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Intracellular Acidosis and pH Regulation in Central Respiratory Chemoreceptors

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Abstract

Dysfunctions of brainstem regions responsible for central CO₂ chemoreception have been proposed as an underlying pathophysiology of Sudden Infant Death Syndrome (SIDS). We recorded respiratory motor output and intracellular pH (pHi) from chemosensitive neurons in an *in vitro* tadpole brainstem during normocapnia and hypercapnia. Flash photolysis of the H⁺ donor nitrobenzaldehyde was used to induce focal decreases in pHi alone. Hypercapnia and flash photolysis significantly decreased pHi from normocapnia. In addition, chemoreceptors did not regulate pHi during hypercapnia, but demonstrated significant pHi recovery when only pHi was reduced by flash photolysis. Respiration was stimulated by decreases in pHi (hypercapnia and flash photolysis) by decreases in burst cycle. These data represent our ability to load the brainstem with nitrobenzaldehyde without disrupting the respiration, to quantify changes in chemoreceptor pHi recovery, and to provide insights regarding mechanisms of human health conditions with racial/ethnic health disparities such as SIDS and Apnea of Prematurity (AOP).

Keywords

pHi; respiration; carbon dioxide; chemoreceptors

Although the rate of infant mortality in the United States has been steadily declining from 26.0 per 1,000 live births in 1960 to 6.9 per 1,000 live births in 2000,¹ the rate has shown no signs of significant improvement for more than a decade.² The United States ranked 30th in the world in infant mortality in 2005, behind many European countries, Australia, Canada, Hong Kong, Israel, Japan, New Zealand, and Singapore.³ Racial/ethnic disparities in infant mortality in the United States indicate that in 2005 the African American mortality rate (13.4 deaths per 1,000 live births) was more than twice the infant mortality rate of non-Hispanic Whites (5.6 deaths per 1,000 live births).⁴

In the United States, Sudden Infant Death Syndrome (SIDS), represents the third leading cause of infant death, following congenital malformations and low birthweight, and is the leading cause of postneonatal (28 days to one year) infant mortality.⁴ It is defined as the

sudden death of an infant less than one year of age that cannot be explained after a thorough investigation is conducted, including a complete autopsy, examination of the death scene, and review of the clinical history. Although the risk factors associated with an increased incidence of SIDS have been described,⁵ the ethnic health disparities associated with SIDS have yet to be explained. The incidence of SIDS in 2005 among U.S., non-Hispanic Whites was 55.4/100,000 live births; the SIDS rate in African Americans is 1.8 times greater (99.4/100,000 live births) and the American Indian/Alaskan Native SIDS rate (111.6/100,000 live births) is more than twice as great.⁴

In 1994, Filiano and Kinney⁶ delineated the Triple-Risk Model which addressed the pathophysiology of SIDS. According to their model, SIDS occurs when three risk factors are met: 1) vulnerability of the infant, 2) a critical developmental period, and 3) exogenous stressor, such as hypoxia or asphyxia.⁷ Vulnerability of the infant refers to an infant with an abnormality in an area of their brainstem that controls respiration, heart rate, temperature, and arousal from sleep. Central respiratory chemoreceptors, specialized neurons that detect changes in blood carbon dioxide, provide a very fast and robust increase in respiration in response to elevated CO₂.⁸⁻⁹ Acidification of the blood and extracellular space of the brainstem is detected by central respiratory chemoreceptor and is hypothesized to lead to an increase in ventilatory drive. Following intracellular acidification, non-chemosensory neurons regulate intracellular pH (pHi) primarily *via* the extrusion of H⁺, whereas neurons located in known chemosensory regions of the brain do not demonstrate regulation of pHi unless only pHi is decreased.¹⁰ The mechanisms by which chemoreceptors regulate pHi, and how changes in pHi correlate to respiration are not known. The development of central respiratory chemoreception function is of considerable interest, as the disruption in the normal development of central respiratory chemoreception has been implicated in the pathophysiology of both Sudden Infant Death Syndrome and Apnea of Prematurity (AOP). Both SIDS and AOP represent either the lack of, or immature development of, respiratory reflexes to hypoxia and hypercapnia.¹¹ Faulty central CO₂ chemoreception has been proposed as a mechanism that could promote sudden death.¹²⁻¹³ The defect in central chemosensitivity would lead to a failure to arouse and a lack of autoresuscitation. Shannon et al.¹⁴ reported dysfunctional CO₂ chemosensitivity during sleep in 11 near-miss SIDS infants, two of whom subsequently died of SIDS. Further support of the importance of functional central CO₂ chemosensitivity stems from the documented deficiencies in SIDS victims in the muscarinic cholinergic, kainite, and serotonergic systems in the ventral medulla in areas analogous to central chemosensitivity in mammals.^{12,15,16}

The overall goal of the current work is to identify the developmental properties and pHi regulation of central respiratory chemoreceptors using the *in vitro* tadpole brainstem preparation of the bullfrog tadpole, *Lithobates catesbeianus*. The brainstem preparation is well oxygenated¹⁷ and exhibits robust, spontaneous fictive breathing and possesses a developmentally dependent ventilatory response to central chemoreceptor stimulation. The *in vitro* tadpole brainstem has many technical advantages over similar reduced mammalian preparations, as synaptic connectivity of respiratory rhythm generators, central respiratory chemoreceptors, and motor neurons remain intact in this preparation. Similarities between respiratory control mechanisms in mammals and the tadpole include respiratory rhythm

generation^{18,19} ventilatory responses to CO₂,^{20–23} the importance of vagal feedback from pulmonary stretch receptors during development,²⁴ and modulation of respiration by serotonin,^{25–27} and adenosine.^{28–31} Torgerson et al.²⁰ and Taylor et al.³² described two discrete locations of chemosensitivity in the tadpole brainstem that were identified as locations that when exposed to focal hypercapnia elicited an increase in respiratory motor output. The two locations of chemosensitivity were both on the ventrolateral medulla, a rostral site adjacent to cranial nerve V and a caudal site located adjacent to cranial nerve X.

Central respiratory chemoreceptors typically remain acidic when exposed to hypercapnia; however, if just pHi is reduced these same chemoreceptor neurons regulate pHi primarily *via* extrusion of H⁺ using a sodium-hydrogen exchanger (NHE).¹⁰ We present data that illustrate our ability to focally reduce pHi in central respiratory chemoreceptors using flash photolysis to “uncage” H⁺ from nitrobenzaldehyde (NBA). We employed focal decreases in pHi while simultaneously recording chemoreceptor pHi and respiratory motor output. To our knowledge, the ability to correlate changes in chemoreceptor pHi, and respiratory motor output has not been performed in any other vertebrate model of respiratory control. We have the ability to demonstrate an association with changes in chemoreceptor properties and ventilation in a developmental fashion by utilizing the novel nature of the tadpole brainstem preparation and the experimental techniques in this work. The capacity to manipulate the developmental progression of an animal in which detailed neuroanatomical and neurophysiological studies can be conducted may provide insights into human problems such as Sudden Infant Death Syndrome, which may be an example of failed or improper developmental control of autonomic functions.^{33,34}

Methods

Animals

Experiments were performed on *Lithobates catesbeianus* tadpoles from developmental stages³⁵ 9–21 (n=5) 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

The brainstem was removed and prepared for electrophysiological recordings as described by Gdovin et al.³⁶ Throughout the dissection, the brainstem was continuously superfused with artificial cerebrospinal fluid (aCSF) bubbled with O₂. The composition of the aCSF was as follows (in mM): NaCl 104; KCl 4.0; MgCl₂ 1.4; D-glucose 10; NaHCO₃ 25; and CaCl₂ 2.4. Following dissection, the brainstem was incubated in 40 μM oxygenated 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester as described below (BCECF-AM; Molecular Probes, Eugene, OR). The brainstem was removed as described above then transferred to a superfusion recording chamber (SHD-26GKIT, Warner Instrument Corporation) and maintained at room temperature, 21–23°C. The brainstem was

superfused with aCSF that had been equilibrated with a CO₂/O₂ gas mixture (5% CO₂ and 95% O₂) 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 CO₂ bubbling into the tonometer. Our previous study indicates that the brainstem preparation dorsal-ventral thickness between cranial nerves V and X aspect was approximately .92 mm at the lateral margin and 1.32 mm in the midline.¹⁷

Whole nerve recordings

Neural recordings of fictive gill and lung ventilation were obtained from the roots of cranial nerve (CN) VII using a glass suction electrode. The suction electrode was made from thin walled borosilicate glass capillaries (OD 1 mm; ID 0.5 mm), and 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 μ m). 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), digitized and recorded on a Pentium 4 PC (Datapac 2000 software). The tadpole brainstem preparation exhibits two distinct patterns of rhythmic burst activities, characterized as either a high-frequency, low-amplitude bursts lacking corresponding activity in spinal nerve II, or a low-frequency, high-amplitude bursts with coincident bursts in spinal nerve II.³⁷ These two bursting patterns corresponded to gill and lung ventilatory burst patterns, respectively, recorded from cranial nerve V and VII and spinal nerve II in the decerebrate, spontaneously breathing tadpoles.³⁸

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 120 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 $\pm$ 25 nm emission filter (Chroma Technology, Brattleboro, VT). Data were collected with an image acquisition software program (MetaMorph/MetaFluor, Universal Imaging Corporation, Downingtown, PA) for offline analysis of BCECF fluorescence. We previously identified glial cells using both glial fibrillary acid protein (GFAP) and a high potassium functional test to confirm the depolarization-induced alkalinization (unpublished data). The combination of the functional test and cell morphology using GFAP allowed us to use two mechanisms to discriminate glial cells from neurons. We confirmed that glial cells were significantly smaller in size than neurons and used these criteria to identify candidate chemoreceptor neurons in this study. In addition, our pHi recordings were obtained from

cells in a known chemosensitive region and the corresponding pHi profiles were characteristic of chemosensitive neurons.

Calibration of BCECF

The ratio of BCECF fluorescence emitted after excitation at 495 and 440 nm was used to calibrate pHi, as described by Boyarsky et al.^{39,40} Briefly, a calibration curve of pHi as a function of normalized fluorescence ratios was generated from neurons perfused with solutions of known pH ranging from 6.5 to 8.5. Through this range of pH values, pHi was measured after equilibration of pHe and pHi using the high K⁺/nigericin technique.⁴¹ The calibration of di-8-ANEPPS fluorescence ratios was performed using the high K⁺ solution protocol previously described by Nottingham et al.¹⁰

Recording of pHi, respiration, and NBA loading

When the brainstem was first placed in the recording chamber it was superfused with aCSF for 60 min to ensure normal respiratory rhythm at hypercapnic bath pH of 7.30 to 7.50. When hypercapnia steady state was achieved, neural recordings of respiratory motor output and optical recordings of pHi were taken for 10 minutes. Following hypercapnic recordings, the bath P_{CO2} was decreased to attain a normocapnic bath pH of 7.80, the plasma pH of anuran amphibians as described by West et al.⁴² Once a steady respiratory rhythm had been achieved a respiratory rhythm recording was then taken for a duration of 10 minutes. The aCSF was then removed and replaced with a 10 μ M concentration of nitrobenzaldehyde (NBA) to aCSF which was superfused for 10 minutes. The NBA solution was removed from the tonometer and replaced with the aCSF thus leaving only NBA within the intracellular space and only aCSF in the extracellular. In order to induce dissociation of intracellular NBA and H⁺, the cells were exposed to 20 seconds of ultraviolet (UV) flash at a wavelength of 351 nm. Optical recordings of pHi were then taken every 30 seconds for up to 40 minutes following the flash photolysis of NBA.

Analysis

In order to determine the effects of NBA on CN VII gill and lung burst rhythmicity and central chemoreception, respiratory burst variables were recorded at hypercapnic acidosis, normocapnia, and normocapnia with UV flash. Respiratory variables included gill and lung burst cycle (the time from the onset of the burst to the onset of the subsequent burst), amplitude (the maximum height of burst reported in arbitrary units, au), burst duration (the time from the onset of the burst to the offset of the same burst), and interburst interval (the time period from the offset of one burst to the onset of the next burst). Mean respiratory variables in response to changes in 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<.05 was the criterion for significance. The mean changes in pHi (pHi) from normocapnia to hypercapnia, and from normocapnia to UV flash were calculated. In addition, linear regression was performed on pHi values after UV flash to quantify regulation of pHi.⁴³ Unless otherwise stated, values reported are mean $\pm$ one standard error of the mean (SEM). Following the recordings of pHi in response to flash photolysis, we recorded pHi ratios while the brainstem was exposed to

an aCSF solution containing nigericin titrated to pH 7.20 in order to calibrate recorded pHi values.

Results

Changes in pHi in response to hypercapnia and uncaging of H⁺ using flash photolysis

We optically recorded pHi from neurons in chemosensitive regions in the tadpole brainstem (n=17) during normocapnia, hypercapnia, and with focal decreases in pHi attained by uncaging H⁺ from nitrobenzaldehyde. Mean pHi from chemosensitive neurons during normocapnia (7.94 ± 0.07) was significantly greater than mean pHi during hypercapnia (7.24 ± 0.03 ; $P < .001$). Following flash photolysis with UV light, we were able successfully to induce a focal intracellular acidification that was significantly lower than normocapnia (7.05 ± 0.03 ; $P < .001$). Changes in pHi during hypercapnia and in response to flash photolysis were normalized as a change in pHi (Δ pHi) from the pHi during normocapnia. The mean Δ pHi from normocapnia to hypercapnia (0.69 ± 0.07 pH units) was not significantly different ($P = .101$) from the mean Δ pHi from normocapnia to focal intracellular acidosis in response to uncaging of H⁺ (0.87 ± 0.10 pH units). Figure 1 illustrates the pHi profile of three neurons located in known chemosensitive regions during hypercapnia, normocapnia, and during decreases in pHi alone *via* the uncaging of H⁺ with UV light.

pHi recovery

pHi did not significantly change during hypercapnia and normocapnia; however 15/17 neurons demonstrated significant pHi recovery ($P < 0.001$) following reductions in pHi attained by uncaging H⁺ with flash photolysis. The lack of pHi recovery during hypercapnia and the evidence of pHi recovery during flash photolysis can be observed in the pHi profiles of three neurons illustrated in Figure 1.

Effects of changes in pHi on gill respiration

We observed significant effects of changes in pHi on respiration, specifically in gill and lung burst cycle, and lung burst interburst interval. Gill burst cycle during hypercapnia (1.54 ± 0.25 sec) was significantly reduced to $80.75 \pm 10.97\%$ of the gill burst cycle at normocapnia (2.11 ± 0.36 sec), as illustrated in Figure 2. Similarly, gill burst cycle during UV flash (1.44 ± 0.18) was significantly reduced to $76.17 \pm 8.53\%$ of gill burst cycle during normocapnia. There were no significant changes in gill burst duration during hypercapnia (1.32 ± 0.24 sec) or UV flash (1.08 ± 0.18 sec) when compared with gill burst duration during normocapnia (1.38 ± 0.23 sec). There were no significant changes in gill burst interburst interval during hypercapnia (0.23 ± 0.03 sec) or UV flash (0.36 ± 0.10 sec) when compared with gill interburst interval during normocapnia (0.73 ± 0.26 sec). There were no significant changes in gill burst amplitude during hypercapnia (0.15 ± 0.02 au) or UV flash (0.13 ± 0.01 au) when compared with gill burst amplitude during normocapnia (0.15 ± 0.02 au).

Effects of changes in pHi on lung respiration

We observed significant effects of changes in pHi on lung burst activities, specifically in lung burst cycle and IBI (Figure 3). Lung burst cycle during hypercapnia (2.80 ± 0.76 sec) was significantly reduced to $25.40 \pm 10.60\%$ of the lung burst cycle at normocapnia

(18.47 ± 3.77 sec). Lung burst cycle during UV flash (19.98 ± 4.13) was not significantly different from lung burst cycle during normocapnia. There were no significant changes in lung burst duration during hypercapnia (1.39 ± 0.17 sec) or UV flash (1.30 ± 0.12 sec) when compared with lung burst duration during normocapnia (1.17 ± 0.06 sec). There were no significant changes in lung interburst interval during UV flash (18.67 ± 4.01 sec) when compared with lung interburst interval during normocapnia (0.73 ± 0.26 sec), however lung interburst interval during hypercapnia (1.44 ± 0.51 sec) was significantly reduced to $16.23 \pm 14.50\%$ of the normocapnic lung interburst interval. There were no significant changes in lung burst amplitude during hypercapnia (0.36 ± 0.06 au) or UV flash (0.29 ± 0.06 au) when compared with lung burst amplitude during normocapnia (0.32 ± 0.05 au).

Discussion

Sudden Infant Death Syndrome (SIDS) is disorder with demonstrated racial/ethnic disparities in the U.S. involving the incomplete development of the neural components of respiratory circuitry. Critical gaps in the field of the neural development of respiratory chemoreception involve detailed understanding of the development of the cellular mechanisms of central respiratory chemoreceptor function and how changes in central chemoreceptors correlate to changes in respiration. We developed a novel approach to characterize central CO_2 chemoreceptors in an animal model well-suited to examine the neural development of respiration, the *in vitro* tadpole brainstem of *Lithobates catesbeianus*. We employed electrophysiology and fluorescence microscopy to perform simultaneous recordings of spontaneous respiratory motor output and pHi from neurons located in known chemosensitive regions and report four significant findings: 1) it is possible to load NBA and BCECF without disruption of respiratory rhythmogenesis and central respiratory chemoreception, 2) we can employ flash photolysis to induce decreases in pHi within physiological limits, 3) it is possible optically to record pHi during changes in pHe and pHi while quantifying pHi regulation, and 4) central respiratory chemoreceptors demonstrate pHi recovery when only pHi is reduced.

The ratiometric pH-sensitive fluorophore BCECF has been used optically to measure intracellular pH.^{43–46} We previously demonstrated that BCECF could be used to label brainstem neurons of the tadpole without the loss of cell membrane integrity while retaining respiratory rhythmicity or central respiratory chemosensitivity.³⁶ This study confirms the ability to record dynamic changes in pHi using BCECF and incorporates the use of a novel technique to induce decreases in pHi by using flash photolysis to “uncage” H^+ from NBA. The ability to perform simultaneous recordings of spontaneous whole nerve respiratory motor output and pHi while manipulating both extracellular pH (pHe) and pHi represents a significant accomplishment in the field of central chemoreceptor function in vertebrates. Hypercapnic acidosis elicits decreases in both pHe and pHi whereas flash photolysis permits us to decrease pHi while holding pHe at normocapnic conditions. With the exception of lung burst cycle and interburst interval, gill and lung burst activities demonstrated hypercapnic responses that were not significantly different from the decreases in pHi associated with flash photolysis of NBA.

The development of flash photolysis of NBA in the tadpole model represents a novel tool to provide a missing “disconnect” from changes in pHi in chemoreceptor to ventilatory responses. Previously, the ammonium chloride prepulse technique has been successfully used to induce decreases in pHi alone,¹⁰ and has been successful in characterizing central chemoreceptor regulation. Our laboratory attempted to use the ammonium chloride prepulse method, however we were unable to induce focal intracellular acidification without disruption of central respiratory motor output. The use of flash photolysis of NBA reported in this study stems from our desire to induce discrete, focal decrease in pHi without disruption of respiratory rhythmogenesis or central chemosensitivity. We were successful in developing a flash photolysis protocol using NBA that allows us to induce decreases in pHi that are similar in magnitude to the decreases in pHi attained with bath applied CO₂. The reductions in pHi associated with the uncaging of H⁺ from NBA are brought to physiological pHi values associated with hypercapnia within 30 seconds of UV light exposure without disruptions to central respiratory rhythmogenesis or chemosensitivity.

We optically recorded pHi in central respiratory chemoreceptors every 30 seconds for up to one hour with no degradation of the BCECF emitted fluorescence. More importantly, we report that central respiratory chemoreceptors in the tadpole do not regulate pHi during hypercapnic acidosis, however the same chemoreceptors will demonstrate significant pHi recovery during decreases in pHi alone. Our finding that tadpole chemoreceptors do not regulate pHi when both pHe and pHi are reduced during hypercapnic acidosis, but show significant pHi regulation when only pHi is reduced represents yet another similarity between the tadpole brainstem preparation to mammals.¹⁰ Like mammals, the stimulus for pHi recovery in tadpoles appears to involve the detection of both pHi and pHe and may represent a chemosensory system that arose with the evolution of terrestriality and remained conserved in air breathing mammals. The use of flash photolysis using NBA will permit us to investigate further the mechanisms of pHi recovery in the tadpole. Central respiratory chemoreceptors in mammals accomplish regulation of pHi via the Na⁺/H⁺ exchanger (NHE) or the Na⁺-driven Cl⁻/HCO₃⁻ exchanger (NDCBE)¹⁰. Recovery of pHi in the tadpole following reductions of pHi alone appear to be driven by an amiloride-sensitive NHE (unpublished data), representing further similarities between the tadpole and mammal chemoreceptor function.

In summary, we provide data that demonstrate an advance in the field of the neural control of respiratory chemoreceptor function. The combination of a novel approach to reduce pHi without disruption of respiration in the tadpole brainstem preparation is well-suited to address two critical gaps in our knowledge of central respiratory chemoreceptor function: 1) connecting the changes in cellular events in central respiratory chemoreceptors to changes in respiration, and 2) using an animal model well-suited to describe the developmental properties of central respiratory chemoreception and their role in respiratory motor output.

Notes

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Figure 1.

pHi profiles from three representative neurons located in chemosensitive regions from a developmental stage 11 tadpole during hypercapnia, normocapnia, and flash photolysis of UV light (downward arrow). pHi during hypercapnia was significantly lower than during normocapnia and the decreases in pHi attained by uncaging H^+ were significantly lower than pHi during normocapnia. Note that during hypercapnia the pHi shows no sign of recovery, as both intracellular and extracellular pH are reduced, however when only pHi is reduced using flash photolysis all three neurons demonstrate significant regulation of pHi.

Figure 2.

Mean changes in gill burst respiratory variables during hypercapnic acidosis (HA) and UV flash photolysis (UV) normalized to normocapnia (NC).

n=5

*indicates slightly different from normocapnia

Figure 3.

Mean changes in lung burst respiratory variables during hypercapnic acidosis (HA) and UV flash photolysis (UV) normalized to normocapnia (NC).

n=5

*indicates slightly different from normocapnia