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ABSTRACT

THE FUNCTIONAL EFFECTS OF BARIUM AND HYPOXIA ON THE IN VITRO RESPIRATORY ACTIVITY

by
Gaofeng Xu

The hypoxic respiratory response in mammals consists of a transit increase in the respiratory frequency (augmentation phase) followed by a decrease in frequency (depression phase). To understand how the central respiratory system contributes to this response, the in vitro transverse brainstem slice model is used, which contains the pre-Bötzinger Complex, which is responsible for respiratory rhythm generation. The in vitro experiments performed for this thesis provide evidence that external barium exposure alters respiratory activity and significantly increases(P<0.001) the voltage of tonic activity under control oxygen conditions (95% FO_2). During severe hypoxia (0% FO_2), respiratory tonic activity is significantly elevatedduring the depression phase (from 0.55 to 0.95, n=6, P<0.001) by external barium, presumablydue to the closing of K^+ channels and a reduction in K^+ conductance.

THE FUNCTIONAL EFFECTS OF BARIUM AND HYPOXIA ON THE IN VITRO RESPIRATORY ACTIVITY

by
Gaofeng Xu

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biology

Federated Biological Sciences Department

May 2012

APPROVAL PAGE

THE FUNCTIONAL EFFECTS OF BARIUM AND HYPOXIA ON THE IN VITRO RESPIRATORY ACTIVITY

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ACKNOWLEDGMENT

Thanks to Thesis Advisor: Dr. Andrew Hill and Committee members: Dr. Jorge Golowasch; Dr. Robert A O’Brien.

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LIST OF SYMBOLS

VRG	Ventral Respiratory Group
preBötC	pre-Bötzinger complex

CHAPTER 1

INTRODUCTION

1.1 Objectives

The objective of this thesis is to study the effect of barium, a non-specific K^+ channel antagonist on respiratory activity generated by the brainstem. K^+ channels are important for setting the resting membrane potential of neurons under normal conditions and are activated to an even larger degree under conditions of stress such as hypoxia. The goal of this thesis is to determine the degree to which barium (1mM) exposure affects baseline of respiratory rhythmic activity during normal conditions (95% FO_2) and during severe anoxia (0% FO_2).

1.2 Background Information

The mammalian respiratory system responds to severe hypoxia in a biphasic manner (Bureau et al., 1984, Haddad and Mellins, 1984, St John and Bianchi, 1985). The hypoxic respiratory response comprises an initial increase in respiratory frequency, called the augmentation phase, followed by a decrease in respiratory frequency, known as the depression phase. Eventually prolonged severe hypoxia may cause respiratory activity to terminate (Richter et al., 1991, Bissonnette, 2000). The mechanisms of hypoxia-mediated depression of the respiratory activity are not completely known (Bissonnette, 2000). K^+ conductance is important for the resting membrane potential of neurons and for rhythmic respiratory activity (Ballanyi, 2004). According to studies of ventral respiratory group (VRG) neurons within the transverse brainstem slices of neonatal brainstem-spinal cord preparation, the range of resting membrane potential values is from -60 to -40 mV (Arata et al., 1993, Onimaru et al.,

1995, Brockhaus and Ballanyi, 1998, 2000). A major hyperpolarization is rapidly caused by anoxia-induced opening of K^+ channels within respiratory neurons of neonatal mice (Ballanyi et al., 1994, Ballanyi et al., 1999). Thus, an increase in K^+ conductance leads to a hyperpolarization of the membrane potential, which, in turn, may lead to a slowing of the respiratory rhythm (Bureau et al., 1984).

Barium, a blocker of K^+ channels, inhibits the delayed rectifier K^+ conductance as well as the inward rectifier K^+ conductance in muscle fibers(Hagiwara and Kidokoro, 1971, Standen and Stanfield, 1978). Ballanyi 2004 demonstrates that external barium exposure inhibits anoxia-induced depression of respiratory rhythm presumably by reducing K^+ conductance within neurons of the in vitro transverse brainstem slices. The degree to which barium affects the in vitro frequency under different oxygen tensions, however, has not been quantified. The goal of this thesis is to determine the degree to which barium (1mM) exposure affects the in vitro respiratory rhythm under conditions of high and low oxygen (95% vs. 0% FO_2).

Transverse brainstem slices are used to quantify the effects of barium on the in vitro respiratory rhythm. These slices contain the pre-Bötzinger complex (preBötC), a region that is essential for generating respiratory rhythmic activity (Ramirez et al., 1996). In addition to rhythmogenic preBötC neurons, a transverse medullary slice also contains premotoneurons, respiratory hypoglossal motoneurons (Rekling et al., 1996, Rekling and Feldman, 1998, Gray et al., 1999), and neuromodulatory neurons that are presynaptic to the neurons of the preBötC (Ramirez et al., 1998). Furthermore, transverse medullary slices provide a convenient way for application of drugs which are added via superfusion to an anaesthetic-free preparation (Ramirez et al., 1998).

CHAPTER 2

METHODS and MATERIALS

2.1 Transverse Brainstem Preparation

Transverse brainstem slice preparations of neonatal mice (P0-P11) are used for the experiments (Smith et al., 1992, Ramirez et al., 1996). Male and female CD-1 neonatal mice bred from founders from Charles River Laboratories (Wilmington, MA) are anaesthetized deeply with isoflurane until there is no withdrawal reflex from toe pinch, then rapidly decapitated at the C3—C4 spinal level. The brainstem and a small part of spinal cord are dissected with ice-cold artificial cerebral spinal fluid (aCSF) including composition (mM): 118 NaCl, 25 $NaHCO_3$, 1 NaH_2PO_4, 1 $MgCl_2 \times 6H_2O$, 3 KCl, 30 D-glucose, 1.5 $CaCl_2$ equilibrated with gas (95% O_2 and 5% CO_2) (Hill et al., 2011). The pH of the aCSF is 7.2 to 7.4 at room temperature (21 ℃). The brainstem and a small part of spinal cord are glued on the agar block with spinal cord above the brainstem and dorsal side of brainstem attached to agar block. Thin slices are cut in serial sections (100 m) from caudal face to rostral face by a vibrating blade microtome (Leica VT 1200) until the caudal border of the preBötC is identified by the presence of the principle loop of the inferior olive. At this rostral-to-caudal level a final 500μm rhythmic slice is cut (Ramirez et al. 1996). The final slice is submerged in oxygenated aCSF for 30 min to recover from the slicing process before the commencement of recordings, then transferred to superfusion chamber (RC-29 Warner Instruments) and also submerged with fluid flow of aCSF solution (temperature, 32 ℃; flow rate,10 mL min^{-1}). The extracellular K^+ concentration is increased from 3mM to 8 mM to augment rhythmic activity of the preBötC (Tryba et al., 2003). Barium chloride is directly added to reservoirsfilled with 100mL aCSF to

achieve a final concentration of 1mM. A solution reservoir heater and an in-line heater system (TC-344B; Warner Instruments) are used to consistently control the temperature of system.

2.2 Electrophysiological Recording

aCSF-filled electrodes are placed on the caudal face of a 500μm thick slice in the area of pre-Bötzinger complex, which is a region of a column of neurons called the ventral respiratory group (VRG), to record the waveforms of respiratory rhythmic activity. These glass electrodes are composed of filamented borosilicate glass (Clarke GC150F), which are etched and broken to an outer tip diameter of about 50-100 μm. An extracellular recording technique is used to observe population activity of preBötC neurons using a 100-fold gain extracellular preamplifier (JFIE 1626) purchased from the James Franck Institute Electronics Laboratory, University of Chicago, in concert with a variable gain amplifier (Model 410; Brownlee Precision).

2.3 Control of O_2 tension

Alteration of the fractional value of oxygen (FO_2) is used to control the partial pressure of oxygen (PO_2) within the superfusion chamber. Using an aeration stone, O_2 is bubbled with a rate of 50 ml/min into a reservoir filled with 100mL aCSF. Under normal conditions the aCSF contains 95% of O_2 and 5% of CO_2. Under hypoxic conditions the aCSF contains 0% O_2 (O_2 replaced by N_2) and 5% of CO_2. One reservoir is filled up with a control gas (95% FO_2); the other reservoir is filled up with a test gas (0% FO_2). Thus the PO_2 can be immediately altered in superfusion bath by changing the stream of aCSF from one reservoir to another reservoir (Hill et al., 2011).

2.4 Normalize voltage of tonic activity and Data Analysis

Bursts frequency data was not collected from enough experiments for statistical analysis (less than 6 groups for each experiment), thus the voltage of tonic activity is used to replace burst frequency data to analyze the respiratory activity in response to hypoxia and barium exposure. Tonic activity is due to neural spiking activity. Application of tetrodotoxin, which blocks of voltage-gated sodium channels, results in a reduction in tonic activity (Ballanyi et al., 1999).This finding demonstrates that the tonic activity is correlated positively to spiking activity. Thus, variation in tonic activity may be used as a general indicator of the degree of spontaneous activity in the slice. Although tonic activity doesn't directly reflect respiratory activity, tonic activity and respiratory frequency often have a positive correlation. During the initial augmentation phase of the response to hypoxia (when the respiratory bursts increase in frequency) tonic activity also increases. During the depression phase (when the respiratory bursts decrease in frequency) tonic activity also decreases (Hill et al., 2011). Accordingly, due to the positive correlation between changes in tonic activity and burst frequency, we use tonic activity as a measure of in vitro respiratory activity.

Tonic activity is a voltage signal that corresponds to a rectified-and-integrated version of the original raw extracellular activity measured from a population of neurons immediately below the tip of an aCSF filled glass electrode. An increase in tonic activity results in a greater deflection of this signal from zero. By lifting the electrodes at the end of an experiment, we are able to distinguish between biological tonic activity due to action potentials of neurons and background electronic noise. Tonic activity (voltage) is measured at various times during whole experiment such as: just before the application of hypoxia (95% FO_2); during the augmentation phase of

hypoxia (0% FO_2); during the depression phase of hypoxia (0% FO_2); and during the recovery phase (95% FO_2). For each preparation, we calculated a "normalized" voltage for tonic activity during each phase. This is done in the following way: let *a* be the mean voltage before exposure to hypoxia and let *b* be the mean voltage after lifting the electrodes to measure the voltage deflection from zero due to background electronic noise, thus the normalized voltage at any time point *c* is then given by the following formula: $c' = (c-b) / (a-b)$. These normalized tonic activity values then can be used to average data across preparations. Note that the normalized values can be higher than 1 if tonic activity increases relative to the baseline level. The reason for doing this normalization is to be able to compare values from different preparations in which the actual voltages may be quite different due to differences in the tip size and degree of sealing between the glass and the slice surface as well as differences in the electronic gain that may occur due to differences in battery strength. Furthermore, all statistical data are given as means $\pm$ S.E.M. Significance ($P < 0.05$) is evaluated with paired and unpaired *t*-tests, as appropriate.

CHAPTER 3

RESULTS

3.1 Barium exposure changes the baseline fictive respiratory activity

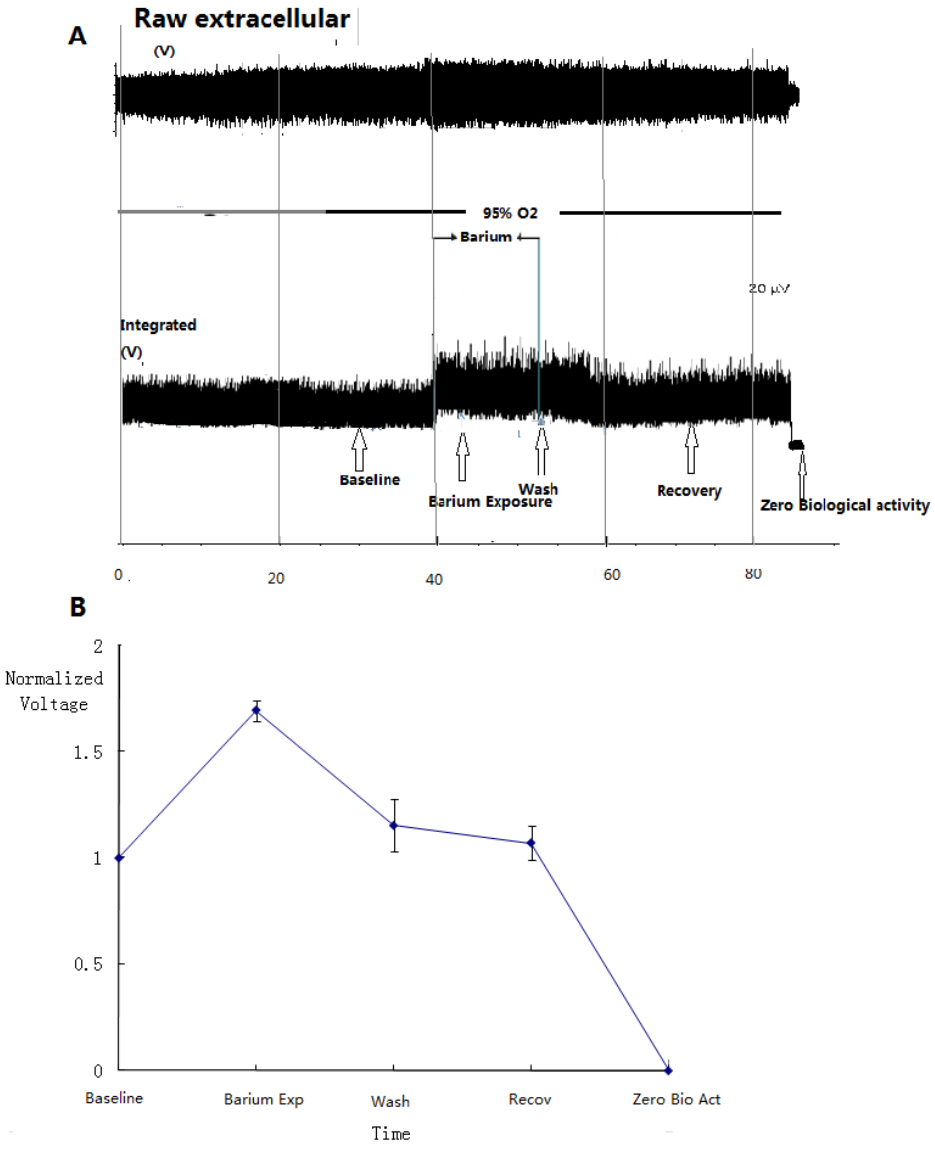

Figure 3.1 **(A)** Integrated extracellular activity from representative slice of P5 mice show changes in voltage of respiratory tonic activity in response to Barium exposure followed by wash with aCSF. **(B)** Changes in normalized voltage of tonic activity during different phases of the response to barium exposure followed by wash with aCSF. Different phases of respiratory activity show respectively: Baseline; After Barium Exposure; Wash; Recovery; Zero Biological Activity. All values are mean values ± S.E.M.

Control O_2 tension (FO_2=95%) is maintained constant throughout the experiment. Voltage of tonic activity after barium bath is significantly increased (P<0.001) in contrast to that of baseline (Figure, 3.1). After 10 min barium exposure, barium is washed out and the normalized voltage of tonic activity during recovery phase returns to the baseline level.

3.2 Respiratory activity responds to hypoxia condition (0% FO_2)

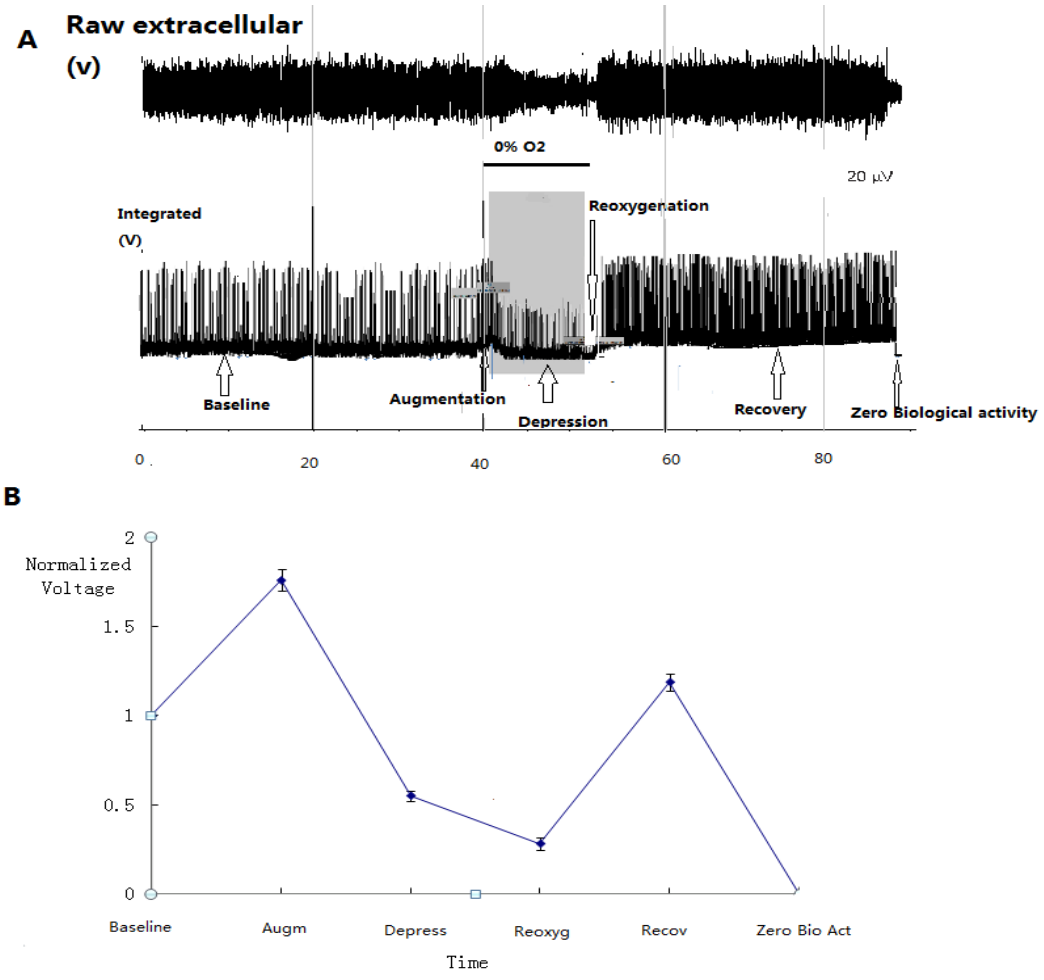

Figure 3.2 (A) Integrated extracellular activity from a representative slice from a P5 mouse show changes in voltage of respiratory tonic activity in response to hypoxia (0% FO_2) followed by reoxygenation (95% FO_2). **(B)** Changes in normalized voltage of tonic activity during different phases in respond to hypoxia (0% FO_2) followed by reoxygenation (95% FO_2). Different phases of respiratory activity show respectively: Baseline; Augmentation; Depression; Reoxygenation; Recovery; Zero Biological Activity. All values are mean values ± S.E.M.

aCSF-filled micro-pipette electrodes are placed on the caudal surface of a 500 μm thick slice in the area of pre-Bötzinger Complex, which is a region of VRG, to record the respiratory rhythmic activity. Anoxia significantly alters the normalized voltage of respiratory tonic activity. The normalized voltage of tonic activity during augmentation phase (1.7, n=6) is significantly increased (P<0.001) in contrast to that of baseline (1, n=6) (Figure. 3.2). The normalized voltage of tonic activity during depression phase (0.55, n=6) is lower (P<0.001) than that of baseline (1, n=6) (Figure. 3.2).

3.3 Barium exposure alters respiratory activity during severe hypoxia (0% FO_2)

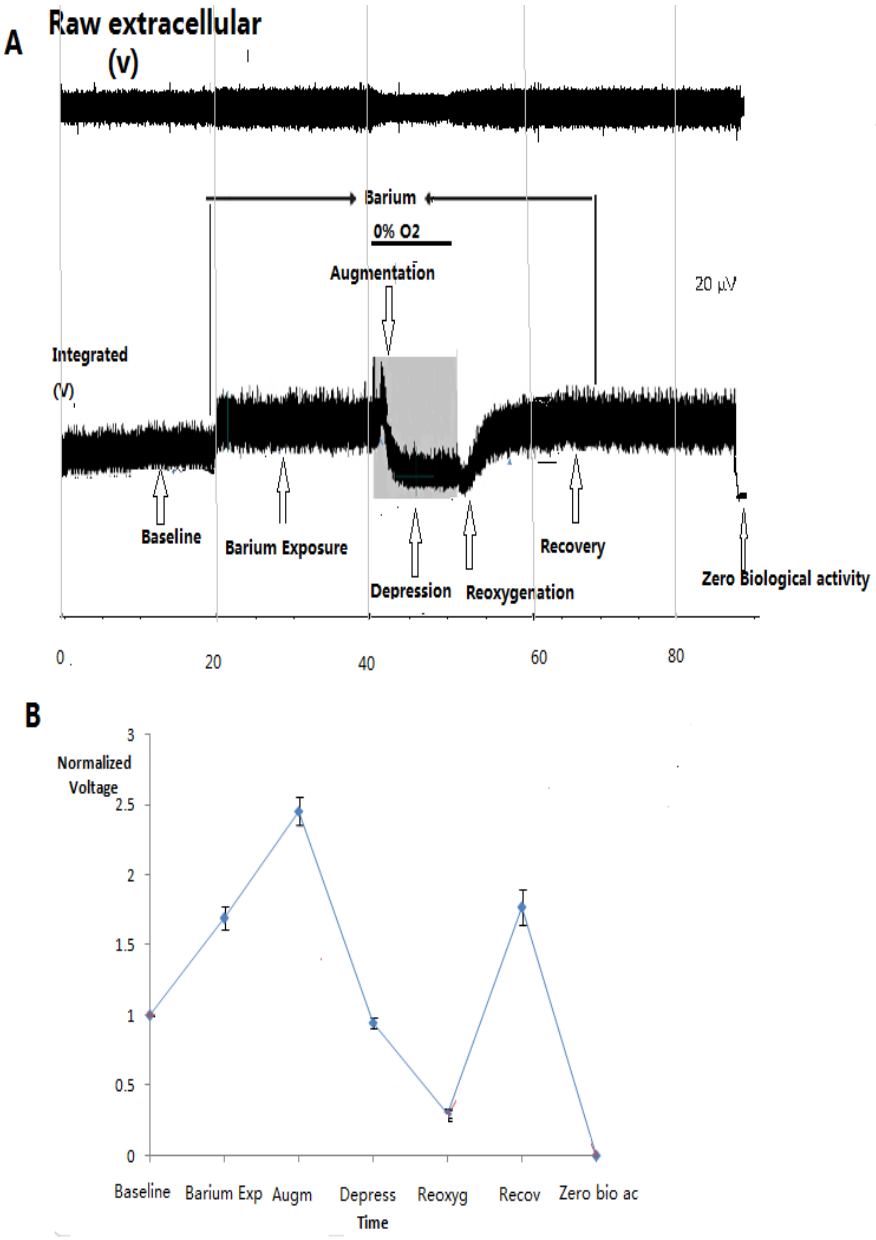

Figure 3.3 (A) Integrated extracellular activity from representative slice of P5 mice show effects of barium exposure on the changes in voltage of respiratory tonic activity in response to and hypoxia (0% FO_2) followed by reoxygenation (95% FO_2). **(B)** Effects of barium exposure on changes in normalized voltage of tonic activity compared to control group during different phases in response to hypoxia (0% FO_2) followed by reoxygenation (95% FO_2). Different phases of respiratory activity show respectively: Baseline; Barium Exposure; Depression; Reoxgenation; Recovery; Zero Biological Activity. All values are mean values ± S.E.M.

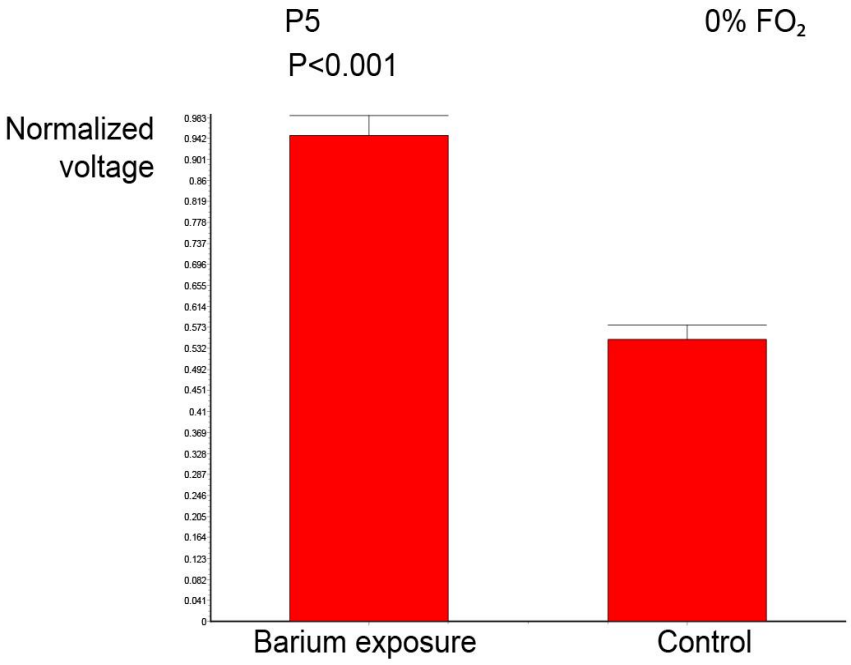

Figure 3.4 Effects of Barium exposure on the normalized voltage of tonic activity in response to hypoxia (0% FO_2) during the depression phase (unpaired *t*-test). All values are mean values ± S.E.M.

During severe hypoxia, the normalized voltage of tonic activity in the presence of barium exposure (0.95, *n*=6) is higher (*P* <0.001) than that without application of barium (0.55, *n*=6) (Figures3.3; 3.4).

CHAPTER 4

DISCUSSION

4.1 Methodological Considerations

In this thesis, only slices from mice within an a range of ages from postnatal day 3 to postnatal day 9 are used for statistical comparisons, since the structure of respiratory system within slices preparation changes with time. Despite neonatal mice of slightly different ages, the transverse brain stem slices are cut to the same thickness (500 μm) in order to keep tissue oxygenation level similar under the control high-oxygen condition (95% O_2). However, a weakness of using mice of slightly different ages is that the structures of respiratory neural network within transverse slices from mice with different ages are not exactly the same. The caudal border of the transverse slice is constant, because caudal surface of the slice is used to record respiratory rhythmic activity. The rostral border, however, may have varied slightly in terms of the extent of inclusion of elements of the respiratory network rostral to the preBötC(Hu et al., 2012). Despite this variability in the inclusion of rostral neural elements, which might be expected to introduce some variability in terms of the response of tonic activity to hypoxia, we nevertheless found significant changes due to treatment with barium (see below).

A question that should be taken into consideration concerns that how relevant in vitro respiratory rhythmic activity is for in vivo eupneic breathing from young mice. The frequency is one order of magnitude lower in vitro compared to respiratory activity from intact mice (Ballanyi, 2004). In vivo bilateral vagotomy in neonatal mice reduces the frequency of respiratory activity (Murakoshi and Otsuka, 1985, Smith et al., 1990). Ballanyi, Onimaru et al. 1999 postulates that the lesion of

peripheral afferent inputs within transverse brainstem slices preparations leads to a decrease in vitro frequency of respiratory activity. Even if the in vitro pattern of respiratory activity is not exactly the same as in vivo respiratory breathing, the in vitro transverse brainstem slice model is essential for performing cellular and molecular analyses of voltage- and ligand-gated receptors associated with respiratory rhythm generation (Ballanyi et al., 1999). Thus, the in vitro brainstem slice preparation from neonatal mice is a good model for the study of respiratory network function.

4.2 Barium exposure significantly alters baseline of respiratory activity

The results suggest that the voltage of tonic activity after barium bath is significantly increased ($P<0.001$) in contrast to that of baseline. This result is consistent with a previous study that shows that bath application of barium lead to an increase in the frequency and amplitude of respiratory rhythmic activity (Ballanyi, 2004). Barium causes a decrease in membrane conductance within inspiratory interneurons of the VRG (Ballanyi, 2004). As previously mentioned, barium inhibits the delayed rectifier K^+ conductance as well as the inward rectifier K^+ conductance (Hagiwara and Kidokoro, 1971, Standen and Stanfield, 1978). Similarly, Solessio, Linn et al. 2000 shows that these two conductances are inhibited by barium via similar processes. Thus, a barium-induced decreased K^+ conductance may be involved in modulation of variations in the baseline of respiratory activity.

However, in contrast, one study suggests that while barium reduces outward K^+ conductance it also increases inward Na^+ current flow and that this combined effect on two ion conductance leads to a prolonged action potential (Richter et al., 1991). Thus, a barium-induced increased inward Na^+ current is likely to play a role in

reducing the threshold for action potentials and for generating action potentials of greater amplitude. This evidence is corroborated by another study showing that inward Na^+ current induced by barium is more important for lowering threshold potential and generating prolonged action potentials than a reduction in K^+ channel conductance (Giebisch and Weidmann, 1971, Vitek and Trautwein, 1971). Taken together, these studies show that barium lowers the action potential threshold and prolongs the duration of action potentials. This phenomenon is consistent with our result that barium significantly increases voltage of tonic respiratory activity.

4.3 Barium exposure reverses the depression of respiratory activity during severe hypoxia (0% FO_2)

In this thesis, during severe hypoxia, the normalized voltage of tonic activity in the presence of barium exposure (0.95, *n*=6) is higher ($P < 0.001$) than that without barium bath (0.55, *n*=6). This result supports the finding that barium exposure reverses the frequency depression of respiratory rhythmic activity (Ballanyi et al. 2004). Barium, as mentioned above, decreases the delayed rectifier K^+ conductance and the inward rectifier K^+ conductance (Hagiwara and Kidokoro, 1971, Standen and Stanfield, 1978). Thus, those results demonstrate that the application of external barium in transverse brainstem slice may reduce greatly the increase in K^+ conductance that normally occurs during severe hypoxia. During the initial reoxygenation phase (undershoot), the normalized voltage of tonic activity is lower than that in the depression phase This phenomenon is in consistent with finding showing that after the cessation of hypoxia and rapid return to 95% FO_2, frequency declines further below the depression frequency. Several studies have shown that neurotransmitters are released during hypoxia and release of reactive oxygen

compounds during reoxygenation may be responsible for decrease of normalized voltage of tonic activity and burst frequency (Cherniack et al., 1971, Richter DW, 1999). This variation suggests that respiratory network is functionally flexible and able to generate various patterns of respiratory activity in respond to different conditions (Marder and Calabrese, 1996, Stein et al., 1997). In other words, the output of the respiratory system is plastic (Mitchell and Johnson, 2003).

The one of major mechanisms for central respiratory network in respond to severe hypoxia (0% FO_2) is to open K^+ channels resulting in an increase in K^+ conductance and subsequently generate a major hyperpolarization (Ballanyi, 2004). Ballanyi et al. 1999 concluded that reaction of central respiratory network to hypoxia is an adaptive mechanism for energy saving instead of showing a pathophysiological result of inhibition of aerobic metabolism. The neuronal activity and transmembrane ion fluxes are decreased greatly by the hyperpolarization caused by opening of K^+ channels during severe hypoxia (Hansen, 1985, Hochachka, 1986). This significant decrease in ion flux leads to reduced ion pump activity and reduces ATP utilization by 50%. Accordingly, if hypoxic K^+ channel-mediated hyperpolarization lasts for a prolonged time, it will have a neuroprotective function for brain during severe hypoxia (0% FO_2) (Ballanyi, 2004). As previously mentioned, the main function of barium is to block K^+ channels and reverse the hypoxia-induced hyperpolarization in respiratory neurons. Thus, the application of external barium may cause increased ion flux and a concomitant need for extra ATP consumption. Thus the tolerance of the respiratory network to severe hypoxia is likely to be greatly decreased in the presence of barium.

In this thesis, quantification of the barium-induced reversal of the depression of respiratory activity is developed by a novel protocol for normalizing the

voltage of tonic activity. This methodology may be used in future studies with antagonists of specific K^+ channels such as K_{ATP} and K_{IR} to determine their relative contributions to the anoxia-induced decrease in tonic activity.

In the presence of barium, anoxia-induced augmentation and depression are still observed relative to the level of tonic activity just prior to hypoxic exposure. The anoxia-induced augmentation may not depend on the activation of K^+ channels. Excitatory glutamate release may play a major role in generation of augmentation (Richter DW, 1999). During hypoxic depression, a certain percentage of K^+ channel may not be blocked by barium. Alternatively, another possible explanation for hypoxic depression in the presence of barium is that inhibitory neurotransmitters such as GABA and glycine result in opening of ligand-gated Cl^- channels which is likely to evoke a major hyperpolarization through increasing inward Cl^- conductance during severe hypoxia (Donato and Nistri, 2000).

CHAPTER 5

CONCLUSIONS

The transverse brainstem slice preparation from neonatal mice is a well established model to study the respiratory activity. The results of this thesis show that external barium exposure alters the in vitro respiratory response to severe hypoxia (0% FO_2). Barium significantly increases the voltage of baseline tonic activity during the depression phase of the hypoxic respiratory response. This apparent reversal of hypoxic respiratory depression is likely due to the closing of K^+ channels and lowering K^+ conductance by extracellular barium.

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