Stochastic activation among inspiratory cells in the pre-Bötzinger complex of the rat medulla revealed by Ca^{2+} imaging

Yoshihiko Oke^{a*}, Dimitri Boiroux^a, Fumikazu Miwakeichi^{b, c}, and Yoshitaka Oku^a

^aDivision of Physiome, Department of Physiology, Hyogo College of Medicine, Mukogawacho 1-1, Nishinomiya, Hyogo, 663-8501, Japan;

^bDepartment of Statistical Modeling, The Institute of Statistical Mathematics, Midoricho 10-3, Tachikawa, Tokyo, 190-8562, Japan

^cDepartment of Statistical Science, School of Multidisciplinary Sciences, The Graduate University for Advanced Studies, Tachikawa, Midoricho 10-3, Tachikawa, Tokyo, 190-8562, Japan

*Corresponding author:

Yoshihiko Oke, Ph.D.

Division of Physiome, Department of Physiology, Hyogo College of Medicine,
Mukogawa-cho 1-1, Nishinomiya, Hyogo, 663-8501, Japan

Phone: +81-798-45-6387

Fax: +81-798-48-9643

E-mail: okey1@hyo-med.ac.jp

Abbreviations: preBötC, pre-Bötzinger complex; aCSF, artificial cerebrospinal fluid;

OGB-1, Oregon Green 488 BAPTA-1 AM

Abstract

In the pre-Bötzinger complex of the ventral medulla (preBötC), a variable pattern of inspiratory neuronal output and synchronous activation of inspiratory cells can be observed. However, it is not well known whether cellular activation patterns among inspiratory cells are variable or fixed. Here, we evaluated the activation sequence of inspiratory cells during individual rhythmic bursts using calcium imaging. Onset timing and peak timing of calcium fluctuations during rhythmic bursts in individual inspiratory cells were used to evaluate the activation sequence. The sequences of both timings changed stochastically in individual rhythmic bursts, although the sequences differed between the two timings even within the same rhythmic burst. The weak correlation between these two timings might indicate that the two parameters reflect different physiological events. Furthermore, a subset of inspiratory cells was found to initially activate in the sequences of successive rhythmic bursts. These results suggest that rhythmic activation of inspiratory cells occurs with a degree of loose regularity but is not invariable with respect to the sequence of either onset or peak timing.

Keywords: Pre-Bötzinger complex, Calcium imaging, Activation sequence, Rhythmic neuronal activity.

Introduction

Medullary slices containing the pre-Bötzinger complex (preBötC) from neonatal rodents exhibit spontaneous rhythmic activity, however the rhythmic bursting pattern of the inspiratory neuronal output from hypoglossal rootlets is not unique in each rhythmic

burst [1]. There are many studies concerning generation mechanisms of rhythmic activity in the preBötC, including studies of cell types, neuronal populations and single neuron kinetics [2, 3, 4, 5, 6]. However, these studies do not explain the observed variability in rhythmic activity. Recently, a multichannel extracellular recording technique was used to determine that respiratory neurons in the preBötC are stochastically activated in each rhythmic burst [7]. Taking this into account with the variability of rhythmic activity, we hypothesized that activation sequences of inspiratory cells, which are closely related to neurotransmission sequences or pathways, change with some regularity in each rhythmic burst. Calcium imaging is known as a useful tool for the simultaneous recording of the activity of multiple cells, thereby enabling the analysis of multi-cell dynamics [8, 9, 10]. In this study, we evaluated the sequence of rhythmic activation among inspiratory cells during rhythmic bursts by imaging intracellular calcium levels with a multi-photon laser scanning microscopy system. We applied two parameters to determine the activation sequence, and showed that the inspiratory cells were activated in a stochastic manner with loose regularity, and that the occurrence of the initial activation was restricted to a certain subset of cells.

Materials and Methods

Animal care and experimental procedures

All experimental procedures were conducted in accordance with the Japanese Physiological Society *Guide for the Care and Use of Laboratory Animals* and followed the guiding principles of the American Physiological Society. This study was also approved by the Committee on Animal Care and Use at Hyogo College of Medicine.

Rhythmic slices were prepared from postnatal day 2-7 Wistar rats (SLC, Shizuoka,

Japan) as described previously [11]. Briefly, rats were decapitated under isoflurane anesthesia and the brainstem was isolated in ice-cold, oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) composed of (mM): 118 NaCl, 3 KCl, 1.2 CaCl₂, 1 MgCl₂, 1 NaH₂PO₄, 25 NaHCO₃, 30 D-glucose (pH 7.4). Transverse slices (550-600 μ m) including the preBötC, the nucleus of hypoglossus and the hypoglossal (XII) rootlets were then cut from the brainstem in ice-cold, oxygenated high osmolarity Ringer solution without calcium (composed of [mM] 124 NaCl, 3 KCl, 2 MgCl₂, 1.3 NaH₂PO₄, 26 NaHCO₃, 10 D-glucose, 200 sucrose, 1 kynurenic acid) [12]. The cutting plane of the slice was positioned so that the preBötC was exposed on the rostral side, according to a previous report [13]. Rhythmic activity in the slice was initiated by incubation (> 20 min at 27 °C) in aCSF, followed by an increase in potassium concentration from 3 to 8 mM in the recording chamber [1].

Calcium imaging and electrophysiological recording

Staining, calcium imaging and electrophysiological imaging were conducted using a previously described method [11]. Briefly, cells at the preBötC in the slice were stained by glass-pipette-loading of 100 μ M Oregon Green 488 BAPTA-1 AM (OGB-1, Thermo Fisher Scientific Inc., MA, USA) for 10 min and subsequently washed with oxygenated aCSF for > 40 min at 27 °C. Calcium imaging was performed using a two-photon laser-scanning microscope based on a mode-locked laser system operating at 800 nm, 80 MHz pulse repeat, 140 fs pulse width (Chameleon Vision II, Coherent, CA, USA) and a laser-scanning system (Leica TCS SP5 MP, Leica Microsystems, Germany) through a 500-550 nm band-pass emission filter. Each frame was captured at a size of 512 $\times$ 200 pixels (= 434.17 $\times$ 169.08 μ m) at depths of up to 80 μ m. Each frame was

imaged by single line scanning at 8000 Hz per line, and images were acquired at 34.5 Hz (29 msec/frame). A clear still image was captured by averaging $48 \times$ scanning of each line before acquiring a movie of calcium imaging.

Raw nerve signals from the hypoglossal rootlets were amplified $100,000 \times$, band-pass filtered (cutoff frequencies: high-pass 50 Hz, low-pass 1 kHz), and digitized at 1 kHz on the PowerLab 4/25T and Chart 5 software (ADInstruments, New South Wales, Australia). The signals were also full-wave rectified and integrated online [time constant (τ) = 100 ms].

Processing of data and detection of onset time and peak time of OGB-1 fluorescence

All signal processing and timing detection were analyzed using MATLAB (the Math Works, MA, USA). Moving time averaging and spatial filtering were applied to calcium imaging data. For moving time averaging of each frame, we determined the unweighted average of the previous and following three frames. The data were spatially filtered using the unweighted-averaging of a 5×5 region around each pixel. The spatial filtering was then conducted again using a 7×7 pixels grid for each pixel. A cross-correlation map was constructed by taking the maximum normalized cross-correlation coefficient between each pixel and the integrated hypoglossal nerve signal. Maxima were identified for time lags between 0 and 50 frames (1.45 s). A region of interest (ROI) of 11×11 pixels was placed on cells that had high cross-correlation values. We double-checked the identity of inspiratory cells by visually confirming synchronization between fluorescence fluctuations and rhythmic burst signals from the hypoglossal nerve rootlet. The data in the ROI were band-pass filtered (cutoff frequencies: high-pass 0.025 Hz, low-pass 1.5 Hz). To determine peak times of OGB-1

fluorescence, the timing of the maximum fluorescence intensity (maximum F) was searched over the 70 frames (2.03 sec) before and after the peak of the integrated hypoglossal nerve signal in a rhythmic burst (see Fig. 1C). We then determined when the maximum slope in OGB-1 fluorescence fluctuation was observed within the 53 frames (1.54 sec) preceding the peak time. As a candidate for onset timing, the time at $dF/dt = 0$ was detected within the 53 frames (1.54 sec) preceding the peak of the maximum F and the time of the maximum slope (see Fig. 1C). As another candidate for onset timing, the time at $\max d^2F/dt^2$ was detected within the same range used to search $dF/dt = 0$. Finally, the onset time and the peak time were inspected manually to remove apparent errors and artifacts.

Results

Activation sequence analysis using calcium imaging

The preBötC was loaded with OGB-1 (Fig. 1A, i). We calculated cross-correlation values between fluctuations in fluorescent signals for each pixel and hypoglossal nerve bursting to identify candidate inspiratory cells (Fig. 1A, ii). In the area of inspiratory cells, fluorescence fluctuations coincided with rhythmic bursts (Fig. 1B). In the three slices analyzed in this paper, 6 - 14 inspiratory cells were detected on each focus plane. The numbers of rhythmic cells are consistent with a prior report [13].

To determine the activation sequence among the inspiratory cells, various parameters were evaluated by visual comparison with calcium imaging movies. The peak timing of fluorescence in inspiratory cells during rhythmic bursts were detected as the time at which the maximum fluorescence was observed within the range of ± 2.03 sec from the peak hypoglossal nerve signal (Fig. 1C). As candidates for the onset

parameter, we examined the minimum fluorescence, the onset of increased fluorescence ($dF/dt = 0$), the maximum slope ($\max dF/dt$), and the maximum acceleration of fluorescence ($\max d^2F/dt^2$). The onset of increased fluorescence and the maximum acceleration of fluorescence were validated by visual inspection of the imaging movies. OGB-1 AM stained both neurons and astrocytes and it is not clear whether the maximal acceleration of calcium dynamics reflects the same intracellular reaction between neurons and astrocytes. In order to avoid the possibility of detecting different intracellular events related to cell-type specific calcium dynamics, we used $dF/dt = 0$ as a parameter to measure the onset time in this paper (Fig. 1C).

General properties of onset and peak times in individual rhythmic bursts

To determine whether onset time or peak time would be sufficient to identify rhythmic activation timing in inspiratory cells, the general properties of both onset and peak times were investigated. The distribution of onset and peak times in all inspiratory cells showed an approximately normal distribution for all three slices (Fig. 2AB; Slice 1; $n = 148$ activations from 10 cells, Slice 2; $n = 52$ activations from 6 cells, Slice 3; $n = 250$ activations from 14 cells). The average onset or peak times seemed to vary throughout 18 rhythmic bursts (Fig. 2C). These results indicate that both onset and peak time of inspiratory cells, relative to the peak of the hypoglossal nerve rootlet signal, might shift with respect to individual bursts. Furthermore, the fluctuation patterns of the averaged onset and peak times did not completely correspond with each other. Therefore, we concluded that both parameters might not be clearly correlated and thus activation sequences of inspiratory cells should be analyzed using two parameters. Correlations were then estimated between onset and peak times for all inspiratory cell activations

(Fig. 2D). The coefficients of determination were 0.1833, 0.0761 and 0.0723 on slices 1 - 3, respectively, which suggests only a weak relationship between onset and peak times. These results suggest that these two parameters might represent different activation sequences, even within the same rhythmic burst. Therefore, we evaluated activation sequences by using both parameters.

Activation sequences vary among individual rhythmic bursts

To examine the possibility that both onset time and peak time might vary over successive rhythmic bursts, activation sequences of inspiratory cells were evaluated in individual rhythmic bursts. Sequence charts of onset timing and peak timing demonstrated the sequence transitions of both timings on the slice shown in Fig. 1A (Fig. 3). The sequences of both timings showed various patterns during individual rhythmic bursts. Additionally, some inspiratory cells occasionally skipped their rhythmic activation. Notably, a sequence of onset timings was also different from that of peak timings even during the same rhythmic burst. This result is consistent with the result in Fig. 2D. Thus, we concluded that the sequence of either onset or peak timing changed stochastically for individual rhythmic bursts.

Characteristics of onset time and peak time in individual inspiratory cells

The sequences of onset timing or peak timing among respective rhythmic bursts seem to exhibit loose regularity, though the order appears to vary from cycle to cycle (Fig. 3). Therefore, we investigated the characteristics of both onset and peak times in individual inspiratory cells. The average onset and peak times relative to the peak of the hypoglossal nerve rootlet signal appeared to vary among individual inspiratory cells

(Fig. 4A). The p -values for the time differences among inspiratory cells were computed from the three slices using one-way analysis of variance. The p -values for onset time differences were 0.002, 0.657 and 0.347 and those for peak time differences were 8.209×10^{-7} , 0.607 and 0.005, respectively (Supplementary data; Table 1). The significant differences in average time among inspiratory cells indicate that the timings of individual inspiratory cells were distinct from those of other inspiratory cells. Therefore, there might be a degree of ordered regularity in the sequences of these timings during a rhythmic burst, which were investigated next. Regarding differences in p -values across different slices, we assumed that more pacemaker cells were present in the focus area of slice 1, compared to the other slices. We found that inspiratory cells appeared to be separated into an early-activation group and a late-activation group (Fig. 4A), and that the average activation timing was earlier in slice 1 compared to other slices (Table. 1).

Next, in order to understand the putative ordered regularities for the activation sequence, we calculated the ratios of the number of occurrences of individual cells initially activated in each activation sequence against the total number of rhythmic bursts during recording. Some inspiratory cells were not initially activated in either onset or peak timing during each rhythmic burst (Fig. 4C). Similar types of inspiratory cells were detected on the other two slices (Data not shown; Peak: 1/6 cells and 2/14 cells, Onset: 1/6 cells and 3/14 cells). Conversely, a subset of inspiratory cells covered a significant amount of the initial activation, for both onset and peak, in the activation sequences (Fig. 4C; Peak: 78% in the areas of F & G and I and J, Onset: 72% in cell B and the area of F & G). On the two other slices, the ratios of the number of initial activations against all rhythmic bursts indicated the same tendency (Data not shown; Peak: 70% and 50 % in 1/6 cells and 4/14 cells respectively, Onset: 50% and 23% in 1/6

cells and 1/14 cells). Thus, rhythmic activation sequences are thought to occur with a degree of ordered regularity during rhythmic bursts, at least regarding the sequence of either onset or peak timing, despite the lack of correlation between onset and peak timing.

Discussion

In this study, we proposed that the activation sequence among inspiratory cells in the preBötC varies stochastically in individual rhythmic bursts; however, a degree of loose regularity was observed. Recently, analysis of cellular firing patterns revealed that intrinsic pacemaker cells are activated stochastically during individual rhythmic bursts [7]. To our knowledge, this is the first report to examine activation sequences among multiple inspiratory cells during rhythmic bursts using simultaneous recordings. Furthermore, we showed that onset timing and peak timing of rhythmic activation in inspiratory cells were only weakly correlated.

To examine the activation sequence among inspiratory cells in the preBötC, we performed calcium imaging using a high-speed multi-photon laser scanning microscopy system. This method has the following advantages: (1) simultaneous recording of multiple cells in a wide area ($> 0.07 \text{ mm}^2$), (2) single-cell detection resolution (in reality, extracellular recording data represents field potentials that include spikes from multiple cells), (3) less mechanical damage than with an electrode-based technique, (4) capability of considering the relative positions among cells observed and (5) validity for statistical processing due to the use of two different kinds of recording, electric signal from the hypoglossal nerve rootlet and calcium imaging. Previously, calcium imaging techniques had the disadvantage of poor time resolution, however the present technology enabled the analysis of dynamics in active cells because the sampling rate has been increased to $> 30 \text{ frames/s}$ in the observation area. Moreover, the multi-photon laser scanning technique dramatically reduces background noise; therefore the reliability of single cell discrimination is higher than with other imaging methods.

We chose two parameters to characterize the activation sequence, onset timing and peak timing of rhythmic activation in individual cells. Only a weak correlation between the two parameters was found. In fact, the sequence of onset timing was different from that of peak timing during the same rhythmic burst. We do not know the mechanism underlying the low correlation between these two parameters; however, it was assumed that this result likely reflects different physiological events rather than artifacts in the data. For instance, the differences in activation sequences between two parameters might be attributed to difference in cell type. Indeed, our method results in the staining of both glia cells and neurons. In the three slices analyzed, almost all inspiratory cells (except cell B in slice 1) had a 600-800 ms time lag between onset timing and peak timing (Fig. 4B and data not shown) and there was no significant difference among the time lags in inspiratory cells except in cell B on slice 1. If we detected onset timing incorrectly, such as detection of calcium fluctuations in non-rhythmic activities immediately before rhythmic activation, the time lags among inspiratory cells would be longer and show more significant differences.

Our observation revealed that the activation sequence of both timings varied stochastically in individual rhythmic bursts. However, the probability of cellular activation occurring at the initial part of a rhythmic burst was not homogeneous among individual inspiratory cells. From a physiological point of view, the respiratory neuronal network in the preBötC might have several signal transmission pathways, because breathing rhythms are easily changed by modulation of external neuronal inputs. Therefore, our results indicate the presence of stochastic alterations of signal transmission pathways in the preBötC. Our results are consistent with a recent report of stochastic activation of respiratory cells [7]. Moreover, this observation does not

exclude the importance of intrinsic pacemaker cells. For instance, the respiratory network might be composed of clusters that include intrinsic pacemaker cells that act as hub cells [14]. Another possibility would be that quasi-periodic activation sequences might exist in the neuronal network of the preBötC, similar to the metastable network in the hippocampal CA3 region [8]. Further mathematical analysis and investigation, including the targeted inhibition of intrinsic pacemaker cells, will be required to explain the relationship between the stochastic activation sequence of inspiratory cells and the spontaneous rhythmic activity in the preBötC.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research B (26280109) from the Japan Society for Promotion of Science and the JST strategic Japanese-German cooperative program in computational neuroscience.

References

- [1] J.C. Smith, H.H. Ellenberger, K. Ballanyi, D.W. Richter, J.L. Feldman, Pre-Bötzinger complex: a brainstem region that may generate respiratory rhythm in mammals, *Science* 254 (1991) 726–729.
- [2] N. Koshiya, J.C. Smith, Neuronal pacemaker for breathing visualized in vitro, *Nature* 400 (1999) 360–363.
- [3] J.L. Feldman, C.A.D. Negro, Looking for inspiration: new perspectives on respiratory rhythm, *Nat. Rev. Neurosci.* 7 (2006) 232–241.
- [4] C. Morgado-Valle, S.M. Baca, J.L. Feldman, Glycinergic pacemaker neurons in preBötzinger complex of neonatal mouse, *J. Neurosci.* 30 (2010) 3634–3639.
- [5] J.A. Hayes, X. Wang, C.A.D. Negro, Cumulative lesioning of respiratory interneurons disrupts and precludes motor rhythms in vitro, *Proc. Natl. Acad. Sci. U.S.A.* 109 (2012) 8286–8291.
- [6] H. Koizumi, N. Koshiya, J.X. Chia, F. Cao, J. Nugent, R. Zhang, J.C. Smith, Structural-functional properties of identified excitatory and inhibitory interneurons within pre-Bötzinger complex respiratory microcircuits, *J. Neurosci.* 33 (2013) 2994–3009.
- [7] M.S. Carroll, J.-M. Ramirez, Cycle-by-cycle assembly of respiratory network activity is dynamic and stochastic, *J. Neurophysiol.* 109 (2013) 296–305.
- [8] T. Sasaki, N. Matsuki, Y. Ikegaya, Metastability of active CA3 networks, *J. Neurosci.* 27 (2007) 517–528.
- [9] C. Schnell, J. Freseemann, S. Hülsmann, Determinants of functional coupling between astrocytes and respiratory neurons in the pre-Bötzinger complex, *PLoS ONE* 6 (2011) e26309.
- [10] Y. Okada, T. Sasaki, Y. Oku, N. Takahashi, M. Seki, S. Ujita, K.F. Tanaka, N. Matsuki, Y. Ikegaya, Preinspiratory calcium rise in putative pre-Bötzinger complex astrocytes, *J. Physiol.* 590 (2012) 4933–4944.
- [11] D. Boiroux, Y. Oke, F. Miwakeichi, Y. Oku, Pixel timing correction in time-lapsed

calcium imaging using point scanning microscopy, *J. Neurosci. Methods* 237C (2014) 60–68.

[12] G.B. Richerson, C. Messer, Effect of composition of experimental solutions on neuronal survival during rat brain slicing, *Exp. Neurol.* 131 (1995) 133–143.

[13] A. Ruangkittisakul, S. W. Schwarzacher, L. Secchia, Y. Ma, N. Boboccea, B. Y. Poon, G. D. Funk, K. Ballanyi, Generation of Eupnea and Sighs by a Spatiochemically Organized Inspiratory Network, *J. Neurosci.* 28(10) (2008) 2447–2458.

[14] N. Hartelt, E. Skorova, T. Manzke, M. Suhr, L. Mironova, S. Kügler, S.L. Mironov, Imaging of respiratory network topology in living brainstem slices, *Mol. Cell. Neurosci.* 37 (2008) 425–431.

Figure legends

Fig. 1. Calcium imaging and analysis of inspiratory cells in the pre-Bötzinger complex (preBötC). (A) Inspiratory cells in the preBötC were visualized by the bolus loading of OGB-1 (green). (i) Cross-correlation map of OGB-1 fluorescence was calculated between individual pixels and the hypoglossal nerve data. Inspiratory cells were defined as OGB-1-stained areas that exhibited high cross-correlation values. The inspiratory cells are labeled alphabetically. White squares on the image (i) indicate the center positions of the regions of interest (ROI) used for subsequent analysis. (B) Spontaneous fluctuation in OGB-1 fluorescence (lower trace) was measured in a ROI of 11×11 pixels ($9.3 \times 9.3 \mu\text{m}$) on a typical inspiratory cell. The integrated signal from the hypoglossal nerve rootlet (XII) was recorded simultaneously (upper trace). (C) Schematic representing the identification of onset and peak times, specifically the onset of increasing fluorescent intensity and the peak fluorescent intensity, during a rhythmic activation of an inspiratory cell. Both intervals of onset time and peak time were measured as differences from the peak timing of the hypoglossal nerve rootlet signal.

Fig. 2. General properties of onset time and peak time of OGB-1 fluorescence fluctuation in inspiratory cells at the preBötC during rhythmic bursts. (A) Distribution of observed onset times. The proportion of rhythmic activation with onset times is calculated for each slice (Slice 1; $n = 148$, Slice 2; $n = 52$, Slice 3; $n = 250$). Slice 1 is the same slice used in Figure 1. The time interval is the same as the sampling interval for calcium imaging (29 msec). (B) The distribution of observed peak times is shown as with onset time. (C) Averages of onset and peak times are indicated for each rhythmic

burst of the hypoglossal nerve rootlet signal. The average $\pm$ SE are calculated from the dataset of inspiratory cells shown in Figure 1A. (D) Correlation between peak time and onset time.

Fig. 3. Activation sequence charts of inspiratory cells in the preBötC during individual rhythmic bursts. The inspiratory cells shown in Figure 1A were analyzed. Inspiratory cells are color-coded as shown at the upper left. Adjacent cells are indicated by the rectangular outlines (C&D, F&G, and I&J). In both charts, the chronological order of cells with rhythmic activity is displayed from the top to bottom for both onset and peak timing.

Fig. 4. Characterization of onset time and peak time in individual inspiratory cells. Inspiratory cells shown in Figure 1A were analyzed. Adjacent cells are indicated by the rectangular outlines. (A) Averages of both onset time and peak time in individual inspiratory cells are shown. (B) Raw data of onset time and peak time for each inspiratory cell are plotted. The symbols shown on the right indicate the rhythmic burst number in which the raw data were obtained. (C) The histogram shows the proportion of initial occurrences where onset timing or peak timing of individual cells was detected in individual activation sequences versus the total number of rhythmic bursts (18 times). Black and gray bars show the ratios of the earliest onset time and the first peak time, respectively.

Fig. 1

Fig. 2

Fig. 3

Fig. 4