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ABSTRACT

THE EFFECTS OF THEOPHYLLINE ON THE IN VITRO RESPIRATORY RESPONSE TO HYPOXIA

by
Ke Geng

The isolated transverse brainstem slice preparation of neonatal mice is employed to investigate the function of theophylline, a competitive nonselective phophodiesterase inhibitor and adenosine receptor antagonist, on the hypoxic ventilatory response. Brainstem slices are isolated from neonatal mice (4-8 days old) and superfused with artificial cerebrospinal fluid (aCSF), equilibrated with a hyperoxic gas mixture (95% O_2, 5% CO_2) as a control, and anoxic mixture (0% O_2, 5% CO_2, 95% N_2) to create severe hypoxia at the tissue level. Using suction electrodes, extracellular population activities of respiratory neurons is recorded from brainstem slices in the region of the pre-Bötzinger Complex (preBötC), a site of inspiratory rhythm generation. One goal of this study is to detect if the theophylline increases in vitro respiratory activity under control oxygen conditions. Another goal is to determine the extent to which theophylline reverses the respiratory depression during severe hypoxia.

THE EFFECTS OF THEOPHYLLINE ON THE IN VITRO RESPIRATORY RESPONSE TO HYPOXIA

by
Ke Geng

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APPROVAL PAGE

THE EFFECTS OF THEOPHYLLINE ON THE IN VITRO RESPIRATORY RESPONSE TO HYPOXIA

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This Thesis is dedicated to My Parents, Zenxun Geng, Jian Lv, My Fiancée, Beibei Li

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CHAPTER 1

INTRODUCTION

Theophylline, which is also called dimethylxanthine, has been widely used as a critical ingredient of many therapeutic drugs for respiratory conditions such as apnea of prematurity, chronic obstructive pulmonary diseases (COPD) or asthma (Thach 2008) (Ruangkittisakul and Ballanyi 2010). Both theophylline and caffeine are members of the xanthine family and they are similar structurally and functionally. Theophylline has two pharmacological effects; it is a competitive nonselective phosphodiestrase inhibitor and adenosine receptor antagonist (Daly, Padgett et al. 1985; Kawai, Okada et al. 1995). Theophylline blocks one or more of the subtypes of the enzyme phosphodiestrase (PDE) and, therefore, prevents the inactivation of the intracellular second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). As a result of an increase of the amount of intracellular cAMP, protein kinase A (PKA) is activated and the production of leukotriene, which plays an important role in inflammation in asthma, is inhibited. In addition to the blocking PDE, theophylline nonselectively blocks adenosine's receptors. Adenosine is an inhibitory neurotransmitter and, therefore, blocking adenosine has a stimulatory effect on the nervous system. (Dunwiddie and Hoffer 1980)

Adenosine (Ado) is a purine nucleoside comprising a molecule of adenine attached to a ribose sugar molecule (ribofuranose) moiety. Generally speaking, adenosine is inhibitory by working presynaptically to depress the release of several of excitatory neurotransmitters such as glutamate, gamma-aminobutyric acid (GABA), acetylcholine, serotonin, 5-hydroxytryptamine (5-HT), and dopamine (Scanziani, Capogna et al. 1992)

(Dunwiddie and Hoffer 1980). Furthermore, adenosine plays a protective role in neuronal tissue. Adenosine is released in large amounts when the brain requirement for energy in the terms of ATP (adenosine triphosphate) outstrips ATP production in pathological conditions such as hypoxia and hypoxia-ischemia (Laudignon, Farri et al. 1990) (Dunwiddie and Masino 2001). Studies have shown that endogenous adenosine release during hypoxia, ischemia, and other stressful conditions could attenuate damage to brain tissue (Dunwiddie and Masino 2001).

It is accepted that the neuroprotective function of adenosine is accomplished mainly by the activation of A_1 receptors (Snyder, Katims et al. 1981; Landells, Jensen et al. 2000) (Runold, Lagercrantz et al. 1989). Adenosine can hyperpolarize neurons, suppressing the activation of voltage-gated Ca^{2+} channels (Dunwiddie and Masino 2001) (Wei, Li et al. 2011). As a result, the excitatory inward flow of calcium ions will be inhibited. These effects help to maintain the intracellular level of ATP and prevent Ca^{2+} influx, which can lead to excitatory damage to tissue (Dunwiddie and Masino 2001).

In response to severe hypoxia, mammals initially increase their rate of ventilation through an increase in tidal volume and breathing frequency (Holowach-Thurston, Hauhart et al. 1974; Kawai, Okada et al. 1995). A short time later, if the hypoxia sustained, the animal will cease to breathe during a period called primary apnea, which is often followed by a period of gasping (Ramirez, Quellmalz et al. 1998). Gasping is characterized by a very low frequency of respiratory movements. It has been hypothesized that such a decrease in respiratory frequency during hypoxia might be due to the release of protective neurotransmitters or neuromodulator such as adenosine (Runold, Lagercrantz et al. 1989). In this study, we have tested the effects of theophylline

on *in vitro* respiratory activity generated within transverse brainstem slices. An advantage of this in vitro system is that the effects of a drug may be ascribed to changes in a relatively limited neural circuit composed of rhythmogenic neurons within the preBötC as well as neurons located in regions such as the raphe obscurus that exert a modulatory influence on the respiratory rhythm (Ptak, Yamanishi et al. 2009). Two hypotheses have been tested: (1) under normal oxygen conditions (95% O_2) theophylline can increase the respiratory activity measured as tonic activity of the preBötC; (2) during severe hypoxia, exposure to theophylline will increase the level respiratory activity measured as tonic activity of the preBötC.

CHAPTER 2

METHODS AND MATERIALS

2.1 Transverse Brainstem Preparation

In this study, transverse brainstem slice preparations of neonatal mice (P4-P8) are employed (Ramirez, Quellmalz et al. 1996) (Smith, Ellenberger et al. 1991). The experimental subjects are male and female CD-1 neonatal mice breed from founders ordered from Charles River Laboratories (Wilmington,MA). During experiments, isoflurane is used to deeply anaesthetize mice until no withdrawal reflex from toe pinch could be detected. Subsequently, mice are decapitated at the C3—C4 spinal level. The brainstem and partial spinal cord are bathed in ice-cold artificial cerebral spinal fluid (aCSF) that is composed of (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., 2010). The pH of the aCSF is 7.2 to 7.4 at room temperature (21 ^oC). The brainstem and spinal cord are glued on the agar block such that the spinal cord was above brainstem. The dorsal side of brainstem is glued with agar block, while the ventral side faces the blade of slicing machine. On the vibrating blade microtome (Leica VT 1200), the brainstem is cut in serial sections (100 μm) in the caudal-to-rostral direction until the "principle loop" that represents the caudal border of the PBC is observed. At this point, a final 500 μm thick slice is cut for measuring rhythmic respiratory activity (Ramirez, Quellmalz et al. 1996). The slice is then bathed in aCSF saturated with 95% O_2 for 30 min at room temperature to recover from the slicing process prior to the beginning of recordings. It is then transferred to superfusion chamber (RC-29 Warner Instruments)

and also submerged with fluid flow of aCSF solution (32 °C; flow rate, 10 ml min^{-1}). The K^+ concentration of the aCSF is increased from 3mM to 8 mM in order to record a regular and long-lasting respiratory rhythm (Tryba, Pena et al. 2003). A solution reservoir heater and an in-line heater system (TC-344B; Warner Instruments) are used to control the temperature of system.

2.2 Electrophysiological Recording

Glass micropipette electrodes filled with aCSF are employed to record the respiratory rhythm. They are placed on the surface of the 500 µm thick slices with the tips of these pipettes touching the specific area of the slice containing the pre-Bötzinger Complex (preBötC), which is a specific region in the rostral-to-caudal axis of the column of neurons called the ventral respiratory group (VRG). Extracellular rhythmic population activity of preBötC neurons is recorded (Fig. 1) (Telgkamp and Ramirez 1999), using a 100-fold gain extracellular preamplifier (JFIE 1626), product of the James Franck Institute Electronics Laboratory, University of Chicago, in series with a variable gain amplifier (Model 410; Brownlee Precision). When electrodes are recording from the appropriate preBötC area, strong rhythmic bursts of extracellular activity can be heard with an audio monitor.

2.3 Control of O_2 Tension

To control the partial pressure of oxygen (PO_2), the fractional value of oxygen (FO_2) is adjusted by bubbling O_2 into a reservoir filled with 100 ml aCSF (gas flow rate: 50 ml/min) with the help of an aeration stone. Under the normal conditions used for the control group experiment, FO_2 is 95% while FCO_2 is 5%. During hypoxia, FO_2 is 0% (O_2 is replaced by N_2) and the fractional value of CO_2 remains 5%, in order to maintain a constant pH. Alteration of PO_2 can be achieved by changing the flow of aCSF between two reservoirs. One reservoir is filled up with a control gas (FO_2 = 95%), whereas the other one is saturated with a test gas in which FO_2 is 0% (for details see Hill et al. 2010).

2.4 Normalize Voltage of Tonic Activity and Data Analysis

Experimental data with respiratory rhythms (bursts) are collected from less than six slices for each section of this study, which is a requirement for proper statistical analysis. Therefore, the voltage of tonic activities are employed to represent respiratory activity under alternative conditions (i,e., 95% O_2 vs. 0% O_2). The use of tonic activity to represent respiratory activity rather than burst frequency data is justified based on a positive correlation between tonic activity and burst frequency (for details see **3.1**). In the experiments, tonic activity, in the form of a rectified-and-integrated version of the raw extracellular voltage is recorded. To analyze the data, an experiment is divided into several phases. For example, baseline represents the time before the application of theophylline, augmentation phase the very beginning of hypoxia, and depression phase the end of hypoxia. Mean voltage values are measured from each phase, followed by a

normalization of these mean values. The normalization is done for two reasons: (1) voltage values for tonic activity are fairly arbitrary; they vary with experimental details such as the degree of sealing between the pipette tip and the slice surface (2) voltage values for tonic activity are influences be the level of on-going background electronic activity of non-biological origin. For these reasons, tonic activity values are normalized when comparisons between different preparations are desired. Normalization of tonic activity is done as follows. Suppose the mean value during baseline phase is *a* and the mean value of voltage after the electrodes are lifted, which is the very end of one experiment, is *b*, then any other value *c* collected from different phases can be normalized as: $c' = (c-b) / (a-b)$. Such normalization is helpful for comparing values across preparations because the raw values can be difficult to analyze directly. All statistical data are given as means ± S.E.M. Significance ($P < 0.05$) is evaluated with either a paired or unpaired *t*-test, as appropriate.

CHAPTER 3

RESULTS

3.1 The Positive Correlation between Variation in Voltage of Tonic Activity and Change in Frequency of Rhythmic Activity

Previous studies conducted with application of tetrodotoxin, a specific blocker of voltage-gated sodium channels, showed that the blockage of voltage-gated sodium channels reduced tonic activity (Ballanyi, Onimaru et al. 1999). Also, it has been proved that tonic activity varied in a similar manner to the respiratory rhythm in response to different experimental conditions. For instance, during the augmentation phase, tonic activity increases simultaneously with an acceleration of the in vitro respiratory rhythm. Similarly, during the depression phase, tonic activity decreases as the respiratory rhythm decelerates. Based on these findings it can be concluded that the tonic activity is positively correlated with respiratory burst frequency. Thus, the alteration of tonic activity during each phase of the experiment can be considered as representative of the frequency of rhythmic outputs.

3.2 The Application of Theophylline Changes the Fundamental Respiratory Activity

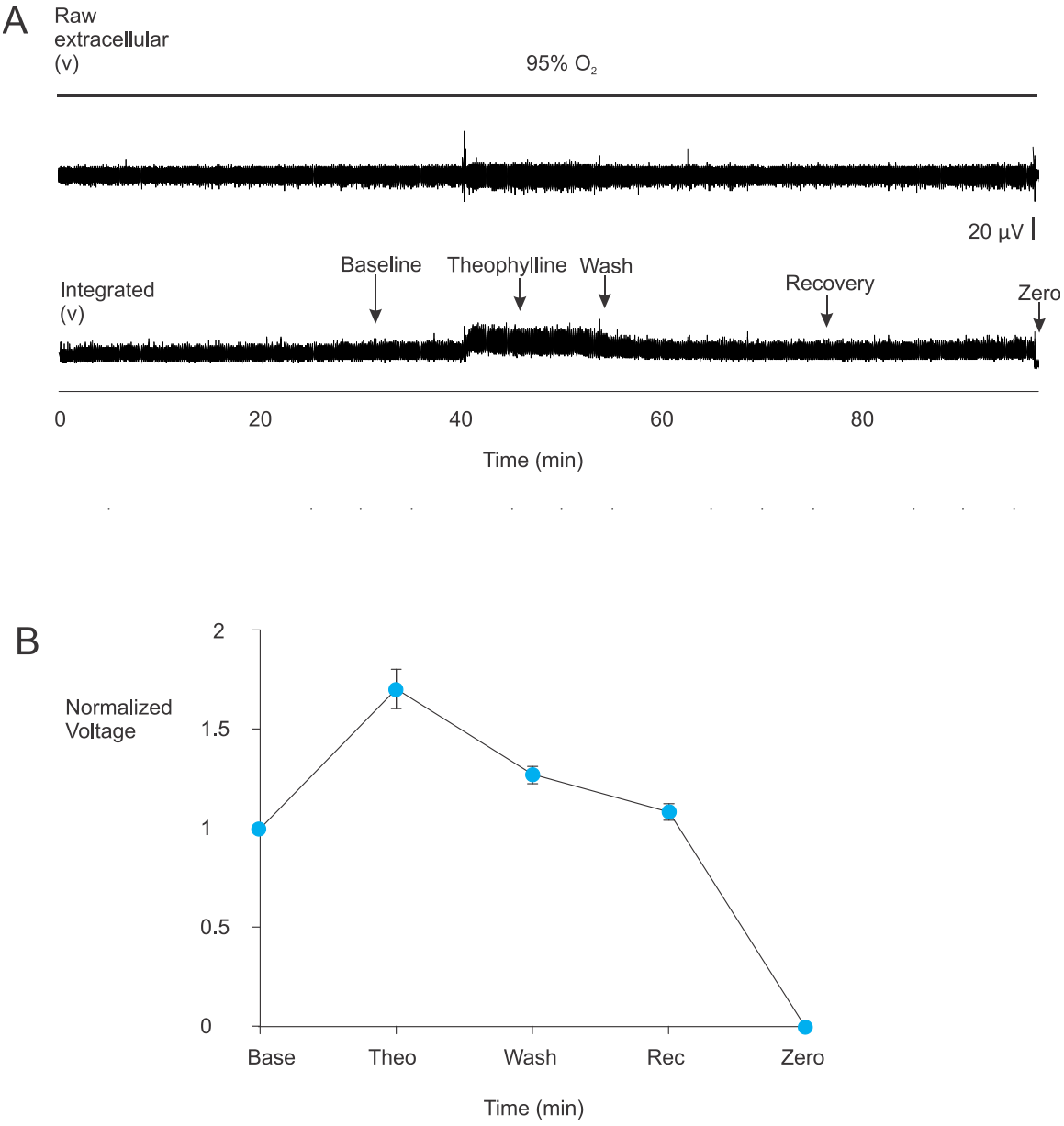

Figure 3.1 Variation in voltage of tonic activity during different phases of an experiment in response to theophylline exposure followed by wash with application of aCSF. "Base", "Theo", "Wash", "Rec", "Zero" represent different phases respectively: Baseline, After Theophylline Exposure, Wash, Recovery, Zero Biological activity. All values are mean values ± S.E.M.

In this experiment, the fractional oxygen concentration is maintained constantly (FO_2=95%). After 20 minutes, theophylline is applied. Theophylline significantly increases tonic activity in compared to the baseline value. Significances are evaluated by a paired t-test afterwards with raw voltage values. (P<0.001). After 10 minutes of bath application of theophylline a wash is performed during which tonic activity recovers to its baseline level.

3.3 Respiratory Activity Responds to Hypoxia Condition (0%FO_2)

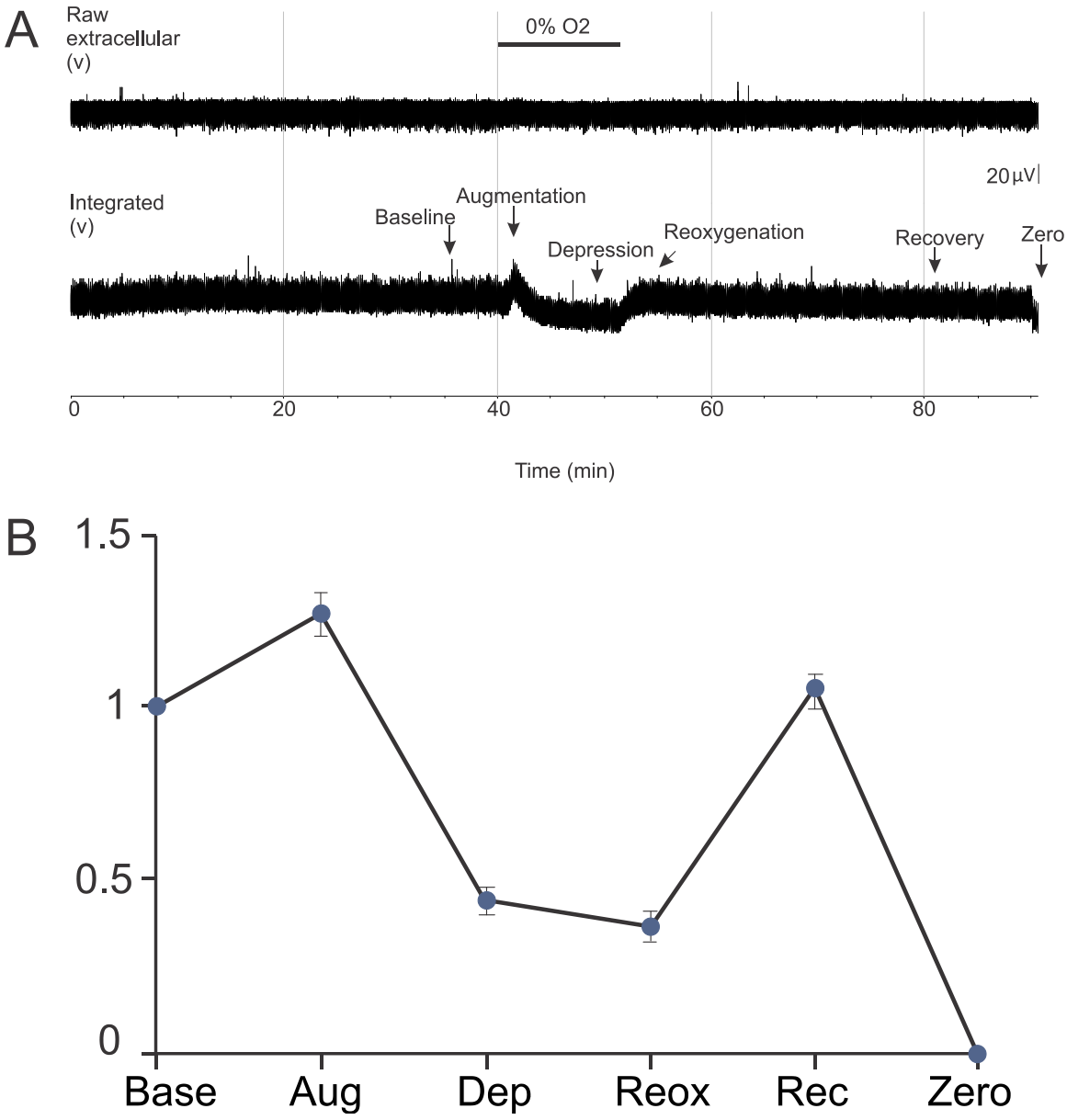

Figure 3.2 Variation in voltage of tonic activity in respond to hypoxia (0% FO_2) during different phases of the experiment followed by reoxygenation (95% FO_2). "Base", "Aug", "Dep", "Reox", "Rec", "Zero" represent different phases respectively: Baseline, Augmentation, Depression, Reoxygenation, Recovery, Zero Biological activity. All values are mean values ± S.E.M.

Respiratory activity (measured as tonic activity) is recorded with glass micropipette electrodes filled with aCSF. The electrodes are placed on a 500 µm thick brainstem slice in the area of the preBötC. There is variation in tonic activity during hypoxia in the control data. During the initial period of hypoxia, which is called the augmentation phase, the average voltage increases compared with the mean voltage value in the baseline phase (1.27, n=6) (P<0.001). The mean voltage during the depression phase under hypoxia is significantly decreased compared with baseline phase (0.44, n=6) (P<0.001). Both significances are evaluated by paired t-tests using raw voltage values.

3.4 Theophylline Exposure Changes Respiratory Activity during Severe Hypoxia ($0\%FO_2$)

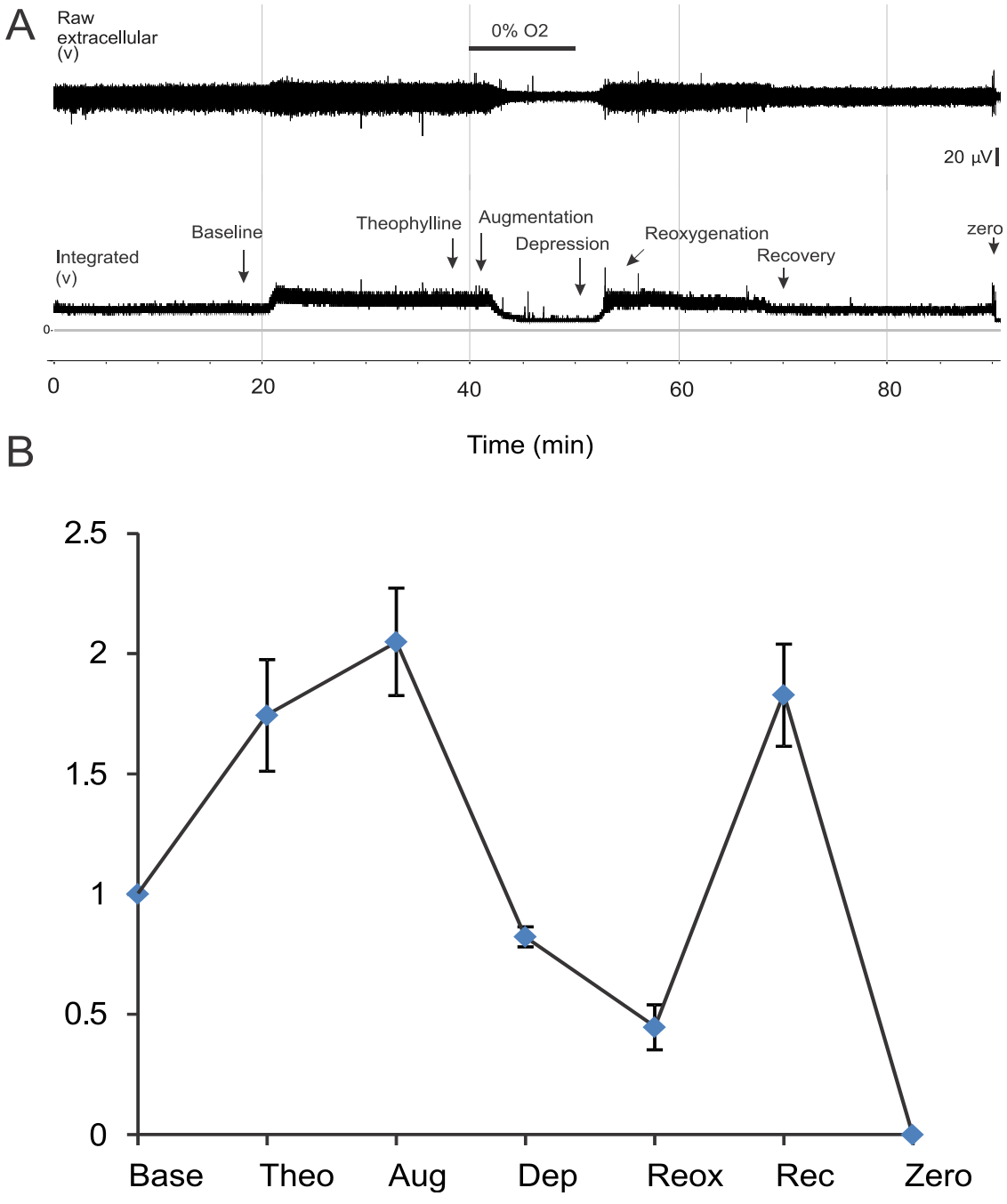

Figure 3.3 Effect of theophylline on the variation in voltage of tonic activity during different phases of an experiment in response to hypoxia (0% O_2). "Base", "Theo", "Aug", "Dep", "Reox", "Rec", "Zero" represent different phases respectively: Baseline,

After Theophylline Exposure, Augmentation, Depression, Reoxygenation, Recovery, Zero Biological activity. All values are mean values ± S.E.M.

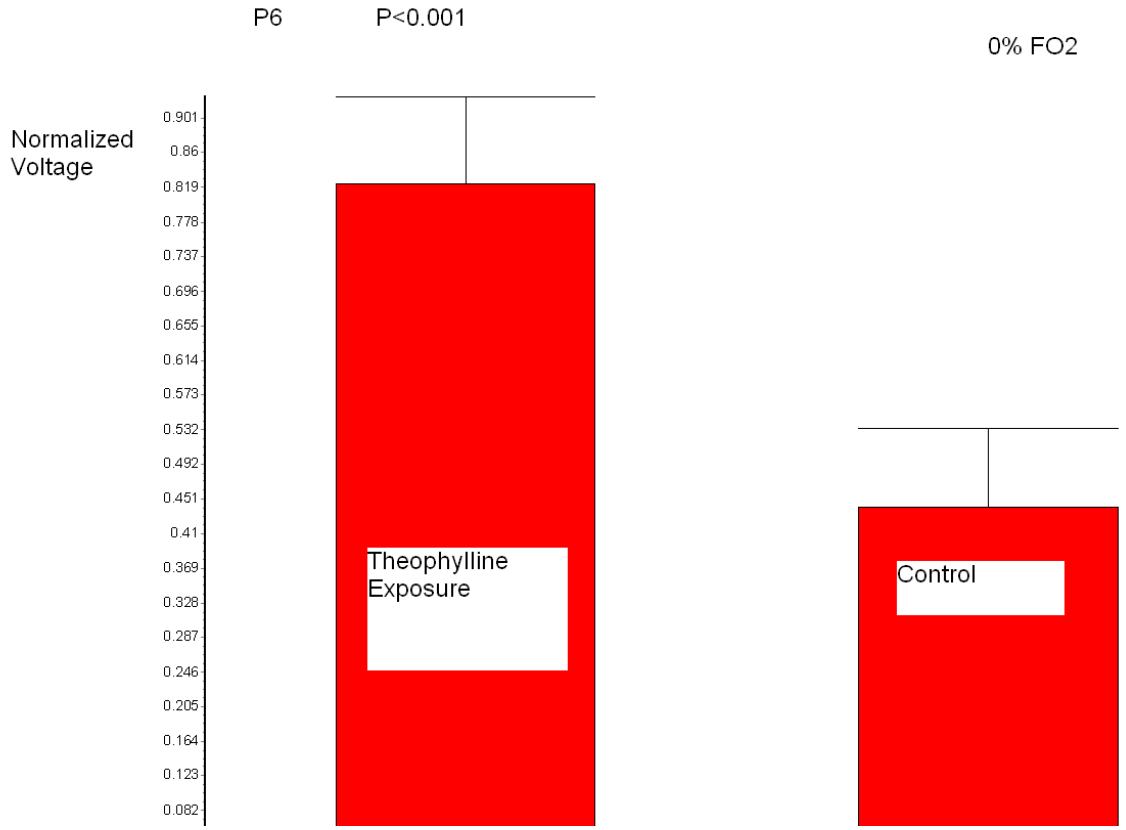

Figure 3.4 Effect of theophylline on changes in voltage of tonic activity in response to hypoxia (0% FO_2). All values are mean values ± S.E.M.

In the 10 minute period in which theophylline is bath applied, the normalized value of the voltage is significantly higher than the average value prior to the drug application (1.74, *n*=6) (P <0.001). Paired t-test is performed to test the significance using raw voltage values. During exposure to severe hypoxia, the normalized tonic activity in the presence of theophylline is increased significantly (0.82, *n*=6) (*P* <0.001) compared to the control group. The significance is evaluated using normalized voltage values by unpaired t-test.

CHAPTER 4

DISCUSSION

It is generally accepted that theophylline can reverse the depression of respiratory activity in response to hypoxia (Ruangkittisakul and Ballanyi 2010). Nevertheless, there are contradictory results of former studies. While hypoxia-induced respiratory suppression is found to be counteracted by theophylline, some other studies show that theophylline fails to reverse the respiratory depression during hypoxia in a double-blind trial in human adults in which the theophylline is orally administrated. (Easton and Anthonisen 1988; Swaminathan, Paton et al. 1992). Given that many previous studies regarding the theophylline's effect on the hypoxic ventilatory response have been done by recording phrenic nerve discharge from C4 roots of rat, the approach used in the present study, using *in vitro* recordings from the preBötC in brainstem slices is novel. An advantage of using this reduced preparation is that the effects of theophylline can be ascribed to a restricted subset of the brainstem respiratory network, consisting of neurons of the preBötC and neuromodulatory neurons that are presynaptic to the preBötC located in areas such as the raphe obscurus and raphe pallidus (Ptak, Yamanishi et al. 2009) (Kobayashi, Fujito et al. 2010).

Although many hypotheses had been proposed, the mechanism underlying hypoxic respiratory depression remains unclear. Several neuromodulators and neurotransmitters are released as a result of maintained low oxygen tension (Richter, Schmidt-Garcon et al. 1999). Among them, adenosine may play a fundamental role in hypoxic respiratory depression. Adenosine inhibits the release of many neurotransmitters and therefore

preventing the arising of action potentials. Then, in principle the inward sodium ion flow is decreased. As a result, the requirement of energy for Na^+ -K^+ ATPase to maintain homeostasis will be largely reduced. Previous studies have proved that adenosine is responsible for regulating many physiological activities in heart and brain (Gomes, Kaster et al. 2011). Overall, adenosine has an inhibitory effect on excitatory neurotransmitter release (Fredholm and Dunwiddie 1988). Experiments had been done to determine how adenosine inhibits the release of neurotransmitters. A well accepted model is G-protein-coupled inhibition of Ca^{2+} channels in nerve terminals. Even though some other hypothesis have been proposed based on adenosine's suppression of spontaneous Ca^{2+}-independent release of neurotransmitter, blockage of inward Ca^{2+} flux still seems to be the principle explanation (Eldridge, Millhorn et al. 1985; Wu and Saggau 1997). Adenosine is capable of hyperpolarizing cells; as a result, there is a reduction in the inward flux of calcium ions because a majority of Ca^{2+} channels are voltage-gated. According to some reports adenosine receptors could also promote neurotransmitter release (Cunha, Milusheva et al. 1994). However, these excitatory effects appear less common compared with the inhibitory function. One of adenosine receptors' features is that they can hyperpolarize the resting membrane potential by activating G-protein-dependent inwardly rectifying K^+ channels (GIRKs) (Dunwiddie and Masino 2001).

Adenosine receptors have been divided into three categories: extracellular A_1 receptors that are inhibitory with high affinity, extracellular A_2 receptors that are excitatory with low affinity, and intracellular P receptors that are depressant (Dunwiddie and Masino 2001). Theophylline, as well as other methylxanthines, is a competitive antagonist of adenosine's extracellular receptors (Eldridge, Millhorn et al. 1985). Furthermore,

adenosine-induced inhibition may be related to the activation of A_1 receptors (Runold, Lagercrantz et al. 1989). Meanwhile, no evidences has been reported indicating such inhibition might be related to the accumulation of cAMP caused by theophylline (Dunwiddie and Masino 2001).

Another aspect that may be worth considering is the relationship between dosages of theophylline and its effects. Under the concentration of 100 μM, methylxanthines acts to attenuate respiratory depression during hypoxia by affecting adenosine receptors, whereas at concentration higher than 100 μM, theophylline also inhibits phosphodiestrase (PDE) (Eldridge, Millhorn et al. 1985). On the contrary, other studies claim that the counteracting role of theophylline and methylxanthines against respiratory depression is largely achieved by its antagonism of adenosine receptors (Dunwiddie and Fredholm 1984). Given that the concentration of theophylline used in the present study was 2.5 mM, further experiments could be designed to investigate the mechanisms by which theophylline reverses respiratory depression. For example, an exclusive inhibitor of PDE, Vinpocetine, can be applied individually to test if it can reverse such depression without affecting adenosine receptors. In the same way, exclusive adenosine inhibitor, for instance, Mesembrine, can be used individually test the effects of adenosine receptor antagonism on hypoxic respiratory depression.

Another concern is about whether the finding from an in vitro study is significantly relevant to an in vivo situation under hypoxic stress. In vivo, the tissue oxygenation is largely uniform, since the estimated maximal distance for oxygen to diffuse to target tissue is 20 μm (Hill, Garcia et al. 2011). On the contrary, for the in vitro slices, the PO_2 within the slice tissue will drop considerably with the depth increases since 500 μm is

quite a large distance compared with 20 μm as mentioned above. According to a previous study, the top and bottom edges of a slice are found to have the highest PO_2 while the central part of the slice had the lowest PO_2 (Hill, Garcia et al. 2011). Despite these difficulties, the tissue is on average hyperoxic even at the center of a slice in 95% FO_2. Thus, switching from 95% O_2 to 0% O_2 will cause a dramatic shift in the oxygenation state of neural tissue *in vitro*. While such as shift from a hyperoxic state to a hypoxic state does not occur *in vivo*, a previous study has shown that the response of the *in vitro* respiratory network to hypoxia is graded (Hill, Garcia et al. 2011). Therefore, despite the more dramatic shifts in tissue oxygenation state that occur in vitro compared to *in vivo*, the responses of the respiratory network may be qualitatively similar.

It may appears strange that theophylline can be used to treat asthma and infant premature apnea given that it is an antagonist of a protective neurotransmitter—adenosine. In fact, the usage of theophylline against apnea of immaturity has received negative comments recently, since the antagonism of adenosine may increase harmful calcium influx during the pathological condition, as described earlier in this paper. The therapeutic effect of theophylline on asthma can be attributed into several factors. First, theophylline relaxes bronchial smooth muscle by inhibiting phosphodiestrase function, so that the intracellular concentration of cAMP increases. Second, both adenosine and adenine phosphate can deteriorate the spasm of bronchis during asthma. Considering this, it is convincible that theophylline can be applied to treat asthma since it is an antagonist of adenosine receptors. At last, asthma is an inflammatory disease that is caused by the entry of inflammatory medium into pulmonary tissue such as histamine, leukotrienes, prostaglandin, and so forth. The accumulation of cAMP, however, can effectively depress

the release of leukotrienes from mastocyte. Again, this can also explain why theophylline is used to treat asthma, based on its ability to increase intracellular amount of cAMP.

CHAPTER 5

CONCLUSIONS

The transverse brainstem slice preparation of neonatal mice is a well established system to serve the investigation of this study. It is shown that under high content of oxygen supply (FO_2 =95%), the exposure to extracellular theophylline is able to significantly increase the overall respiratory activity (measured as the tonic activity of the preBötC). Furthermore, during severe hypoxia (FO_2 =0%), the theophylline group displays increased respiratory activity during the depression phase, compared with the control group in which no theophylline is applied.

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