Employing a pH Sensitive Fluorophore to Measure Intracellular pH in the *In Vitro* Brainstem Preparation of *Rana catesbeiana*

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Abstract: We developed an *in vitro* tadpole brainstem preparation in order to investigate the development of central respiratory chemoreception and rhythmogenesis. pH sensitive fluorescent dyes have been utilized to record intracellular pH (pHi) optically in central respiratory chemoreceptive regions in mammals. Our goal in this study was to develop the ability to record pHi optically while simultaneously recording respiratory motor output in the superfused tadpole brainstem preparation. We developed a dye-loading protocol that demonstrated our ability to adequately load the majority of brainstem neurons. The presence of the dye was not disruptive to ongoing respiratory rhythmogenesis or the respiratory response to central respiratory chemoreceptor stimulation. The tadpole brainstem is an excellent model to study the development of the neural control of respiration, as it is well oxygenated and retains synaptic connectivity among respiratory central pattern generators, central respiratory chemoreceptors, and respiratory motor neurons. Validating of the use of the pH sensitive dyes to record pHi optically in central respiratory chemoreceptors in this preparation will permit further characterization of the pH regulatory responses of central respiratory chemoreceptors.

Keywords: pH, respiration, carbon dioxide, chemoreceptors.

INTRODUCTION

The ontogeny of respiration in the frog is of considerable interest since respiratory gas exchange occurs at the skin, gills, and lungs throughout development, and the interdependence and balance among gas exchange sites change during development [1-3]. Besides developmental changes in the contribution of these sites to gas exchange, the transition from aquatic to aerial respiration is associated with changes in the respiratory motor pattern as well as the sensitivity of the ventilatory response to hypoxia and CO_2 [4-6]. A similar pattern of ontogenesis may occur in the mammalian fetus given that the anatomical structures of the gill arches in the early fetus resemble the comparable amphibian structures. Hence, the metamorphic progression apparent in the development of central respiratory control mechanisms may enhance and guide our understanding of mammalian neural development.

Ventilatory responses to stimulation of central respiratory chemoreceptors change as a function of development (for review see [7]). The tadpole exhibits a developmentally dependent respiratory response to stimulation of central respiratory chemoreceptors that has been described in the *in vitro* brainstem preparation [8]. Fictive respiration does not respond to chemoreceptor stimulation in the earliest developmental stages of tadpole brainstem preparations. As metamorphosis progresses, however, a central respiratory response to chemoreceptor stimulation emerges. Attempts to locate chemosensitive regions have implicated a caudal medullary site in the pre-metamorphic tadpole and a more rostral medullary site in the post-metamorphic tadpole, thus indicating a developmental translocation of chemosensitive elements [9, 10]. It is not known what prompts this apparent translocation or if similar mechanisms underlie chemosensitivity in the caudal and rostral sites.

Although respiratory rhythm generation and central chemoreception are currently being studied in reduced mammalian preparations, the inaccessibility of mammalian fetal life makes ontogenetic studies in mammals difficult. In addition, mammalian brain slice preparations that exhibit respiratory burst activity must be relatively thin to facilitate adequate oxygenation of the tissue. Reducing the mammalian brainstem to a thin slice in order to maintain oxygenation eliminates any possibility of simultaneously studying rhythm generation and central chemoreception given that many of the respiratory chemoreceptors are widely distributed throughout the brainstem [11, 12], but are absent in brain slices. The *in vitro* tadpole brainstem preparation lets us investigate in an ontogenetic fashion both respiratory rhythmogenesis and central chemoreception simultaneously, as synaptic connectivity of these central networks and respiratory motor neurons is intact. The production of the gill respiratory rhythm in the *in vitro* tadpole preparation appears to rely upon reciprocal inhibition, whereas the lung rhythm appears to possess more pacemaker like properties. Shao and Feldman [13] described the genesis of respiratory rhythm in mammals does not require, but is modulated by reciprocal inhibition mediated by glycinergic neural transmission.

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1874-3366/09

Characterization of the cellular mechanisms involved in CO_2/H^+ chemoreception has been the focus of recent research [7, 14]. Unequivocally identifying a chemosensitive cell as being respiratory in nature continues to be problematic when studying central respiratory chemoreception. It is difficult to establish firm causal relationships between stimulus, effector elements and responses in intact preparations because each neuron makes only a small contribution to the overall response – different patterns of neuronal activity can only be correlated with particular responses. In reduced preparations, causal links may be established between sequential neural elements, but the biological importance of any particular response is moot since the neural activities are frequently unconnected to meaningful physiological responses. Brain areas involved in respiratory chemosensitivity have been identified by the discrete application of CO_2/H^+ [10, 12, 15] and differences in rates of pHi regulation, the neurons ability and rate of extrude H^+ in response to an acid load, between putative chemosensitive and non-chemosensitive cells have been proposed. In addition, separate studies using pH-sensitive dyes and whole cell recordings have found significant correlations between pHi and neuronal firing rate [15]. While these studies provide valuable information regarding the effects of CO_2/H^+ on cellular mechanisms, it has not been possible to attribute changes in respiratory motor output to the change in CO_2 or pH in any particular cell or specific cell population. The ability to directly correlate decreases in pHi in chemoreceptive neurons to increases in ventilation in a phylogenetic fashion may shed light on the evolution of air breathing and terrestriality. Addressing this issue requires simultaneous measurements of pHi dynamics and whole nerve respiratory output in a single preparation. The development of the *in vitro* tadpole brainstem preparation and simultaneous measurement of pHi using 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) provides a novel model to determine if there is a relationship between changes in pHi in specific cells and neural activity related to ventilation.

Fluorescein diacetate derivatives have been previously used to determine the integrity of cellular membranes, the presence of intracellular esterases, intracellular pH, and more recently to elucidate mechanisms of pHi regulation [14-18]. BCECF-AM is an analog of the widely used fluorescein. It is synthesized from carboxyfluorescein by adding two extra carboxylate groups *via* short alkyl chains and has a pKa of 6.97 making it ideal for reporting pHi in physiological conditions [19]. The addition of the acetoxymethyl (-AM) as an ester makes BCECF temporarily membrane permeable. Therefore, it is able to passively enter all cells [20]. Once inside the cell, hydrolysis of the acetyl ester linkage by enzymatic cleavage regenerates the less permeable, fluorescent, original compound [16]. BCECF is a dual excitation ratio metric dye that when excited at 440 nm is pH insensitive and when excited at 495 nm is pH-sensitive [21]. BCECF reports only cytoplasmic changes in pH and is sufficiently sensitive to respond to rapid kinetic changes of pH as small as 0.01 pH units [22].

To determine if the use of the pH sensitive dye BCECF can be incorporated to study pHi changes in the *in vitro* tadpole brainstem preparation, several important criteria must be established. First, we must determine if cells on the ventral surface of the tadpole brainstem contained the intracellular esterases necessary for cleavage of the –AM portion of the dye. Second, we must develop a dye loading protocol that will allow us to load and visualize an adequate representation of brainstem neurons. Finally, since hydrolysis of the acetyl ester linkage is followed by the spontaneous release of formaldehyde [20], it was also important to determine if the loading and/or excitation of BCECF has any toxic effects on respiratory rhythmogenesis or central respiratory chemoreception.

Here we describe a method to perform optical recordings of pHi from neurons in the *in vitro* tadpole brainstem preparation. Whereas valuable information regarding the development of respiratory control has been provided by other animal models, such as the *en bloc* neonatal rodent preparation, we chose to develop the optical recording of pHi the *in vitro* tadpole brainstem preparation because it has several advantages over mammalian preparations. The *in vitro* tadpole brainstem preparation is well-oxygenated [23] and, more importantly that the synaptic connectivity of respiratory central pattern generators, central chemoreceptors and respiratory motor neurons remains intact in this preparation [8, 24-28]. The ability to monitor whole nerve respiratory activity and changes in pHi simultaneously provides a powerful tool to dissect the mechanistic connections among the neural elements involved in respiratory responses to CO_2, which is not currently possible in mammalian preparations. Optically monitoring pHi continuously during various acid challenges, such as hypercapnic acidosis, will allow investigators to quantify pHi recovery *via* the extrusion of H^+. Validation of the technique to monitor optically pHi described in this manuscript may open the door for other researchers interested in investigating pHi regulation in other amphibian and/or non-mammalian preparations.

METHODS

In Vitro Brainstem Preparation

Animals

Experiments were performed on *Rana catesbeiana* tadpoles of various developmental stages of either sex obtained from a commercial supplier (Sullivan, TN, USA). All tadpoles were housed in aquaria and were maintained at 24 – 26˚C with a 12h:12h light:dark photoperiod. Tadpoles were fed on a daily basis (Fish Flakes, Wardly, Phoenix, AZ) as needed so that food was not a limiting resource. Animal care and experimental protocols were approved by the University of Texas at San Antonio Institutional Animal Care and Use Committee.

Dissection

Tadpoles (n = 10) were anesthetized in 3-aminobenzoic acid ethyl ester (MS 222; Sigma-Aldrich, St. Louis, MO) dissolved in distilled water (1:10,000) until unresponsive to tail pinch and weighed. Using a dissecting microscope, the dorsal cranium was removed, and the cranial and spinal nerves were severed at their ostia. After the dura and arachnoid were removed dorsally and ventrally, the brainstem was transected just caudal to the level of the second spinal (hypoglossal) nerve root and just rostral to cranial nerve V. Throughout the dissection, the brainstem

was continuously superfused with artificial cerebrospinal fluid (aCSF) bubbled with O_2. The composition of the aCSF was as follows (in mM): NaCl 104; KCl 4.0; $MgCl_2$ 1.4; D-glucose 10; $NaHCO_3$ 25; and $CaCl_2$ 2.4. Following dissection, the brainstem was incubated in oxygenated 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxy-methyl ester as described below (BCECF-AM; Molecular Probes, Eugene, Oregon). The brainstem was removed as described above then transferred to a superfusion recording chamber (SHD-26GKIT, Warner Instrument Corporation) maintained at room temperature, 21-23˚C. The brainstem was superfused with aCSF that had been equilibrated with a CO_2/O_2 gas mixture in an external tonometer at room temperature. The superfusate entered the recording chamber through an inflow aperture at a rate of 2 ml/min and exited from the opposite end *via* a vacuum. The aCSF pH was measured in the tonometer (Orion, 420A), and aCSF pH was adjusted by changing the amount of CO_2 bubbling into the tonometer.

Whole Nerve Recordings

Neural recordings of fictive gill and lung ventilation were obtained from the roots of cranial nerve (CN) VII using glass suction electrodes. Suction electrodes were made from thin walled borosilicate glass capillaries (OD 1 mm; ID 0.5 mm), pulled to a fine tip with a horizontal micropipette puller (Flaming/Brown, P-97). Pipettes were cut with a diamond pen and smoothed by heating on an open flame to achieve inner tip diameters that fit the cranial nerve of interest (approximately 220 to 315 μm). A 0.25 mm diameter silver grounding wire was wrapped around the outside of the pipette down to the tip of the electrode. Suction electrodes were filled with filtered aCSF solution described above. Efferent neural activity was amplified (10,000X; AM Systems, 1700), filtered (10 Hz to 500 Hz), simultaneously averaged with a moving time averager (CWE, MA-821) and digitized and recorded on a Pentium 4 PC (Datapac 2000 software).

Pre-Dye Neural Recordings

In order to investigate the central CO_2 chemoreceptive response before BCECF was loaded, the tadpole brainstem was randomly exposed to normocapnic (pH = 7.8) and hypercapnic conditions (pH = 7.4 in froglets and pH 7.4 and 7.2 in tadpoles). The superfusate was equilibrated in an external tonometer with gas having a P_{CO2} range from approximately 13 – 18 mm Hg, 31 – 38 mm Hg, and 55 – 64 mm Hg (balance O_2) to achieve pH values of 7.8, 7.4, and 7.2, respectively. Within two minutes after a stable pH was achieved in the tonometer, the pH in the recording chamber was equal to the tonometer pH ± 0.01 pH units [23, 24]. Each experiment began after the brainstem had been positioned in the recording chamber and superfused with oxygenated aCSF at pH 7.8 for at least 60 minutes, by which time stable rhythmic bursting nerve activities were well established. The pH of the aCSF was changed in random order between pH 7.4 and 7.8, and nerve activities were recorded from CN VII for 10 minutes. After each 10 minute recording, the P_{CO2} was adjusted to the new target pH followed by a five-minute period to permit equilibration of the recording chamber pH and stabilization of the brainstem response to the new superfusate pH. After the last recording, CN VII was detached from the suction electrodes and the rubber base and brainstem were removed from the recording chamber to be subsequently loaded with BCECF dye. It is important to note that at no time during any of the experiments was the brainstem preparation exposed to either the 440 or 495 nm wavelengths of light. By eliminating the exposure to these two excitatory wavelengths of light, we removed any potential effects of the light alone on respiratory rhythmicity and/or central respiratory chemoreception.

Post-Dye Neural Recordings

Immediately following the 30 minute aCSF wash, the brainstem was placed in the recording chamber where CN VII was reattached to the suction electrodes for subsequent recordings of respiratory motor output. The post-dye protocol began after the brainstem had been superfused with aCSF in the recording chamber for at least 60 minutes (as previously described for pre-dye recordings). Nerve activities were recorded from CN VII following the same normocapnic and hypercapnic protocol used for the pre-dye recordings

Analysis

In order to determine the effects of BCECF on CN VII gill and lung burst rhythmicity and central chemoreception, respiratory burst variables for pre-dye and post-dye brainstems were compared at pH 7.4 and 7.8 in froglets, and pH 7.2, 7.4, and 7.8 in tadpoles. Respiratory variables included gill and lung frequency (bursts per minute), amplitude (maximum height of burst), duration (onset to offset of burst), cycle (time period from the onset of one burst to the onset of the next burst), and interburst interval (time period from the offset of one burst to the onset of the next burst). Mean respiratory variables in response to dye and pH were compared using repeated measures analysis of variance (ANOVA). If data did not meet the assumptions of parametric statistical tests, a nonparametric Mann-Whitney Rank Sum Test was used. $P \leq 0.05$ was the criterion for significance. Unless otherwise stated, values reported are mean $\pm$ one standard error of the mean (SEM).

Dye Loading

BCECF-AM stock 0.004 M (Molecular probes, Eugene, OR) was made by dissolving 1 mg BCECF-AM in dimethyl sulfoxide (DMSO; 201 μL). We incubated the *in vitro* tadpole brainstem in 10 ml of 20, 40, or 60 μM solutions by diluting the BCECF stock solution in the tadpole aCSF. The tadpole brainstem was incubated in BCECF, prepared as described above, for 30 minutes in the dark. Following dye incubation, the brainstem was washed for 30 minutes in oxygenated aCSF with a pH of 7.8 attained by adding carbon dioxide to the aCSF .

Imaging of BCECF-Loaded Neurons

The superfusion chamber containing the brainstem was placed under an upright microscope (Eclipse E600FN Nikon, Melville, NY) mounted with a charge-coupled device (CCD) camera (MicroMAX, Roper Scientific, Trenton, NJ) connected to a Pentium computer (Hewlett Packard Company, Palo Alto, CA). Neurons were excited for 200 – 400 msec with light from a 175-W xenon arc lamp (Sutter

Instrument, Novato, CA) that was filtered to 495 or 440 nm using a high speed filter changer (Lambda DG-4, Sutter Instrument, Novato, CA). Emitted light passed through a dichroic mirror with a high pass cut off of 515 nm and a 535 ± 25 nm emission filter (Chroma Technology, Brattleboro, VT). To determine the percentage of cell loading in the brainstem was sectioned into 500 μm slices, and differential interference contrast (DIC) microscopy was used to capture an image of cells in the brainstem slices. The same brainstem section was then exposed to the 440 nm excitation wavelength, making visible all cell bodies that were loaded with BCECF. To avoid potential photobleaching of BCECF, we only exposed each section to enough light to capture one DIC image and one subsequent 440 nm excitation wavelength of light (200-400 msec). Data were collected with an image acquisition software program (MetaMorph/MetaFluor, Universal Imaging Corporation, Downingtown, PA) for offline analysis of cell counts for both DIC and BCECF fluorescence. We maintained bath pH = 7.8 and chose to excite the cells with 440 nm light as this wavelength is pH insensitive and serves as a control to assess cell membrane integrity. The percent loading was calculated for each brainstem at a given concentration of BCECF and then averaged to report a mean percent loading for each concentration of BCECF.

RESULTS

Dye Loading

An overlay of the DIC and BCECF fluorescence is shown in Fig. (**1**). Differential interference contrast and fluorescence microscopy were performed on 500 μm slices of the brainstem following incubation in 20, 40, or 60 μM concentrations of BCECF. The results of this dye-loading study indicated that the 20 μM (n = 6 tadpoles) concentration yielded significantly lower BCECF loading (386 fluorescent cells of 1161 total cells; 33.2% ± 10.7%) than the 40 μM (n = 8 tadpoles; 350 fluorescent cells of 420 total cells, 83.4% ± 13.6%) or 60 μM (n = 6 tadpoles; 840 fluorescent cells of 932 total cells; 90.1% ± 4.1%) concentrations, while there were no significant differences between the 40 μM and 60 μM concentrations (Fig. **2**). Between 83% and 90% of cells in each slice contained BCECF. This loading efficiency is similar to experiments in mammalian preparations that yield 89% to 90% loading with BCECF [28].

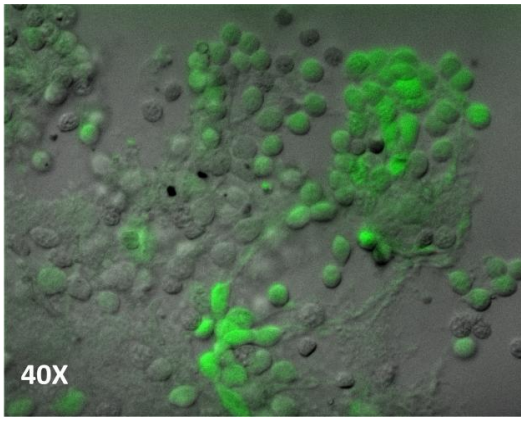

Fig. (1). Overlay image of DIC (gray) and 440 nm excitation (green) with 40 μM BCECF. Images were used to calculate BCECF percent loading.

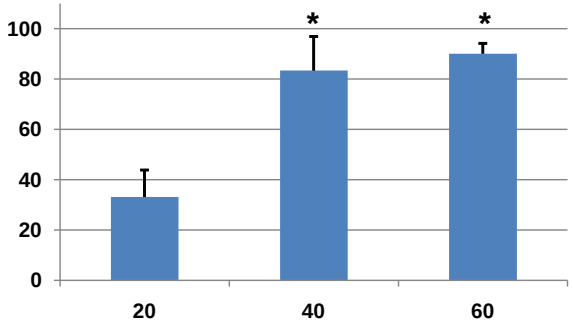

Fig. (2). The percentage of BCECF cell loading in the tadpole brainstem preparation as a function of the concentration of BCECF. The 40 μM (n = 420) and 60 μM (n = 932) concentrations yielded significantly greater (*) percent loading than the 20 μM (n = 1161) concentration, but were not significantly different from each other.

Effects of BCECF on Respiratory Rhythmogenesis and Central Respiratory Chemoreception

Ester hydrolysis produces the spontaneous release of formaldehyde. To ensure that BCECF concentrations used do not have toxic effects on ventral medullary cells that would affect respiratory chemosensitivity or rhythm, whole nerve respiratory responses were monitored during control and hypercapnic solutions.

Pre Dye Neural Recordings: Respiratory Response to CO_2

Mean gill and lung burst frequency in response to changes in bath pH before and after BCECF loading are summarized in Table **1**. Gill burst frequency during normocapnia (pH 7.8) was 39.2 ± 6.7 burst·min^{-1}, which was not significantly different from bath pH 7.4 (33.1 ± 5.4 burst·min^{-1}), but was greater than the gill burst frequency during hypercapnia (pH 7.2 and 25.6 ± 3.9 burst·min^{-1}). Gill interburst interval at bath pH 7.2 was 0.7 ± 0.1 sec^{-1}, which was significantly greater than the normocapnic gill interburst interval (0.3 ± 0.0 sec^{-1}) at bath pH of 7.8, was not different from the interburst interval at bath pH 7.4 (0.4 ± 0.1 sec^{-1}). There were no effects of bath pH on gill burst amplitude, cycle, or duration.

Lung burst frequency during normocapnia (bath pH 7.8) was 1.2 ± 0.3 burst·min^{-1}, which was not significantly different from the lung burst frequency (1.7 ± 0.5 burst·min^{-1}) when the bath pH was 7.4. Lung burst frequency (8.4 ± 1.7 burst·min^{-1}) was significantly greater during hypercapnia when the bath pH was 7.2 compared to normocapnia. There were no significant differences between normocapnia (pH 7.8) and hypercapnia (pH = 7.4) for lung burst duration (0.95 ± 0.11 sec vs. 0.97 ± 0.10 sec), interburst interval (6.1 ± 1.0 sec vs. 4.2 ± 0.8 sec), or cycle (7.0 ± 1.1 sec vs. 5.2 ± 0.9 sec). Lung burst amplitude during normocapnia was not significantly different from the lung burst amplitude during hypercapnia (amplitude during hypercapnia was 92.2 ± 6.1% of the normocapnic value).

Post Dye Neural Recordings: Respiratory Responses to CO_2 and BCECF

Gill and lung burst activities were retained in all brainstem preparations following incubation with 40 μM

Table 1. Gill and Lung Burst Frequency in Response to Changes in bath pH Prior to and after Loading with BCECF

Bath pH	Prior to BCECF		After BCECF	
	Gill frequency	Lung frequency	Gill frequency	Lung frequency
7.2	25.6 ± 3.9 (min^{-1})*	8.4 ± 1.7 (min^{-1})*	20.9 ± 1.8 (min^{-1})*	6.7 ± 1.8 (min^{-1})*
7.8	39.2 ± 6.7 (min^{-1})	1.2 ± 0.3 (min^{-1})	34.5 ± 3.9 (min^{-1})	1.3 ± 1.4 (min^{-1})

*Indicates significantly different from the corresponding 7.8 pH value.

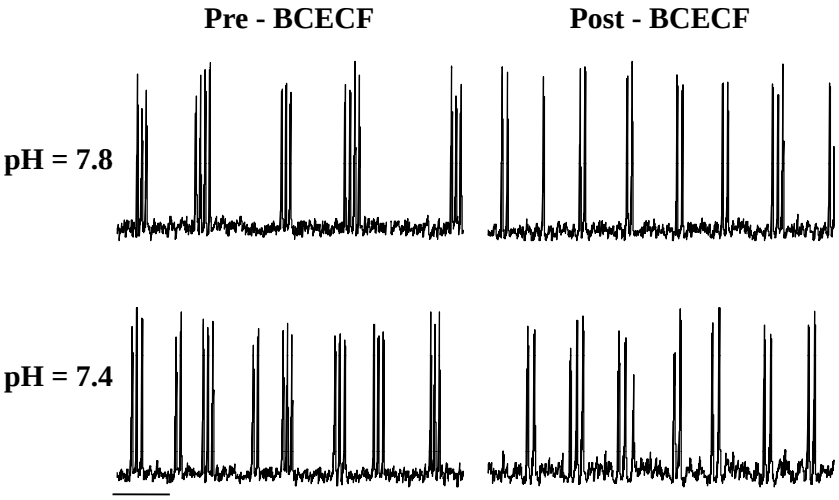

Fig. (3). Gill and lung burst activities recorded from cranial nerve VII at control (bath pH = 7.8) and hypercapnic acidosis (bath pH = 7.4) prior to BCECF (left column) and following 40 μM BCECF incubation (right column). Scale bar = 10 seconds.

BCECF, as illustrated in Fig. (**3**). Buccal burst frequency during normocapnia (34.5 ± 3.9 burst·min^{-1}) was not significantly different from the bath pH 7.4 condition (26.4 ± 3.6 burst·min^{-1}), but was significantly greater during hypercapnia at bath pH 7.2 (20.9 ± 1.8 burst·min^{-1}). Following BCECF incubation, buccal burst amplitude at bath pH 7.2 was significantly greater (133 ± 13.9% of the pH 7.8 burst amplitude) than burst amplitude at bath pH 7.8. The buccal burst amplitude at bath pH 7.4 (113.8 ± 5.4% of control) was not significantly different from bath pH 7.8. Buccal burst cycle length at bath pH 7.2 (2.8 ± 0.2 cycle·sec^{-1}) was significantly greater than buccal burst cycle at bath pH 7.8 (1.8 ± 0.2 cycle·sec^{-1}). The buccal burst amplitude at bath pH 7.4 was not significantly different from bath pH 7.8.

Lung burst frequency during normocapnia (1.3 ± 1.4 burst·min^{-1}) was not significantly different from the burst frequency at bath pH 7.4 (2.9 ± 0.5 burst·min^{-1}); lung burst frequency was significantly greater than the normocapnic value during hypercapnia (bath pH 7.2 and burst frequency 6.7 ± 1.8 burst·min^{-1}). Lung burst frequency during control and hypercapnia normalized to the control bath pH of 7.8 are illustrated in Fig. (**4**). There were no effects of bath pH on lung burst amplitude, cycle, interburst interval or duration.

At each bath pH condition, there were no significant differences in gill burst frequency or lung burst frequency prior to BCEFC and after BCECF loading. Gill burst frequency at bath pH 7.8 prior to BCECF (39.2 ± 6.7 (min^{-1}) was not significantly different from gill burst frequency at bath pH 7.8 after BCECF loading 34.5 ± 3.9 (min^{-1}). Similarly, lung burst frequency at bath pH 7.8 prior to BCECF 8.4 ± 1.7 (min^{-1}) was not significantly different from lung burst frequency at bath pH 7.8 after BCECF loading 6.7 ± 1.8 (min^{-1}).

DISCUSSION

The results of this study validate the use of the fluorescence pH indicator BCECF to record pHi optically in the *in vitro* tadpole brainstem preparation. In addition, the

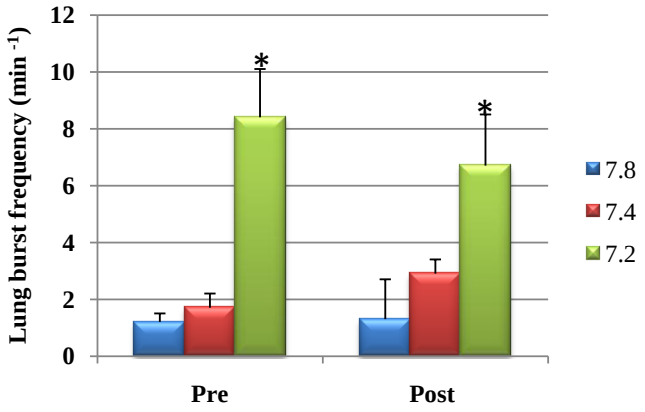

Fig. (4). Lung burst frequency (min^{-1}) during normocapnia (pH 7.8), hypercapnia (pH 7.4), and hypercapnia (pH 7.2) before (Pre) and after (Post) incubation with BCECF. There were significant (*) increases in lung frequency in response to decreases in bath pH before and after BCECF (n=10).

use of BCECF will permit further insight regarding the regulation of pHi in response to changes in extracellular carbon dioxide. The *in vitro* tadpole brainstem preparation offers a unique opportunity to investigate the neural development of respiratory rhythmogenesis and central respiratory chemoreception, as the synaptic connections of central respiratory pattern generators, central respiratory chemoreceptors, and respiratory motor neurons remain intact in this preparation throughout all developmental stages. To demonstrate the validity for using BCECF to record pHi in the tadpole brainstem preparation, we report four important findings. First, we were successful determining the percent loading of cells with BCECF across three concentrations of BCECF. The 40 μM BCECF solution loaded over 83% of cells in the tadpole preparation, similar to BCECF loading efficiencies in mammalian preparations, which range from 89% to 90% loading of BCECF [29]. Second, following the incubation period and washout, DIC and fluorescence microscopy indicate that BCECF undergoes cleavage of the –AM portion of the dye making it membrane impermeable, indicating that the tadpole brainstem neurons contain the appropriate intracellular esterases. Moreover, BCECF is a vital dye, and the presence of a high loading efficiency indicates that a large majority of cells in the tadpole brainstem preparation are viable. Third, the presence of BCECF within brainstem cells did not alter respiratory rhythmogenesis, as gill and lung burst activities were not significantly different before or after BCECF loading. Finally, we observed significant increases in lung burst frequency and decreases in gill burst frequency in response to central respiratory chemoreceptor stimulation before and after dye loading cells with BCECF. The increases in lung frequency and decreases in gill frequency in response to hypercapnia observed in this study are consistent with respiratory responses previously reported in the *in vitro* brainstem preparation [8-10, 23, 24, 26, 27, 30]. In addition, we demonstrated that loading over 83% of the brainstem cells with BCECF did not alter central respiratory chemoreceptor function, as the respiratory response to hypercapnia was retained following BCECF. More importantly, the magnitude of the gill and lung respiratory responses to hypercapnia was not different before and after BCECF. Additional studies in our laboratory indicate that BCECF loaded neurons retain pH sensitivity [31] and support the use of BCECF in the *in vitro* tadpole brainstem as a viable technique to optically monitor pHi and more importantly, changes in pHi as a measure of a neurons ability to regulate intracellular pH.

ACKNOWLEDGEMENTS

This work was supported by the National Centers for Research Resources (NCRR) Research Centers in Minority Institutions (RCMI) grant 5G12 RR013646-08 awarded to MJG. Technical assistance was provided by Blas Ortiz, and Vonnie Jackson.

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Received: May 06, 2009 Revised: June 08, 2009 Accepted: October 29, 2009