FFA [13]. Remarkably, the bursting of type I pacemaker neurons is not eliminated by FFA and type II pacemaker bursting persists in the presence of riluzole [13]. Both drugs were used to test the role of specific pacemaker neurons in the activity of the PBC *in vitro* and the results showed that in normoxic conditions riluzole does not abolishes the generation of fictive eupnea [2,13], but abolishes fictive gasping generation [13]. On the other hand, FFA application alone does not abolish fictive eupnea or fictive gasping [13]. Interestingly, when both drugs are present, no rhythmic activity is recorded in the PBC *in vitro* [3,13]. One report showed that in those conditions, after blocking both pacemakers populations, substance P can reestablish rhythmic activity [3], however this finding could not be reproduced in similar conditions [27]. Overall, these findings led to the hypothesis that both types of pacemaker neurons participate in

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eupnea generation in normoxia; whereas riluzole-sensitive pacemaker neurons seem to be the sole drivers of gasping [13,12,27]. This last finding has clinical relevance since gasping is a major component of the autoresuscitation process that follows hypoxia [4,5,24], and both mechanisms seem to be altered in victims of sudden infant death syndrome [17,22,24].

Using the *in situ* preparation, it was confirmed that riluzole blocks fictive gasping generation but does not affect fictive eupnea [10], and it has been recently shown that riluzole does not abolish eupnea *in vivo* [23]. However, so far it has not been tested, in these preparations, if coapplication of riluzole and FFA abolish eupnea generation and whether or not either riluzole or FFA affect gasping generation and concomitantly autoresuscitation. We aimed to answer these questions by performing plethysmographic recordings in neonate mice, which might be a better model of SIDS than adults. We observed that eupnea activity is abolished when both FFA and riluzole are coadministered but not when either of them is administered independently. Gasping is abolished only with riluzole and this reduction compromised the ability of mice to autoresuscitate. Apnea produced by coapplication of riluzole and FFA could not be reverted following application of either substance P or forskolin.

The experiments were performed using postnatal day 9–13 (P9–P13) neonatal mice belonging to the Swiss-Webster mouse strain. Adequate measures were taken to minimize pain and discomfort of experimental animals. The experimental procedures were approved by The Local Committee of Ethics on Animal Experimentation (CICUAL-Cinvestav) and followed the regulations established in the Mexican Official Norm for the Use and Care of Laboratory Animals (NOM-062-ZOO-1999). The animals were barely anesthetized with ether before transferred to a plethysmographic chamber and left to recover at least for 30 min.

Respiratory signals elicited by displacement of the chest were acquired in restrained animals placed in a size-appropriate head-out plethysmographic chamber by using the barometric method [5], such that gas mixtures could be rapidly changed as needed. For all experiments, environmental temperature was maintained constant, slightly below the thermoneutral range for rat pups ($31 \pm 1\,^{\circ}C$). Pressure changes in the chamber due to the inspiratory and expiratory displacements were measured by using a high-gain differential pressure transducer (Grass Instruments, Quincy, MA, USA). The signal was amplified and filtered (low pass 0.25 kHz, high pass 0.3 Hz) with a DC amplifier (Grass Instruments, Quincy, MA, USA). Control breathing was recorded for 10 min with mice breathing humidified room air. Hypoxic conditions were applied for 2 min with mice breathing a humidified gas mixture containing 97% N_2 and 3% CO_2. This condition has been previously reported to be effective for the induction of gasping followed by hypoxic-apnea and to be useful for the evaluation of autoresuscitation as well [4,5]. After the hypoxic period mice were left to recover by breathing humidified room air and full recovery of eupneic activity was considered as autoresuscitation [4,5]. Both riluzole and flufenamic acid (SIGMA-RBI, St. Louis, MO), were solubilized in dimethylsulfoxide (DMSO, SIGMA-RBI, St. Louis, MO) and applied intracisternally (i.c., in 5 μl) at different doses using a precision syringe (Rainin, Oakland, CA). Susbtance P or forskolin (SIGMA-RBI, St. Louis, MO), solubilized in artificial cerebrospinal fluid [11], were intracisternally injected as well.

All recordings were stored on a personal computer using a program designed by Luis Carrillo-Reid and José Bargas at Instituto de Fisiología Celular, UNAM (México) in a LabView environment (National Instruments Austin, TX, USA), using an acquisition system from National Instruments (Austin, TX, USA). Data analysis was done offline using customized analysis software written with IGOR Pro (Wavemetrics, Lake Oswego, Oregon). We obtained the irregularity score (S) of the respiratory cycle using the following formula: $S_n = \mathrm{ABS}(P_n - P_{n-1})/P_{n-1}$ with S_n = score of the nth cycle, P_n being its period, P_{n-1} the period of the preceding burst and ABS the absolute value. Data are expressed as mean ± S.E. Statistical differences were assessed by Student's t-test for unpaired samples.

Under control conditions (breathing room air) neonate mice (P9–P13), administered i.c. with vehicle (5 μl DMSO), show typical eupnea breathing (Fig. 1A, $n = 7$), with a mean respiratory frequency of 2.36 ± 0.17 Hz; inspiratory duration (measured at 50% of the maximal amplitude-half duration) of 123.7 ± 11.8 ms and an irregularity score of 0.19 ± 0.09 (Fig. 1B). When these same animals are subjected to hypoxic conditions for 2 min, animals show the typical respiratory response to hypoxia which consist of an initial acceleration of breathing frequency followed by a depression during which gasping activity is observed (Fig. 2A upper trace). Gasping can be easily distinguished from eupnea due to a clear pattern change that includes a shortening of inspiratory duration (from 0.13 ± 0.01 s to 0.07 ± 0.01 s) and rise time (measured as the time from the 20% to the 80% of maximal amplitude, from 0.08 ± 0.02 s to 0.03 ± 0.01 s) [7,13,27]. Gasping frequency in these animals was 0.25 ± 0.09 Hz (Fig. 2B left graph). As previously reported [4,5,7,24], gasping eventually disappears and hypoxic apnea is observed in all animals tested. Breathing is reassumed after 3.05 ± 0.37 min of switching to normal room air breathing in all tested animals (100% of autoresuscitation, Fig. 2B right graph).

When riluzole is administered i.c. at several doses (0.4, 2.0, 4.0 and 6.0 nmol), breathing is slightly affected in control conditions (breathing room air, Fig. 1A). In general, an increase in breathing frequency is observed, but this is not dose-dependent. There was a 46% increase in respiratory frequency with riluzole following application of 0.4 nmol ($n = 11$); 37% increase with 2.0 nmol ($n = 8$); 32% increase with 4.0 nmol ($n = 8$) and 53% increase with 6.0 nmol ($n = 11$, Fig. 1B right graph). Inspiratory half-duration was reduced only at the maximal riluzole dose of 6.0 nmol (to 62% of control, Fig. 1B left graph). Irregularity of the rhythm was not significantly altered at any of the tested doses (irregularity score changed to 97%, 89%, 74% and 100% of control, with the doses of 0.4, 2.0, 4.0 and 6.0 nmol, respectively, $P > 0.05$). In contrast, the effect of riluzole is more dramatic in hypoxic conditions. With the exception of the smallest dose of 0.4 nmol riluzole, applied in all cases 5 min before hypoxia, dramatically reduces gasping frequency to 12% of control at the dose of 2.0 nmol; to 28% of control at the dose of 4.0 nmol; and to 12% of control at the dose of 6.0 nmol (Fig. 2A