

Role of intracellular Ca^{2+} and pH in CO_2/pH chemosensitivity
in neuroepithelial cells of the zebrafish (*Danio rerio*) gill
filament

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
Δ	Delta
μM	Micromolar
$[\text{Ca}^{2+}]_i$	Intracellular Ca^{2+} concentration
5-HT	5-hydroxytryptamine or serotonin
ACh	Acetylcholine
ACTZ	Acetazolamide
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
BCECF	2',7'-bis(2carboxyethyl)-5-(and-6)-carboxygluorescein
CA	Carbonic anhydrase
Ca^{2+}	Calcium
Ca_2Cl	Calcium chloride
CB	Carotid body
CCAC	Canadian Council on Animal Care
Cd^{2+}	Cadmium
CO_2	Carbon dioxide
CO	Carbon monoxide
CSN	Carotid sinus nerve
DMSO	Dimethyl sulfoxide
EDTA	Ethylenedinitrietetraacetic acid
f_H	Bradycardia
FCS	Fetal calf serum
H^+	Proton
h	Hour
H_2O	Water
HCl	Hydrochloric acid
HCO_3	Bicarbonate
IP_3	Inositol trisphosphate
K^+	Potassium
K_{ir}	Inwardly rectifying K^+ channel
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
L	Litre
Mg_2Cl	Magnesium chloride
min	Minute
mM	Millimolar
MS222	Ehly 3-aminobenzoate methanesulfonate salt
NaCl	Sodium chloride
Na_2HPO_4	Sodium phosphate dibasic
NECs	Neuroepithelial cells
NH_4^+	Ammonia
Ni^{2+}	Nickel

nm	Nanometers
NR	Neutral red
O ₂	Oxygen
P _a CO ₂	Arterial PCO ₂
PASMC	Pulmonary arterial smooth muscle cells
PBS	Phosphate-buffered saline
pH _i	Intracellular pH
pH _o	Extracellular pH
P _w CO ₂	Water PCO ₂
s	seconds
S.E.M	Standard error of the mean
TASK	Two-pore acid sensing K ⁺ channel
TRP	Transient receptor potential
V _{amp}	Ventilation amplitude
V _f	Ventilation frequency

ABSTRACT

Neuroepithelial cells (NECs) of the zebrafish gill filament have been previously identified as bimodal O_2 and CO_2/H^+ sensors that depolarize in response to chemostimuli via inhibition of background K^+ channels. To further elucidate the signaling pathway underlying CO_2/H^+ chemoreception in the NECs we employed microspectrofluorometric techniques to examine the effects of hypercapnia on $[Ca^{2+}]_i$ and pH_i . NECs increased their $[Ca^{2+}]_i$ in response to acidic hypercapnia (5% CO_2 , pH 6.6) and isocapnic acidosis (normocapnia, pH 6.6), but not to isohydric hypercapnia (5% CO_2 , pH 7.8). The acid-induced increase in $[Ca^{2+}]_i$ persisted in the absence of extracellular Ca^{2+} , and Ca^{2+} channel blockers (Cd^{2+} , Ni^{2+} and nifedipine). NECs exhibited a rapid and reversible drop in pH_i in response to acidic hypercapnia and isohydric hypercapnia. Isocapnic acidosis also induced intracellular acidification within NECs, but it was less severe than the drop in pH_i elicited by acidic hypercapnia and isohydric hypercapnia. The rate and magnitude of intracellular acidification was reduced by the CA-inhibitor, acetazolamide, without effect on the acid-induced increase in $[Ca^{2+}]_i$. Acetate was used to investigate the relationship between pH_i and $[Ca^{2+}]_i$. Acetate induced intracellular acidification without augmentation of $[Ca^{2+}]_i$. The results of this thesis demonstrate that (1) extracellular acidification, but not CO_2 , is critical to the hypercapnia-induced increase in $[Ca^{2+}]_i$; (2) the increase in $[Ca^{2+}]_i$ is independent of the drop in pH_i ; (3) the increase in $[Ca^{2+}]_i$ is not mediated by the influx of Ca^{2+} across the plasma membrane.

RÉSUMÉ

Les cellules neuroépithéliales (CNEs) des branchies des poissons zèbres ont été précédemment identifiées comme senseurs bimodaux à l'O₂ et le CO₂ et se dépolarisent via l'inhibition de leurs canaux potassiques de fond en réponse à des stimulus chimiques. Afin d'élucider le parcours de signalisation qui mène à la chimioréception du CO₂ dans les CNEs, nous avons utilisé des techniques de microspectrofluorométrie pour examiner les effets de l'hypercapnie sur [Ca²⁺]_i et le pH_i. Les CNEs ont augmenté leur [Ca²⁺]_i en réponse à de l'hypercapnie acide (5% CO₂, pH 6.6) et de l'acidose isocapnique (normocapnie, pH 6.6), mais n'ont pas eu de réaction face à de l'hypercapnie isohydrique (5% CO₂, pH 7.8). Cette augmentation de [Ca²⁺]_i causé par l'acide a persisté en présence d'une solution sans-Ca²⁺ et en présence d'une variété d'inhibiteurs de canaux de Ca²⁺ (Cd²⁺, Ni²⁺ and nifedipine). Les CNEs ont démontré une chute rapide et réversible de pH_i en réponse aux hypercapnies acide et isohydrique. L'acidose isocapnique a aussi induit une acidification intracellulaire dans les CNEs, cependant cette chute de pH_i n'était pas comparable à celles obtenues dans les traitements d'hypercapnies acide et isohydrique. Le taux et l'ampleur de l'acidification intracellulaire ont été réduits par l'inhibiteur-CA, acetazolamide, sans affecter l'augmentation de [Ca²⁺]_i causé par l'acide. L'acétate a été utilisé pour examiner la relation entre le pH_i et [Ca²⁺]_i. L'Acétate a induit une acidification intracellulaire sans augmenter [Ca²⁺]_i. Les résultats de cette thèse démontrent que (1) l'acidification extracellulaire est critique afin d'obtenir l'augmentation de la [Ca²⁺]_i induit par l'hypercapnie, (2) l'augmentation de [Ca²⁺]_i est indépendante de la chute de pH_i, (3) l'augmentation de [Ca²⁺]_i n'est pas facilité par l'afflux de Ca²⁺ à travers la membrane plasmique.

1. GENERAL INTRODUCTION

When confronted with hypercapnia (elevated levels of CO₂), vertebrates typically exhibit a suite of cardioventilatory responses aimed at maintaining homeostasis. The majority of studies examining the physiological responses to hypercapnia have been performed on mammals, reflecting the long held notion that peripheral CO₂ sensing in vertebrates first evolved in air breathers. More recently, however, it has become increasingly apparent that CO₂ sensing is present in water-breathing vertebrates, as is evident by the cardioventilatory responses evoked in fish in response to hypercapnia.

In order to initiate these CO₂-mediated cardiorespiratory adjustments, vertebrates have respiratory chemoreceptors that are capable of detecting changes in their environment. Within mammals, type I cells of the carotid body are the principle peripheral chemoreceptors (Gonzalez *et al.*, 1994; Lopez-Barneo, 2008; Nurse, 2010). In fish, they have been identified as the neuroepithelial cells (NECs) of the gill filament (Jonz *et al.*, 2004; Qin *et al.*, 2010). Both type 1 cells and NECs have been characterized as bimodal O₂ and CO₂ sensors. In fact, the fish gill, and particularly the first gill arch is believed to be the evolutionary precursor of the carotid body (Milsom and Burleson, 2007; Jonz and Nurse, 2009).

The purpose of this introduction is to provide a review of the cardiorespiratory consequences of hypercapnia in the fish species examined to date, the proposed benefits of such reflexes, and the mechanisms by which they are induced. The peripheral chemoreceptors (type I cells and NECs) will be discussed in detail, and the current understanding of the chemotransduction cascade initiated by hypercapnia will be provided. It should be noted that the focus of this study is on the effects of hypercapnia,

and not hypoxia, on chemosensing in the fish. As such, the information provided herein will focus almost exclusively on the physiological effects of hypercapnia.

1.1 Cardiorespiratory effects of CO₂

1.1.1 Cardiovascular effects

Within the limited number of species examined to date, the cardiovascular responses to hypercapnia are highly species-specific. With few exceptions, a reduction in cardiac frequency (f_H ; bradycardia) appears to be the most profound cardiac reflex in response to hypercapnia in several species, such as Spiny dogfish (Mckendry *et al.*, 2001; Perry and McKendry, 2001), rainbow trout (Perry *et al.*, 1999; Mckendry and Perry, 2001), Atlantic salmon (Perry and McKendry, 2001), Pacific sandab (in Gilmour and Perry, 2006), channel catfish (Burlison and Smatresk, 2000), traíra (Reid *et al.*, 2000), tambaqui (Sundin *et al.*, 2000) and jeju (Boikink *et al.*, 2010). Several other species, including the white surgeon (Crocker *et al.*, 2000) and the larval zebrafish (in Perry and Abdallah, 2010), display marked tachycardia. Of the remaining species investigated to date, the eel (in Gilmour and Perry, 2006), bullhead (in Gilmour and Perry, 2006), and goldfish (Tzaneva *et al.*, 2011) lack a pronounced cardiovascular response. The source of interspecific variation in the cardiac responses to hypercapnia remains unresolved and may reflect a blunted sensitivity to CO₂ among species and/or differing thresholds for stimulating the chemoreceptors responsible for the cardiovascular response across species.

As with heart rate, the effects of hypercapnia on arterial blood pressure, vascular resistance and cardiac output are species-specific. Hypercapnia in the Pacific sandab (in Gilmour and Perry, 2002), eel (in Gilmour and Perry, 2002) and channel catfish

(Burlleson and Smatresk, 2000) was without effect on arterial blood pressure. In the spiny dogfish (McKendry *et al.*, 2001; Perry and McKendry, 2001) and traira (Reid *et al.*, 2000) it produced hypotension, and in the remainder of the species studied to date, i.e. the rainbow trout (Perry *et al.*, 1999; McKendry and Perry, 2001), Atlantic salmon (Perry and McKendry, 2001), white sturgeon (Crocker *et al.*, 2000) and jeju (Boijink *et al.*, 2010) it resulted in hypertension. The mechanism by which hypercapnia affects arterial blood pressure is through its combined impact on cardiac output and systemic vascular resistance (see review by Gilmour and Perry, 2006). Hypercapnia-induced hypertension is commonly attributed to an increase in vascular resistance; whereas hypercapnia induced hypotension is associated with a reduction in vascular resistance (see reviews by Gilmour and Perry, 2006; Milsom, 2012). As with cardiac frequency and blood pressure, however, such changes in vascular resistance are not always so unambiguous. In the sturgeon, for example, hypercapnia produces hypertension despite a decrease in vascular resistance (Crocker *et al.*, 2000, see review by Milsom, 2010).

1.1.2 Ventilatory effects

The most frequently reported ventilatory response to hypercapnia is hyperventilation that may be attributed to an increase in ventilation amplitude (V_{amp}) and/or frequency (V_f). The magnitude of the hyperventilatory response is species-specific, with some fish displaying relative insensitivity, e.g. carp (Soncini and Glass, 2000), goldfish (Tzaneva *et al.*, 2011) and eel (in Gilmour, 2001), and others exhibiting pronounced elevations in V_{amp} (e.g. zebrafish; Vulsevic *et al.*, 2006), V_f (e.g. carp; Soncini and Glass, 2000; McKendry, 2000) or both (spiny dogfish, Perry and Gilmour, 1996; spotted dogfish, McKendry, 2000; Atlantic skate, Graham et al., 1990; rainbow

trout, Thomas *et al.*, 1983, Gilmour and Perry, 1994; Pacific sanddab, McKendry, 2000; Atlantic salmon, McKendry, 2000; traira, Reid *et al.*, 2000; channel catfish, Burleson and Smatresk, 2000; and jeju, Boijink *et al.*, 2010). The significance of increasing V_{amp} versus V_f remains unclear, and it may simply reflect the variability in (1) the sensitivity for CO_2 sensing across species or (2) the variability in the CO_2 levels selected for experimental investigation (see Gilmour, 2001).

1.1.3 Physiological benefits of the cardiorespiratory responses to hypercapnia

The physiological benefits of the hypercapnia-induced cardioventilatory responses within fish have been questioned for decades (Dejours, 1973). In fact, it was originally believed that the hypercapnia-induced hypoxemia, as a consequence of the Root and Bohr effects, was solely responsible for mediating the cardioventilatory responses by indirectly triggering peripheral O_2 -sensitive chemoreceptors (see review by Randall, 1982). By examining the cardioventilatory responses to hypercapnia in the presence of hyperoxia (Smith and Jones, 1982; Burleson and Smatresk, 2000) and in fish lacking the Root and Bohr effects (reviewed by Gilmour, 2001), the view has shifted gradually to include a role for CO_2 , independent of blood O_2 status, in the hypercapnia-induced cardioventilatory responses.

Based on theory, it has been proposed (Randall and Daxboeck, 1984), albeit with little conclusive experimental evidence, that bradycardia would increase the diffusion capacity and augment gas transfer at the gill. As discussed in a review by Gilmour and Perry (2006), this is due to:

- (1) lamellar recruitment (increased functional surface area) as a result of (i) increased pulsatility and (ii) an elevation of mean ventral aortic pressure or a rise in systolic pressure
- (2) reduced blood-to-water diffusion distance as a consequence of an elevation of ventral aortic pressure

Similarly, the hypercapnia-induced hyperventilation has also been proposed to play a beneficial role in maintaining homeostasis by:

- (1) minimizing the extent of the associated acidosis, primarily owing to the log-linear relationship between PCO_2 and pH
- (2) enhancing O_2 uptake and increasing arterial PO_2 by increasing perfusion of the gills
- (3) acting as an early “warning system” in the face of ensuing hypoxia (given that hypoxia and hypercapnia usually occur at the same time)

1.1.4 Are fish responding to CO_2 or pH

Aquatic hypercapnia is associated with an increase in water PCO_2 ($P_w\text{CO}_2$) and a decrease in water pH; and exposure to hypercapnia results in an elevation in arterial PCO_2 ($P_a\text{CO}_2$) together with a depression in arterial pH across all species of fish examined to date (see Table 2 in Gilmour, 2001). Consequently, the proximate stimulus responsible for triggering the cardioventilatory responses may be the change in $P_w\text{CO}_2$, $P_a\text{CO}_2$, water pH, blood pH, or any combination of these variables. Because the fish gill has been identified as the principle site for CO_2 sensing (see below), the chemoreceptors mediating the cardioventilatory responses are therefore externally oriented to sense changes in water CO_2/pH , internally oriented to sense changes in blood CO_2/pH , or both

externally and internally oriented. With the exception of Qin *et al.* (2010; see below), the available evidence for internally versus externally oriented CO₂ versus pH chemoreceptors in the cardioventilatory responses is based on indirect approaches (i.e. by monitoring the cardioventilatory responses after changing water or blood CO₂ and/or pH levels).

Of the species examined to date, the cardiovascular responses to hypercapnic stimuli seem to be dominated by externally oriented branchial receptors that respond to changes in water CO₂ and not to the accompanying changes in water pH (see reviews by Gilmour and Perry, 2006; Milsom, 2012). Similarly, the bulk of evidence suggests that the ventilatory responses to hypercapnia are mediated by externally oriented CO₂ sensing chemoreceptors. In fact, ventilatory responses to environmental acidosis in the absence of elevated P_wCO₂ are nonexistent in the literature (see review by Gilmour, 2001). The potential contribution of internally oriented receptors in sensing P_aCO₂ and blood pH changes, however, has not been excluded. The case for internally oriented receptors has been argued on the basis of the sustained ventilatory responses following exhaustive exercise. Post-exercise acidosis, and to a lesser extent, post-exercise elevations in P_aCO₂, have been proposed to be stimulating internally oriented receptors ultimately triggering the post-exercise ventilatory responses (Graham *et al.*, 1990; Wood *et al.*, 1990; Wood and Munger, 1994).

1.2 Chemoreceptors

In vertebrates, a chemoreceptive reflex must be present in order to trigger a hypercapnia-induced cardioventilatory response. Typically:

- (1) the signal (CO_2/pH) is detected by specialized sensory elements, namely chemoreceptors, and subsequently transmitted to the
- (2) respiratory control center of the central nervous system where the signal is integrated and processed leading to changes in the
- (3) efferent nerve activity to effector organs which modulate cardioventilatory function (as discussed above)

Owing to the high capacitance of water for CO_2 relative to O_2 , water-breathing fish maintain P_aCO_2 levels ($\sim 2\text{-}3$ mmHg) that are an order of a magnitude lower than those exhibited by mammals ($\sim 30\text{-}40$ mmHg). Consequently, the hypercapnic signal (whether it be CO_2 or pH) that is detected by the chemosensitive cells of the fish, unlike mammals, must be attuned to sense very small deviations within their environment (whether it be internal or external). The chemosensitive receptors mediating the hypercapnic chemotransduction cascade in fish are considered to be reminiscent of the peripheral chemoreceptors in mammals. Therefore, hypercapnic chemosensing is now thought to have evolved from water-to air breathers (see review by Milsom, 2002).

Little is known about the central pathways through which CO_2/pH sensory information is processed. In mammals, respiratory centers are localized to the surface of the ventral medulla, and extend into several other sites throughout the brainstem and the cerebellum (Nattie, 1999; Nattie and Li, 2006). In fish, the respiratory rhythm generators responsible for coordinating rhythmic breathing movements are also located in the medulla (Fritsche and Nilson, 1993). In both mammals and fish, the excitatory amino acid glutamate has been implicated as a neurotransmitter at the afferent terminals exerting

its effects on NMDA receptors leading to the central processing of sensory input from peripheral chemoreceptors (see review by Gilmour and Perry, 2006).

1.2.1 Central versus peripheral chemoreceptors

As alluded to earlier, the function of chemosensitive cells is to detect changes in environmental and/or blood gas tensions. Within mammals and (possibly) within fish there exist two distinct populations of chemosensitive cells: (1) central chemoreceptors and (2) peripheral chemoreceptors.

- (1) Central chemoreceptors; neurons located within various regions of the brainstem and as described by Putnam *et al.* (2004) they (i) should be intrinsically responsive to changes in O₂ and CO₂/pH, (ii) possess axonal projections to the respiratory control center and (iii) alter cardioventilatory responses when stimulated by changing levels of O₂, CO₂/pH
- (2) Peripheral chemoreceptors include, but are not limited to (i) type 1 cells (glomus cells) of the carotid body (CB) in mammals, which are responsive to altered O₂ and CO₂/pH, are innervated by the carotid sinus nerve and serve to control ventilation (Nurse, 2010) (ii) neuroepithelial bodies (NEB) within the mammalian lungs that are responsive to changes in O₂ (Cutz and Jackson, 1999; Domnik and Cutz, 2011) and (iii) neuroepithelial cells (NECs) within the fish gill which are highly innervated, and respond to changes in O₂ and CO₂/pH (Jonz and Nurse, 2003; Jonz *et al.*, 2004; Qin *et al.*, 2010)

In mammals, the presence of central chemoreceptors and their quantitative role in mediating ventilatory responses to hypercapnia is universally accepted. In fish however,

evidence for central chemosensitivity remains elusive (Milsom, 2010a,b). In fact, the presence of central CO₂/pH chemoreceptors within strictly water breathing species has been questioned. Indeed, the limited available data suggest that central CO₂/pH chemoreceptors are present only in air breathing fish (see Gilmour and Perry, 2006).

NECs were first identified as candidate chemoreceptors by Dunel-Erb *et al.*, (1982). NECs have been found on the gill filaments of all fish species examined to date where they are typically distributed along the entire length of the filament (see review by Perry *et al.*, 2009; Porteus *et al.*, 2012). NEC identification (see review by Porteus *et al.*, 2012) and electrophysiological characterization (Jonz *et al.*, 2004; Qin *et al.*, 2010) has further substantiated the gill filament NEC as the primary site for chemoreception. NECs of the first branchial arch have been proposed to be the evolutionary pre-cursors of the type 1 cells in mammals (Milsom and Burleson, 2007), primarily due to similarities in embryonic origin, morphology, biochemistry, innervation and response to chemostimuli (Milsom and Burleson, 2007; Jonz and Zaccane, 2009; Porteus *et al.*, 2012). Consequently, an understanding of the type 1 cells of the carotid body may shed light on the chemotransduction cascade within NECs of the fish gill filament.

1.2.2 Type I cells of the carotid body

The carotid body is an organ of neural crest origin (see review by Kumar, 2009) whose role in chemosensing was first postulated by Heymans and Heymans (1927). Since then, extensive studies have unequivocally characterized the carotid body as a peripheral chemoreceptor capable of sensing changes in blood O₂, CO₂, pH and glucose (see reviews by Kumar, 2009; Nurse, 2010). The carotid body is located at the bifurcation of

the carotid artery and is comprised, predominantly, of type 1 cells (see reviews by Kumar, 2009; Nurse, 2010).

Type 1 cells are found in clusters, are highly innervated and receive exceptionally high blood flow patterns relative to other tissue of the same size (see review by Kumar, 2009). Type 1 cells receive afferent innervation from the carotid sinus nerve (CSN) that originates as a branch of the IXth cranial (glossopharyngeal) nerve. Efferent innervation has also been identified, and is comprised of both sympathetic and parasympathetic fibers (Kumar, 2009). Type I cells contain carbonic anhydrase (CA), an enzyme which catalyzes the hydration reaction of CO₂ ($\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3 + \text{H}^+$) (Ridderstrale and Hanson, 1984; Rigual *et al.*, 1985; Nurse, 1990). Numerous organelles, including a number of Golgi apparatus-associated vesicles are also found within type 1 cells (Kumar, 2009). The secretory vesicles are packed with neurotransmitters, most notable of which are ATP, dopamine, acetylcholine (ACh) and serotonin (5-hydroxytryptamine or 5-HT). Upon sensing a change in blood gas tensions, type 1 cells modulate their discharge to the CSN, which alters efferent output of the brainstem and thus promotes ventilatory adjustments.

1.2.2.1 Mechanisms of CO₂ sensing in type I cells

The mechanism by which type 1 cells modulate their discharge to hypercapnia is a topic that has received much attention. A comprehensive understanding of the chemotransduction cascade, however, is still unavailable, primarily owing to the multitude of stimuli associated with hypercapnia and to the complexity of type I cells.

As described earlier, aquatic hypercapnia in fish is comprised of an increase in CO₂ and a decrease in extracellular (blood or water) pH (pH_o). The presence of CA

within mammalian type I cells also suggests a role for intracellular pH (pH_i).

Consequently, hypercapnia may be mediating its effects on type I cells via molecular CO_2 , pH_o , pH_i , HCO_3 , or any combination of these stimuli. In order to elucidate the contribution of each of these stimuli to the chemotransduction cascade, electrophysiological characterization of type I cells in response to acidic hypercapnia (increase in CO_2 and a decrease in pH_o), isohydric hypercapnia (increase in CO_2 while maintaining pH_o) and isocapnic acidosis (maintaining CO_2 and decreasing pH_o) have been employed. Such studies have unequivocally revealed that in response to acidic hypercapnia, isohydric hypercapnia and isocapnic acidosis type I cells:

- (i) depolarize via an inhibition of background K^+ channels (Buckler and Vaughan-Jones, 1994)
- (ii) decrease pH_i (Buckler *et al.*, 1991a,b) specifically via a CA mediated mechanism (in response to acidic hypercapnia, Buckler *et al.*, 1991a)
- (iii) increase intracellular calcium ($[\text{Ca}^{2+}]_i$) via an influx of Ca^{2+} through voltage gated L-type Ca^{2+} channels (Buckler and Vaughan-Jones, 1993 & 1994)
- (iv) release neurotransmitters onto the CSN (see review by Iturriaga and Alcayaga 2004; Nurse 2010)

The precise sequence and mechanism within which each of the aforementioned events occurs have not been fully elucidated. The acidic shift in pH_i has been proposed to be a critical component of the chemotransduction cascade because;

- (i) it is the single unifying factor in the hypercapnic response (see review by Peers and Buckler, 1995)

- (ii) CA inhibition, and consequently, a reduced rate of intracellular acidification (Buckler *et al.*, 1991b) has been correlated with reduced:
 - a. rate and magnitude of intracellular acidification in type I cells (Buckler *et al.*, 1991a)
 - b. action potential frequency in the CSN (Rigual *et al.*, 1991) and,
 - c. chemosensory discharge from the CSN (Iturriaga *et al.*, 1991)

Based on these findings, Buckler and Vaughan-Jones (1994) proposed a model in which the hypercapnia induced intracellular acidification inhibits background K^+ channels leading to membrane depolarization and Ca^{2+} entry via voltage gated L-type Ca^{2+} channels. This elevated increase in $[Ca^{2+}]_i$ is proposed to increase neurosecretion from type I cells ultimately leading to an increased firing rate of the CSN.

Indeed, several K^+ channels in type I cells are inhibited by acidic stimuli, including: two-pore acid sensing K^+ (TASK) channels and inwardly rectifying K^+ (K_{ir}) channels (see review by Putnam *et al.*, 2004). TASK channels are strictly inhibited by changes in extracellular pH, whereas K_{ir} channels, depending on the channel subtype, are inhibited by extracellular and intracellular changes in pH (Putnam, 2004). Therefore, although there are data to support the proposed pH_i induced background K^+ inhibition, the evidence is equivocal and does not exclude the contribution of pH_o to membrane depolarization in response to hypercapnia (Putnam, 2004; Trapp *et al.*, 2011).

Similarly, the proposed increase in $[Ca^{2+}]_i$ as a secondary effect to changes in pH_i have also been questioned. Summers *et al.* (2002) investigated the relationship between pH_i and transmembrane Ca^{2+} current by inducing intracellular acidification (using acetate) in the absence of changes in pH_o and CO_2 . It was noted that the acetate induced

drop in pH_i did not mimic the effects of hypercapnia on Ca^{2+} current, leading the authors to conclude that the effects of hypercapnia on Ca^{2+} current are not secondary to changes in pH_i .

Clearly, at present, an exhaustive explanation of the chemotransduction cascade within type 1 cells in response to hypercapnia is lacking, yet a multifactorial model (i.e. combined effect of multiple stimuli), as proposed by Putnam *et al.* (2004), may be the most pragmatic explanation.

1.2.3 NECs of the fish gill

NECs are innervated cells of neural crest origin, whose role in chemosensing was first proposed by Dunel-Erb *et al.* (1982). Filament NECs are 7-10 μM in diameter, distributed along the entire length of the filament epithelium with particularly high density in the distal regions (Jonz *et al.*, 2003; Saltys *et al.*, 2006; Coolidge *et al.*, 2008; Tzaneva and Perry, 2010; Zhang *et al.*, 2011). In zebrafish, NECs receive extrinsic innervation that may be of afferent or efferent origin and intrinsic innervation that projects to the efferent filament artery (Jonz and Nurse, 2003). The precise orientation of NECs is poorly understood, as they may be “open-type cells” (having direct contact with the external environment) or “closed-type cells” (located on the basal lamina and lacking direct contact with the external environment) (Zaccone *et al.*, 1997). As in type I cells, NECs exhibit CA activity, mitochondria, endoplasmic reticulum and golgi-apparatus associated dense cored vesicles (Dunel-Erb *et al.*, 1982; Bailly *et al.*, 1992; Goniakowska-Witalinska *et al.*, 1995; Qin *et al.*, 2010). NEC identification at the ultrastructural level is based on the presence of these dense-cored vesicles, which appear to hold a secretory function (Dunel-Erb *et al.*, 1982). More recent studies have used

immunohistochemical techniques to identify the equivalent synaptic vesicles (Jonz and Nurse, 2003; Saltys *et al.*, 2006).

Immunohistochemical analysis has revealed that bioactive amines, particularly 5-HT, are present within NECs (Dunel-Erb *et al.*, 1982; Bailly *et al.*, 1992; Zaccone *et al.*, 1992, 1997; Goniakowska-Witalinska *et al.*, 1995; Sundin *et al.*, 1998a,b; Jonz and Nurse, 2004; Saltys *et al.*, 2006; Tzaneva and Perry, 2010). The neurochemical content of NECs, however, is not uniform. Non-5-HT containing NECs and NECs exhibiting variable immunoreactivity for leu-5-enkephalin, met-5-enkephalin and endothelin have also been identified (Zaccone *et al.*, 1992, 1996; Goniakowska-Witalinska *et al.*, 1995; Jonz and Nurse, 2003; Saltys *et al.*, 2006). Moreover, smaller NECs confined to the lamellar epithelium have been described in only three species examined to date: zebrafish, bowfin and goldfish (Goniakowska-Witalinska *et al.*, 1995; Jonz and Nurse, 2003; Saltys *et al.*, 2006; Tzaneva and Perry, 2010). The variability in neurochemical composition of NECs suggests the presence of multiple NECs populations.

Recently, cellular characterization of zebrafish NECs via patch-clamp techniques has confirmed their O₂ and CO₂ sensitivity and revealed that their chemosensory mechanism are analogous to type I cells (Jonz *et al.*, 2004; Qin *et al.*, 2010). At present, however, data illustrating a direct relationship between NECs and cardioventilatory responses to chemostimuli are sparse. Burlison and Milsom (1993) demonstrated afferent neuronal activity in the sensory receptors innervated by the glossopharyngeal nerve in the isolated gill arch of the trout. Furthermore, in developing zebrafish, innervation of gill NECs coincides with a dramatic increase in the hyperventilatory response to hypoxia (Jonz and Nurse, 2005). These studies suggest, especially when compared to type I cells,

that NECs may modulate the cardiorespiratory responses to hypercapnia via a chemotransduction mechanism similar to that of type I cells of the carotid body.

1.2.3.1 Mechanisms of CO₂ sensing in NECs

Electrophysiological characterization of the cellular response to hypercapnia has been studied in NECs of the zebrafish gill filament (Qin *et al.*, 2010). As in type I cells, NECs depolarize via inhibition of background K⁺ channels upon stimulation with acidic hypercapnia, isohydric hypercapnia and isocapnic acidosis (Qin, 2007; Qin *et al.*, 2010). This depolarization appears to involve a CA-mediated mechanism as the rate and magnitude of depolarization is reduced after the application of acetazolamide (a CA inhibitor; Qin *et al.*, 2010).

1.3 Model and hypothesis

1.3.1 Zebrafish as a model system

Zebrafish have emerged as an important vertebrate model owing to their short generation time, transparent embryos/larvae and fully mapped genome. They have been used for the study of sensory and nervous system development (Eisen, 1996; Fishman, 1999; Bröckerhoff *et al.*, 1998; Starr, 2004) and represent the only non-mammalian species for which O₂ chemoreceptors have been characterized at the cellular level (Jonz and Nurse, 2006). Because NECs were localized and characterized as CO₂ chemoreceptors in the zebrafish (Qin *et al.*, 2010), I chose to pursue studies of CO₂ chemoreception in the same species.

1.3.2 Thesis goals

The overall goals of this study were to: (1) establish microspectrofluorometric techniques for measuring $[Ca^{2+}]_i$ and pH_i in isolated NECs of zebrafish and (2) to apply this technique to elucidate the effects of hypercapnia on $[Ca^{2+}]_i$ and pH_i in order to understand the cellular mechanism of CO_2 sensing in gill chemoreceptors.

1.3.3 Microspectrofluorometric techniques

Microspectrofluorimetry is a technique which utilizes fluorescent indicators, such as Fura-2 and BCECF, in order to measure free intracellular concentrations of Ca^{2+} and H^+ , respectively. Fura-2 and BCECF are dual-wavelength ratiometric dyes which exhibit changes in their fluorescence excitation wavelength upon changes in intracellular Ca^{2+} or pH without changes in the emission spectra. Fura-2 is excited at 340 and 380 nm (510 nm emission) whereas BCECF is excited at 495 and 440 nm (535 nm emission). The indicators are loaded into the cell by incubating the cells in a dilute solution (e.g. 5-10 μM) of the acetoxymethyl (AM) ester form of the indicator to improve uptake across the plasma membrane. Once the AM ester enters the cell, intracellular esterases cleave the AM group, trapping the polyanionic form of the indicator in the cell.

1.3.4 Hypothesis

On the basis of morphological and electrophysiological similarities between gill NECs and type I cells of the carotid body, the following hypotheses and predictions were tested:

- (1) Chemoreception in response to changes in CO_2 and/or pH is mediated by changes in $[Ca^{2+}]_i$ and pH_i . It is predicted that:

- i. In response to acidic hypercapnia (elevated PCO_2 with accompanying fall in pH), isohydric hypercapnia (increased PCO_2 at constant pH) and isocapnic acidosis (constant PCO_2 at reduced pH) pH_i will decrease and $[\text{Ca}^{2+}]_i$ will increase.
 - ii. The increase in $[\text{Ca}^{2+}]_i$ will be due to the influx of Ca^{2+} from the extracellular environment.
 - iii. The increase in $[\text{Ca}^{2+}]_i$ will be dependent on changes in pH_i
- (2) CA participates in CO_2 sensing. It is predicted that inhibition of CA will reduce the magnitude of intracellular acidification and the rise in $[\text{Ca}^{2+}]_i$ elicited by hypercapnia

2. Methods

2.1 Animals

Adult zebrafish (*Danio rerio*) were obtained from a commercial supplier (MIRDO, Montreal, Canada) and transported to the University of Ottawa Aquatic Care Facility where they were allowed to recover for at least 4 weeks in acrylic tanks (4 L) supplied with well-aerated dechloraminated City of Ottawa tapwater at 28°C. Fish were maintained at a constant 14 h: 10 h light:dark photoperiod. All procedures for animal use were approved by the University of Ottawa Animal Care and Veterinary Services (protocol BL-226) and carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

2.2 Ventilatory responses to acute hypercapnia

Adult zebrafish were anaesthetized with 0.1 mg ml⁻¹ tricaine mesylate (MS 222) dissolved in dechloraminated City of Ottawa tapwater (pH 7.4). Ventilatory responses to

acute hypercapnia (1-5% CO₂) were assessed by recording breathing frequency using a 3CCD MTI camera (Michigan, USA). Fish were placed in a recording chamber that was constructed by carving a small well out of Petri dish coated with Sylgard (Dow Corning, Corp., Midland, MI, USA) and filled with ~ 20 ml of water. The chamber was placed on the stage of a Nikon SMZ 1500 microscope and continuously perfused (15ml min⁻¹) under gravity. Zebrafish were allowed 15 min to recover from handling stress before measurements were made under control (normocapnic) conditions followed by exposure to hypercapnia in a stepwise manner (from 1-5% CO₂ in 1% increments). Hypercapnia was achieved by gassing the perfusion reservoir (2 L beaker in 28°C water bath) with mixtures of CO₂ and air that were provided by a Cameron Gas Mixer (model GF-3/MP, Port Aransas, TX, USA). Fish were exposed to each level of hypercapnia for 15 min before initiating recordings. In each case, 3 min of breathing data were acquired for each level of hypercapnia using CyberLink Power Director software (Karlsruhe, Germany). The videos were analyzed at a slower speed using VLC media player software, and ventilation frequency measurements were obtained by counting the number of opercular movements per minute.

The effect of acidified normocapnic water on breathing frequency was also examined to distinguish between the effects of acidosis (reduced water pH) and hypercapnia (elevated P_wCO₂ coupled with reduced pH) on breathing behaviour. The ventilation frequency of zebrafish at a pH of 5.7 was monitored as described above. A pH of 5.7 was chosen because it corresponded to the pH reached at the highest level of hypercapnia (5% CO₂; P_wCO₂= 37.5 mmHg) tested. A pH of 5.7 was achieved by titrating normocapnic dechloraminated tapwater with HCl.

2.3 Cell isolation

To prepare NECs for imaging, cells were isolated as previously described by Jonz *et al.* (2004). Adult zebrafish were stunned by a sharp blow to the head and killed by decapitation. All NEC isolation procedures were performed under sterile conditions in a laminar flow hood. Gill baskets were excised and rinsed in wash solution [2% penicillin-streptomycin (Sigma-Aldrich, Oakville, ON, Canada)] in phosphate-buffered saline (PBS) for 10 min. PBS contained the following: 137 mM NaCl, 15.2 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄ at pH 7.8 (Bradford *et al.*, 1994; Jonz *et al.*, 2004). All eight gill arches were separated and thoroughly cleaned of blood and mucous. Distal filaments rich in NECs were selectively removed and placed in 0.25% trypsin/EDTA (Invitrogen Corp.) for 1 h at 28°C. Tissue was then subjected to mechanical dissociation with fine forceps and triturated in a 15ml centrifuge tube with a Pasteur pipette for 3 min. The trypsin reaction was stopped with the addition of 10% fetal calf serum (FCS) and undigested tissue was allowed to settle at the bottom of the centrifuge tube. The cell suspension was centrifuged (130 *g*) for 5 min and the pellet triturated in filter-sterilized PBS. The cell suspension was centrifuged once more with PBS and the pellet re-suspended in Leibovitz's (L-15, with L-glutamine, Gibco, Grand Island, NY, USA) culture medium supplemented with 1% penicillin-streptomycin and 2% FCS. Cells were plated in 0.2 ml L-15 on 35 mm glass bottom culture dishes (MatTek Corp., Ashland, MA, USA) coated with 0.1 mg ml⁻¹ poly-L-lysine (Sigma-Aldrich) and Matri-Gel (Becton Dickenson, Mississauga, ON, Canada). Cells were supplemented with 2 ml L-15 16-18 h after isolation, and imaging was carried out 4-5 h thereafter.

2.4 $[Ca^{2+}]_i$ and pH_i measurements

The fluorescent indicators Fura-2-LeakRes-AM (Teflabs, Ausitn, TX, USA) hereafter referred to as Fura-2, and 2',7'-bis(2carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; Invitrogen) were used to monitor intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and pH (pH_i), respectively. Cells that adhered to the culture substrate were incubated in a solution of 5 μ M Fura-2 or BCECF in normal Ringer's solution (containing in (mM) 135 NaCl, 5 KCl, 2 Mg_2Cl , 2 Ca_2Cl , 10 glucose and 10 HEPES, pH 7.8) and 0.1% v/v of 10% w/v Pluoronic F-127 (Invitrogen) at 28°C for 1 h. Cells were subsequently washed in indicator-free Ringer's for an additional 30 min at 28°C. NECs were identified using 4 mg ml^{-1} Neutral Red (NR; Sigma), a vital marker used to identify serotonergic cells (Stuart *et al.*, 1974; Youngson *et al.*, 1993), including NECs (Jonz *et al.*, 2004).

Ratiometric imaging was performed using a Nikon Eclipse microscope equipped with a Lambda DG-5 high-speed wavelength changer (Sutter Instruments, Nocate, CA, USA), a Nikon 40x water-dipping objective lens with a numerical aperture of 0.8 and a digital CCD camera Q Imaging camera (Surrey, BC, Canada). Dual images at 340 and 380 nm excitation (510 nm emission) for Ca^{2+} and 495 and 440 nm excitation (535 nm emission) for pH were acquired every 2 s and ratiometric data were obtained using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON, Canada). Data are represented as raw ratios ($R_{340/380}$ and $R_{495/440}$) and thus indicate relative changes in $[Ca^{2+}]_i$ and pH_i , respectively.

2.5 Perfusion system

Dishes containing NR, Fura/BCECF positive cells were fitted with a perfusion chamber insert (Warner Instruments Inc., Hamden, CT, USA) and mounted on the microscope stage. The recording chamber was continuously perfused under gravity (3-4 ml min⁻¹) with Ringer's solution at room temperature (22-24°C). Multiple perfusion reservoirs (50 ml syringes) were connected to a single gas impermeable tube that was used to deliver the perfusate to the recording chamber. A three-way valve was used to switch between control (Ringer's bubbled with air) and experimental treatments. The solution within the reservoir was continuously pumped out through the outflow opening of the perfusion chamber via a variable flow mini-pump (Fisher scientific). The volume of Ringer's solution within the perfusion chamber never exceeded 400 µL.

2.6 Solutions and drugs

Acidic hypercapnia was generated by pumping CO₂ and air through a Cameron Gas Mixer (model GF-3/MP, Port Aransas, TX, USA) into the perfusion reservoir (5% CO₂ – 95% air, pH ~ 6.6). Isocapnic acidosis was generated by titrating Ringer's solution with HCl until pH 6.6 was reached. Isocapnic solutions were continuously bubbled with air, and are considered to be normocapnic (i.e. atmospheric CO₂). Finally, isohydric hypercapnia was achieved using a 5% CO₂ – 95% air mixture while maintaining pH ~ 7.5-7.8 using NaOH.

The contribution of extracellular Ca²⁺ to the hypercapnia induced rise in [Ca²⁺]_i was examined by perfusing NECs in Ca²⁺-free Ringer's or by using three Ca²⁺ channel blockers. Cd²⁺ (10 and 100 µM) and Ni²⁺ (25 µM) were used as non-selective inhibitors of voltage-gated Ca²⁺ channels, while nifedipine (25 and 50 µM) was used to inhibit L-

type Ca^{2+} channels. The final working CdCl_2 and nifedipine concentrations were prepared from a 100 mM stock solution dissolved in water, and 50 mM stock solution dissolved in DMSO, respectively. NiCl_2 was dissolved in the Ringer's solution immediately before each experiment. In all Ca^{2+} -free experiments, Ca^{2+} was omitted from the Ringer's solution, and 1mM EGTA was added. Sodium acetate (20 mM) was used to induce intracellular acidification, and was prepared by equimolar substitution with NaCl. The membrane-permeant carbonic anhydrase (CA) blocker, acetazolamide (ACTZ; 50 μM) was used to assess the potential role of CA activity in CO_2 -mediated Ca^{2+} and pH signaling. All drugs were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.7 Statistical analysis

All $[\text{Ca}^{2+}]_i$ data are reported as means \pm standard error of the mean (S.E.M), where the mean is the peak $R_{340/380}$ during the application of the stimulus minus the averaged $R_{340/380}$ over the 30 s preceding the stimulus (i.e. baseline control). Where a peak $R_{340/380}$ was unattainable (e.g. in isohydric hypercapnia and acetate), the average $R_{340/380}$ during application of the stimulus was used. All pH data are reported as means \pm standard error of the mean (S.E.M), where the mean is the average of the last 30 s during the application of the stimulus minus the averaged $R_{495/440}$ baseline over the 30 s preceding the stimulus. In these experiments, the final 30 s was chosen because it represented the period when $R_{495/440}$ was most stable. The steepest rate of decline in $R_{495/440}$ before and after ACTZ treatment was calculated using MATLAB software (Mathworks Inc., USA). The raw $R_{495/440}$ data were smoothed using a Hodrick-Prescott (smoothing) filter within MATLAB. The rate of change of the smoothed data was then calculated using a first order finite difference scheme, namely $(\text{Ph}(i+1) - \text{Ph}(i)) / (t(i+1) -$

t (i)). The max slope is simply the steepest rate of change during the pH_i drop. Where appropriate, data were analyzed by one-way ANOVA followed by a Bonferroni post-test for multiple comparison test or by paired or unpaired Student's t-tests. Statistical analyses were performed in GraphPad Prism 5 (Graphpad Software Inc., San Diego, CA).

3. Results

3.1 Effects of acute hypercapnia on breathing frequency

During acute progressive hypercapnia, breathing frequency increased and became significantly different when water CO_2 reached 3% (P_{CO_2} =22.5 mmHg, Fig. 1A) and stabilizing at 4% (P_{CO_2} =30 mmHg). Although not studied in detail, it was noted that 2 of the 8 fish tested exhibited episodic breathing in normocapnic water. Episodic breathing consisted of periods of breathing interspersed with intervals of apnea, which were sustained throughout 1% CO_2 exposure but absent at higher levels of CO_2 .

To determine whether acidification of the water during hypercapnia was contributing to the increase in ventilation frequency, fish were exposed to acidified water at constant CO_2 . Fish that were exposed to a change in water pH from 7.4 to 5.7 (the reduction in pH associated with 5% CO_2) did not increase breathing frequency (Fig. 1B).

3.2 Type I and type II $[\text{Ca}^{2+}]_i$ responses of NECs to acidic hypercapnia

NECs were identified prior to data acquisition under bright field optics based on a Neutral Red staining pattern as previously described by Jonz *et al.* (2004). As summarized in Table 1, of the 64 NECs examined in Normal Ringer's solution, 37 cells responded to acidic hypercapnia and exhibited one of two distinct $[\text{Ca}^{2+}]_i$ profiles (Fig. 2A). Under these conditions, 27 cells (43.75%) displayed an abrupt transient increase in $[\text{Ca}^{2+}]_i$ upon exposure to acidic hypercapnia (5% CO_2 , pH ~6.6) with recovery typically

occurring well before reperfusion with normocapnic solution (termed a type I response). In a subset of NECs (10/64, 15.7%) acidic hypercapnia elicited a slow gradual increase in $[Ca^{2+}]_i$ throughout the duration of the stimulus that was followed by a slow recovery upon reperfusion with normocapnic solution (termed a type II response). Type I and II responses were observed in Ca^{2+} -free (containing 1 mM EGTA) and persisted in the presence of various Ca^{2+} channel blockers (Table 2). As illustrated in Fig. 2B, however, type II responses did not always exhibit full recovery, and were rarely (1/30, 3.33%) observed in response to isocapnic acidosis (normocapnia, pH ~6.6). As such, the primary focus of this study was on type I responses.

3.3 Dose-dependent effects of hypercapnia on $[Ca^{2+}]_i$ and pH_i in NECs

Figure 3A shows typical $[Ca^{2+}]_i$ and pH_i responses of a NEC sequentially exposed to 1% and 5% CO_2 . As summarized in Fig. 3B, acidic hypercapnia evoked an increase in $[Ca^{2+}]_i$ and a decrease in pH_i in a dose-dependent manner (paired Student's t-test, $p < 0.05$).

3.4 Effects of chemostimuli on $[Ca^{2+}]_i$ and pH_i in NECs

The effects of acidic hypercapnia (5% CO_2 , pH ~6.6), isocapnic acidosis (normocapnia, pH ~6.6) and isohydric hypercapnia (5% CO_2 , pH ~7.8) on $[Ca^{2+}]_i$ are illustrated in Fig. 4 A, B and C (top panels). Acidic hypercapnia elicited a transient increase in $[Ca^{2+}]_i$ with recovery typically occurring well before reperfusion with normocapnic solution (Fig. 4A). Isocapnic acidosis elicited a similar transient increase in $[Ca^{2+}]_i$ with $R_{340/380}$ returning to baseline levels before the end of the isocapnic acidosis exposure period (Fig. 4B). However, isohydric hypercapnia did not elicit a marked change in $[Ca^{2+}]_i$ (Fig. 4C). In fact, the mean $\Delta R_{340/380}$ (0.08 ± 0.01) in response to

isohydric hypercapnia was not statistically significant than zero (Table 1; one sample t-test). The mean $\Delta R_{340/380}$ observed during acidic hypercapnia and isocapnic acidosis were 8.05 ± 1.79 and 8.66 ± 2.82 respectively (Table 1). To ensure that the absence of a response to isohydric hypercapnia was unrelated to the cell viability, all NECs exposed to isohydric hypercapnia were exposed to either acidic hypercapnia or isocapnic acidosis (Fig. 4C, Table 1). Of the 18 cells exposed to isohydric hypercapnia 10 were subsequently exposed to acidic hypercapnia of which 5 responded with a transient increase in $\Delta R_{340/380}$ (11.52 ± 6.24) and 8 were subsequently exposed to isocapnic acidosis of which 5 responded with a transient increase in $\Delta R_{340/380}$ (13.64 ± 8.13). Clearly, this protocol demonstrates that the ability of NECs to respond to isohydric hypercapnia differed from their ability to respond to acidic hypercapnia and isocapnic acidosis. It should also be noted, that ~ 30 s after reperfusion with normocapnic solution, 3 of the 18 cells exposed to isohydric hypercapnia exhibited a small transient increase in $[Ca^{2+}]_i$ and 4 of the 18 cells exhibited elevated baseline $R_{340/380}$ (supplementary Fig. 1).

The effects of acidic hypercapnia, isocapnic acidosis and isohydric hypercapnia on pH_i are illustrated in Fig. 4 A, B and C (bottom panels). Acidic hypercapnia induced a sharp drop in pH_i that was sustained throughout the stimulus period (Fig. 4A). Upon reperfusion with normocapnic solution, pH_i returned rapidly to baseline $R_{495/440}$. Isocapnic acidosis evoked a gradual decrease in pH_i that never reached a stable value (Fig. 4B). Upon reperfusion with normocapnic solution, pH_i recovered slightly but was rarely observed to return to baseline $R_{495/440}$. Isohydric hypercapnia (5% CO_2 , $pH \sim 7.8$) induced a sharp drop in pH_i that was typically sustained throughout the stimulus period (Fig. 4C). Three of the 8 cells tested, however, exhibited partial pH_i recovery before

reperfusion with normocapnic solution. The mean $\Delta R_{495/440}$ in response to isocapnic acidosis (0.10 ± 0.02) was significantly different from that observed during acidic hypercapnia (0.45 ± 0.07) and isohydric hypercapnia (0.33 ± 0.03) (ANOVA-Bonferroni test, $P < 0.05$).

Intracellular acidification preceded the increase in $[Ca^{2+}]_i$ in response to chemostimuli as exemplified by the vertical dashed lines in Fig 4 A, B, and C. In fact, intracellular acidification occurred almost immediately after the application of chemostimuli whereas the peak $R_{340/380}$ was achieved 52.3 ± 3.8 s and 59.7 ± 7.3 s after the application of acidic hypercapnia and isocapnic acidosis, respectively. In response to acidic hypercapnia and isocapnic acidosis, NECs increased their $[Ca^{2+}]_i$ in a similar manner despite markedly different pH_i responses. In response to isohydric hypercapnia, pH_i decreased without a substantial change in $[Ca^{2+}]_i$ (Fig. 4C). These observations suggest that the mechanism(s), whereby NECs increase their $[Ca^{2+}]_i$ in response to hypercapnic acidosis, is independent of changes in pH_i .

3.5 Effects of multiple bouts of chemostimuli on $[Ca^{2+}]_i$ and pH_i

Figure 5A and B show typical $[Ca^{2+}]_i$ (top panel) and pH_i (bottom panel) responses of NECs exposed to repeated bouts of acidic hypercapnia (5% CO_2 , $pH \sim 6.6$) and isocapnic acidosis (normocapnia, $pH \sim 6.6$), respectively. NECs were held in a 3 min resting period between recovery from the first stimulus and application of the second stimulus. In response to a second bout of acidic hypercapnia (Fig. 5A) and isocapnic acidosis (Fig. 5B) NECs exhibited a suppressed $R_{340/380}$ response by 5.6 ± 1.5 and 5.6 ± 2.5 respectively. Note that $[Ca^{2+}]_i$ ($\Delta R_{340/380}$) in response to the second stimulus was significantly smaller than the first (two-tailed, paired Student's t-test, $P < 0.05$), whereas

pH_i ($\Delta R_{495/440}$) was unaffected (Fig. 5C). Combined data for all chemostimuli based on measurements of $[Ca^{2+}]_i$ ($R_{340/380}$) and pH_i ($R_{495/440}$) responses are summarized in Table 1.

3.6 Effect of Ca²⁺-free conditions or Ca²⁺ channel blockers on $[Ca^{2+}]_i$ and pH_i responses to chemostimuli

To elucidate the contribution of extracellular Ca²⁺ to the increase in $[Ca^{2+}]_i$, NECs were exposed to acidic hypercapnia (5% CO₂, pH ~6.6) in a Ca²⁺-free solution containing 1mM EGTA, or in the presence of various Ca²⁺ channel blockers. The representative recordings depicted in Fig. 6 A-D demonstrate that the typical acidic hypercapnia – induced increase in $[Ca^{2+}]_i$ persisted in Ca²⁺-free solution, 10 μ M Cd²⁺, 25 μ M nifedipine, and 2mM NiCl₂, respectively. Mean $\Delta R_{340/380}$ in response to acidic hypercapnia under Ca²⁺-free conditions or in the presence of all Ca²⁺ channel blockers did not differ significantly from controls (Fig. 6E). The $[Ca^{2+}]_i$ response to isocapnic acidosis (normocapnia, pH ~6.6) in Ca²⁺-free solution was also examined (Fig. 7); it was not significantly different from controls (i.e. response to isocapnic acidosis in normal Ringer’s solution; Fig. 7). These observations suggest that the increase in $[Ca^{2+}]_i$ in response to acidic hypercapnia and isocapnic acidosis does not result from an influx of extracellular Ca²⁺ across the plasma membrane but may be a consequence of Ca²⁺ release from intracellular stores.

3.7 The role of carbonic anhydrase in mediating the $[Ca^{2+}]_i$ and pH_i response to acidic hypercapnia

Naïve NECs were selected for Ca²⁺ imaging because it was previously determined that a single exposure to acidic hypercapnia depresses the magnitude of the $\Delta[Ca^{2+}]_i$ response to a subsequent exposure (Fig. 5A). NECs were pre-incubated in 50 μ M

acetazolamide ACTZ (a CA inhibitor) for 5 min and subsequently exposed to acidic hypercapnia. Fig. 8 reveals that 50 μ M ACTZ did not significantly affect the typical acidic hypercapnia –induced rise in $[Ca^{2+}]_i$ or the time to reach peak $R_{340/380}$.

Because repeated bouts of acidic hypercapnia did not influence pH_i (Fig. 5), the effect of acidic hypercapnia on pH_i before and after ACTZ was examined on the same NECs. In response to acidic hypercapnia, control NECs exhibited a typical rapid and reversible fall in pH_i (Fig. 8C). After ACTZ administration, acidic hypercapnia exposure resulted in a similar but significantly slower drop in pH_i (Fig. 8C). After ACTZ, the decrease in pH_i by acidic hypercapnia was 50% complete in 65.8 ± 8.2 s, which was significantly different from controls not exposed to ACTZ (31.5 ± 4.4 s Fig. 8D). ACTZ treatment also reduced the magnitude of the absolute decrease in $R_{495/440}$ (Fig. 8D). The difference in the rate of intracellular acidification before and after ACTZ administration is clearly visible in Fig. 8E, where the initial drop in pH_i before (solid line) and after ACTZ (dashed line) treatment from the cell in Fig. 8C are superimposed. The rate of recovery after acidic hypercapnia exposure also was decreased by CA inhibition (Fig. 8 G and H). The contrasting effects of ACTZ on $[Ca^{2+}]_i$ and pH_i responses to acidic hypercapnia further support the conclusion that the mechanism(s) whereby NECs increase their $[Ca^{2+}]_i$ in response to acidic hypercapnia are independent of changes in pH_i .

3.8 The effect of acetate on $[Ca^{2+}]_i$ and pH_i in NECs

Acetate was used to induce intracellular acidification to further elucidate the relationship between the decrease in pH_i and the increase in $[Ca^{2+}]_i$ in response to acidic hypercapnia. CO_2 -sensitive NECs were first identified by exposing them to acidic hypercapnia and responsive NECs were subsequently exposed to 20 mM acetate (Fig. 9A

bottom panel). As illustrated in Fig. 9A (top panel), NECs responsive to acidic hypercapnia displayed the typical rise in $[Ca^{2+}]_i$ but were unresponsive to the application of acetate. All NECs (N=8) responded to a second exposure of acidic hypercapnia after the administration of acetate, confirming that the absence of an increase in $[Ca^{2+}]_i$ was related to the inability of acetate to induce a Ca^{2+} response via intracellular acidification, as opposed to desensitization of the NEC to repeated stimuli. Figure 9A clearly shows that, despite the absence of a Ca^{2+} response, acetate induced a reduction in NEC pH_i (bottom panel). The drop in $R_{495/440}$ induced by acetate was significantly different than the response to acidic hypercapnia (Fig. 9B, bottom panel). The conflicting effects of acetate on pH_i and $[Ca^{2+}]_i$ further strengthen the conclusion that the drop in pH_i in response to acidic hypercapnia does not cause the increase in $[Ca^{2+}]_i$.

Figure 1. The effects of hypercapnia or isocapnic acidosis on ventilation frequency in zebrafish, *Danio rerio*. (A) Zebrafish (N=8) were immersed in control system water bubbled with air (control) and subsequently exposed to acute hypercapnia (1-5% CO₂) in a stepwise manner. Ventilation frequency (V_f) was significantly elevated when $P_w\text{CO}_2$ reached 3% ($\text{PCO}_2 = 22.5$ mmHg). Different letters denote significant differences between mean values (means \pm S.E.M., repeated measures ANOVA; Bonferroni multiple comparison test, $P < 0.05$). (B) A different group of zebrafish was subjected to a change in water pH from 7.4 (control) to 5.7. A pH of 5.7 was chosen because it corresponded to the pH reached at the highest level of hypercapnia tested in A (5%, $\text{PCO}_2 = 37.5$ mmHg). Ventilation frequency was not significantly affected by a change in water pH (N=5, two-tailed paired Student's t-test, $p > 0.05$). In (B) horizontal line denotes the means and the error bars represent the S.E.M.

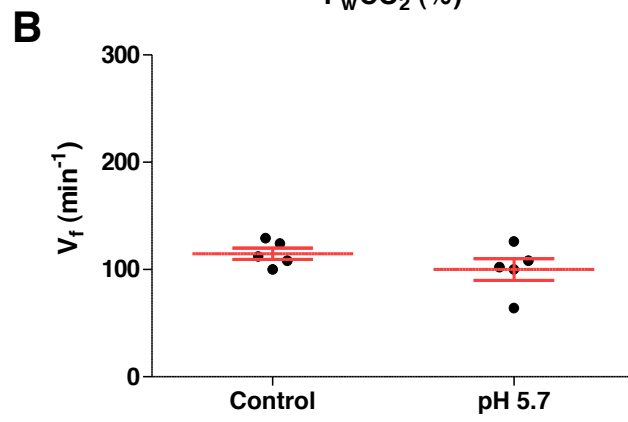
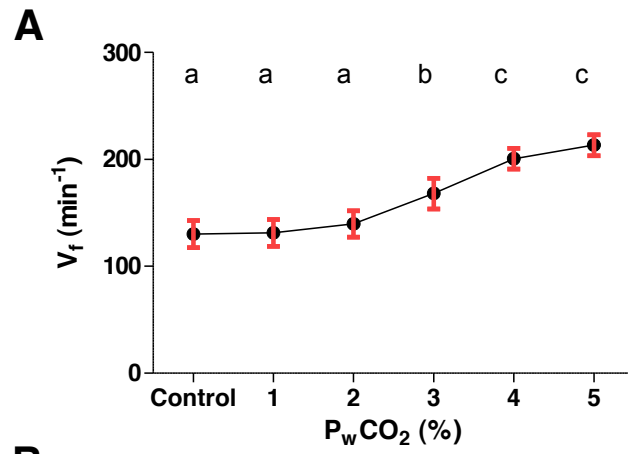


Figure 2. Hypercapnic acidosis elicits type I and type II $[Ca^{2+}]_i$ responses in isolated neuroepithelial cells (NECs) of zebrafish, *Danio rerio* (A) $[Ca^{2+}]_i$ responses (as indicated by changes in $R_{340/380}$) to acidic hypercapnia (5% CO_2 , pH 6.6) from two NECs recorded at the same time from the same dish. Note that type I responses (solid line) were characterized by a marked but transient change in $[Ca^{2+}]_i$, while type II responses (dashed line) consisted of smaller and continuous elevation in $[Ca^{2+}]_i$ throughout the hypercapnic exposure. (B) Typical type II $[Ca^{2+}]_i$ responses to hypercapnia (5% CO_2 , pH 6.6) under Ca^{2+} -free + 1 mM EGTA conditions. Cells exhibiting type II responses did not always exhibit full recovery.

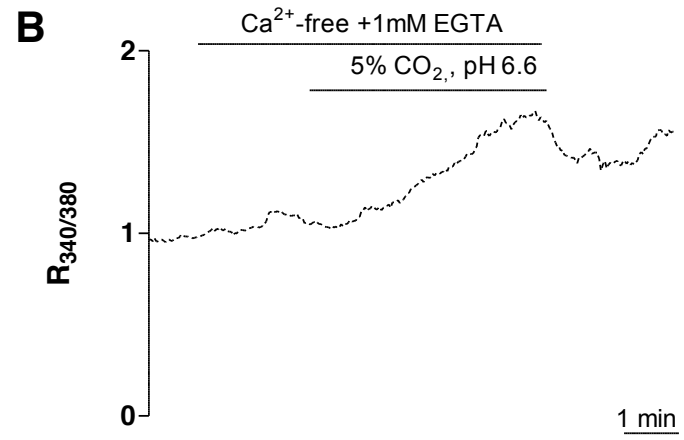
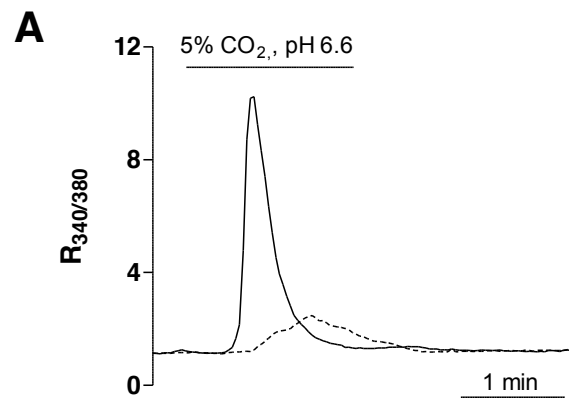


Figure 3. Dose-dependent effects of hypercapnia on $[Ca^{2+}]_i$ and pH_i in isolated neuroepithelial cells (NECs) of zebrafish, *Danio rerio*. (A) Representative traces of $[Ca^{2+}]_i$ (as indicated by changes in $R_{340/380}$; top panel) and pH_i (as indicated by changes in $R_{495/450}$; bottom panel) responses of NECs during perfusion (delineated by dashed lines) with solutions of 1% ($PCO_2 = 7.5$ mmHg) or 5% CO_2 ($PCO_2 = 37.5$ mmHg). (B) Summary of data from multiple cells showing $\Delta[Ca^{2+}]_i$ (N = 7; top panel) and ΔpH_i (N = 3; bottom panel) to 1% and 5% CO_2 . Means (horizontal line) \pm S.E.M. (bars) are indicated. Note that an increase in $R_{340/380}$ denotes an increase in $[Ca^{2+}]_i$ and a decrease in $R_{495/440}$ denotes a decrease in pH_i . Asterisks indicate a significant difference between treatments (two-tailed, paired Student's t-test, $P < 0.05$).

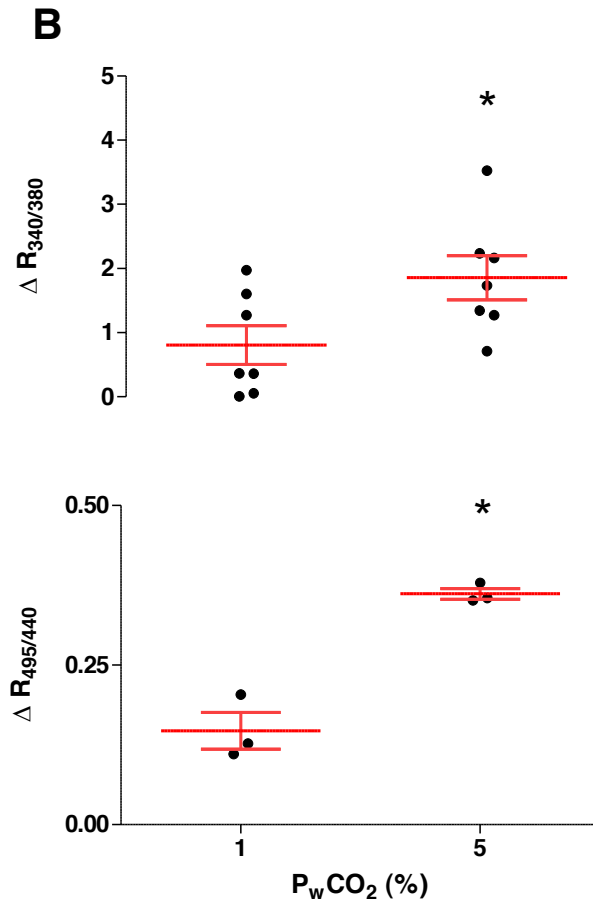
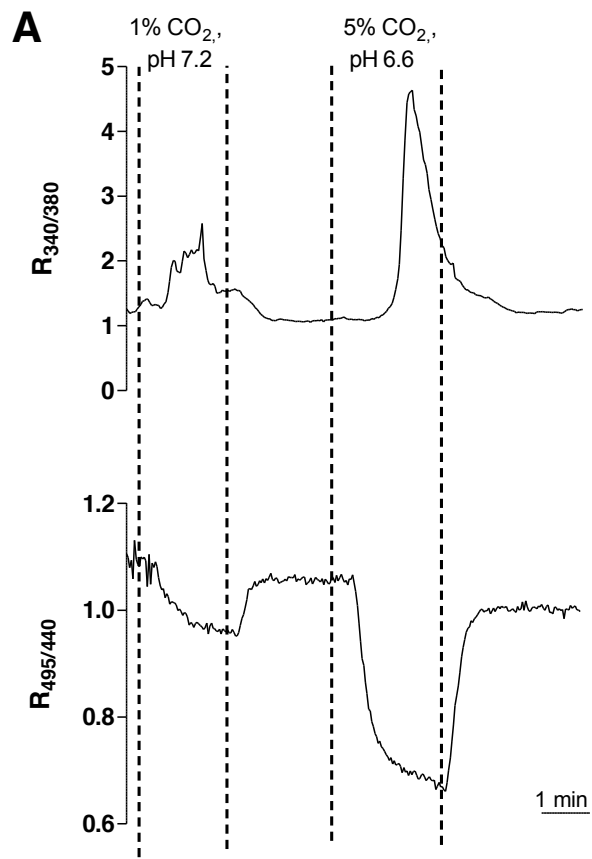


Figure 4. $[Ca^{2+}]_i$ and pH_i responses of isolated cultured neuroepithelial cells (NECs) of zebrafish, *Danio rerio* to chemostimuli. Representative traces of $[Ca^{2+}]_i$ (as indicated by changes in $R_{340/380}$; top panel) and pH_i (as indicated by changes in $R_{495/450}$; bottom panel) responses of a NECs during exposure (delineated by dashed lines) to (A) acidic hypercapnia (5% CO_2 , $pH \sim 6.6$) (B) isocapnic acidosis (normocapnia, $pH \sim 6.6$) (C) isohydric hypercapnia (5% CO_2 , $pH \sim 7.6$). Acidic hypercapnia and isocapnic acidosis elicited a transient change in $[Ca^{2+}]_i$ that was absent under isohydric hypercapnia. Cells that were exposed to isohydric hypercapnia were also exposed to either hypercapnic acidosis or isocapnic acidosis (C). Note that an increase in $R_{340/380}$ denotes an increase in $[Ca^{2+}]_i$ and a decrease in $R_{495/440}$ denotes a drop in pH_i .

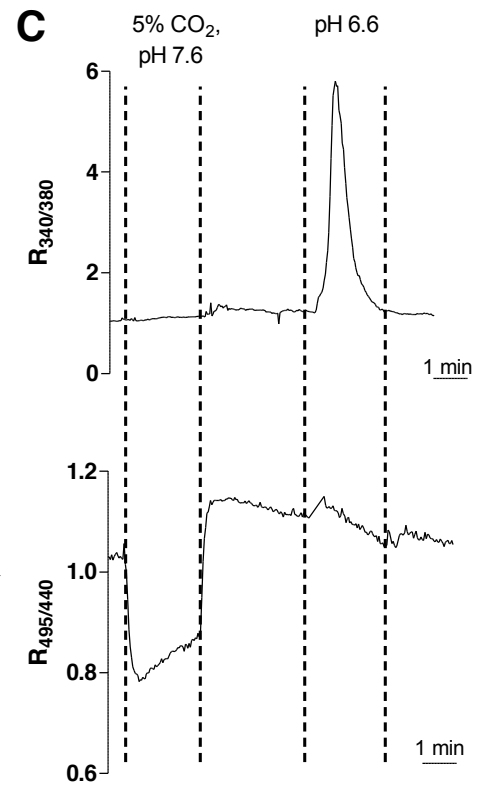
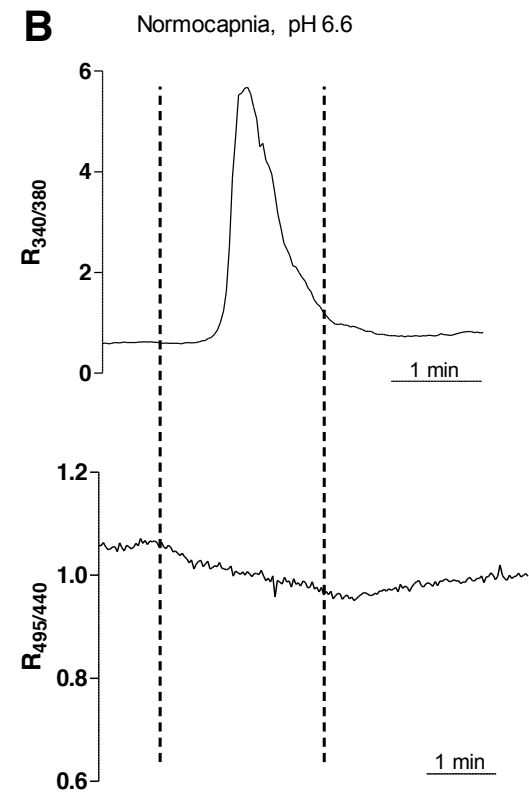
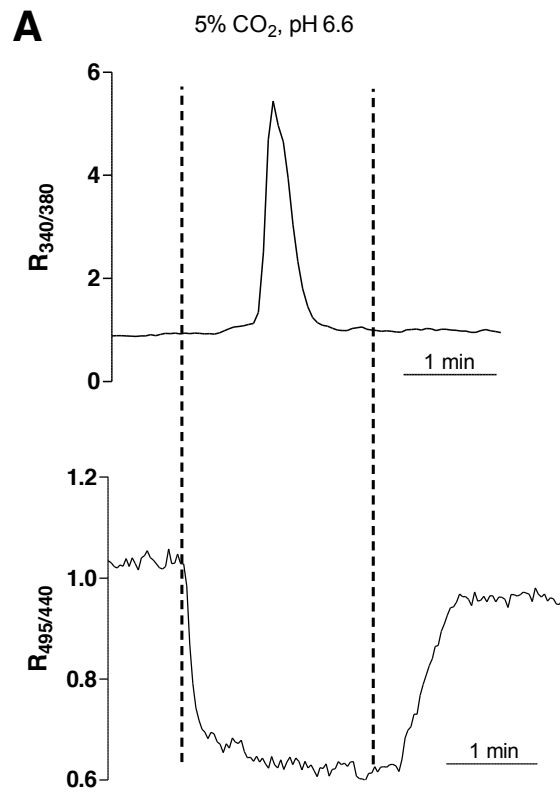


Figure 5. The effects of repeated bouts of chemostimuli on $[Ca^{2+}]_i$ and pH_i responses of isolated cultured neuroepithelial cells (NECs) of zebrafish, *Danio rerio*.

Representative traces of $[Ca^{2+}]_i$ (as indicated by changes in $R_{340/380}$; top panels) and pH_i (as indicated by changes in $R_{495/440}$; bottom panels) responses of a NECs during exposure (horizontal lines) to (A) acidic hypercapnia (5% CO_2 , $pH \sim 6.6$) and (B) isocapnic acidosis (normocapnia, $pH \sim 6.6$).

NECs were allowed to recover for ~ 3 min before the second chemostimulus was applied. (C) Summary of data from all responsive cells

showing $\Delta[Ca^{2+}]_i$ (top panel) responses to acidic hypercapnia (N= 18) or isocapnic acidosis (N = 10) and $\Delta[pH_i]$ (bottom panel) responses to acidic hypercapnia (N= 8) or

isocapnic acidosis (N = 8). Filled and unfilled circles represent the first and second bouts of chemostimuli, respectively. Means (horizontal line) \pm S.E.M. (bars) are indicated.

Note that an increase in $R_{340/380}$ denotes an increase in $[Ca^{2+}]_i$ and a decrease in $R_{495/440}$ denotes a drop in pH_i . Asterisks indicate a significant difference between the first and second bout of chemostimuli (two-tailed, paired Student's t-test, $P < 0.05$).

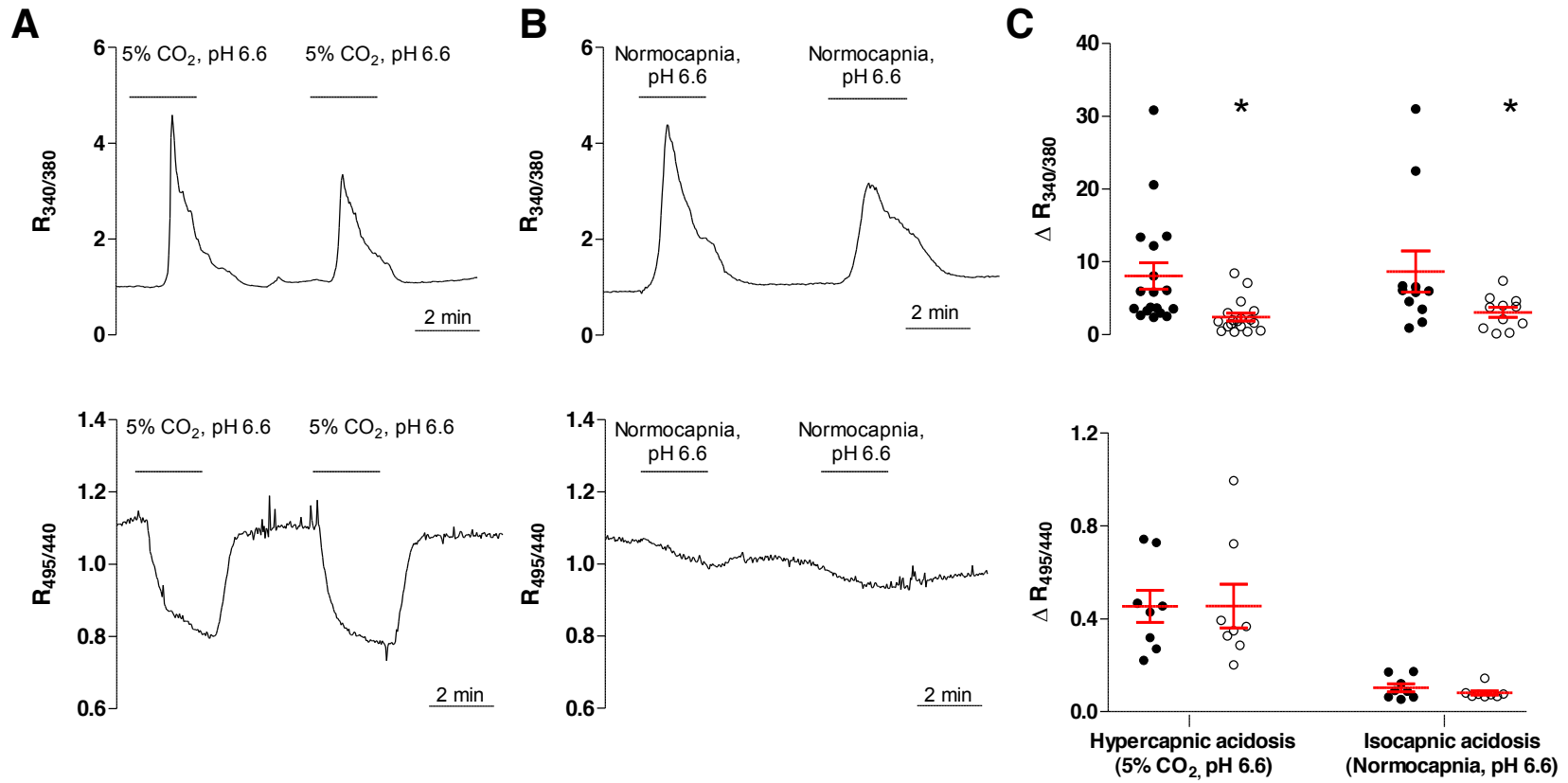


Table 1. Summary of $\Delta[\text{Ca}^{2+}]_i$ ($R_{340/380}$) and ΔpH_i ($R_{495/440}$) from responsive neuroepithelial cells (NECs) of the zebrafish, *Danio rerio* gill filament to chemostimuli.

	5% CO ₂ , pH~6.6		Normocapnia, pH~6.6		5% CO ₂ , pH ~7.5-7.8		
	First bout	Second bout	First bout	Second bout	First bout	Second bout (5%CO ₂ , pH 6.6)	Second bout (normocapnia, pH 6.6)
ΔCa^{2+} ($R_{340/380}$)							
Mean \pm S.E.M.	8.05 \pm 1.79	2.41 \pm 0.53 ‡	8.66 \pm 2.82	3.05 \pm 0.68 ‡	0.075* \pm 0.01	11.52 \pm 6.24	13.93 \pm 8.02
% Responsive Type I	43.75	100	56.67	100	0	50	62.5
% Responsive Type II	15.7	0	3.33	0	0	0	0
N (total)	64	18	30	10	18	10	8
ΔpH ($R_{495/440}$)							
Mean \pm S.E.M.	0.45 \pm 0.07	0.45 \pm 0.09	0.10 \pm 0.02*	0.08 \pm 0.009	0.33 \pm 0.03	---	---
% Responsive	100	100	100	100	72.72	---	---
N (total)	8	8	8	8	11	---	---

* Significant difference across groups (comparing first bouts only, ANOVA-Bonferroni test, $P < 0.05$)

‡ Significant difference within groups (comparing first and second bout, two-tailed, paired Student's t-test, $P < 0.05$)

Figure 6. Ca^{2+} -free solutions or Ca^{2+} channel blockers do not attenuate the $[\text{Ca}^{2+}]_i$ response to acidic hypercapnia in isolated neuroepithelial cells (NECs) of zebrafish, *Danio rerio*. Ca^{2+} -free media and blockers were applied for ~ 2 min before the application of acidic hypercapnia (5% CO_2 , pH-6.6). Representative traces of $[\text{Ca}^{2+}]_i$ responses (as indicated by changes in $R_{340/380}$) of NECs during exposure (horizontal lines) to acidic hypercapnia after the application of (A) Ca^{2+} -free +1 mM EGTA solution, (B) 10 μM Cd^{2+} (C) 25 μM nifedipine or (D) 2 mM NiCl_2 . (E) Cumulative data of $\Delta[\text{Ca}^{2+}]_i$ responses to acidic hypercapnia following administration of Ca^{2+} blockers. Means (horizontal lines) \pm S.E.M. (bars) are indicated. Responses to acidic hypercapnia in the presence of Ca^{2+} blockers did not differ from controls (ANOVA, Bonferroni). Sample sizes are indicated in parentheses. Note that an increase in $R_{340/380}$ denotes an increase in $[\text{Ca}^{2+}]_i$.

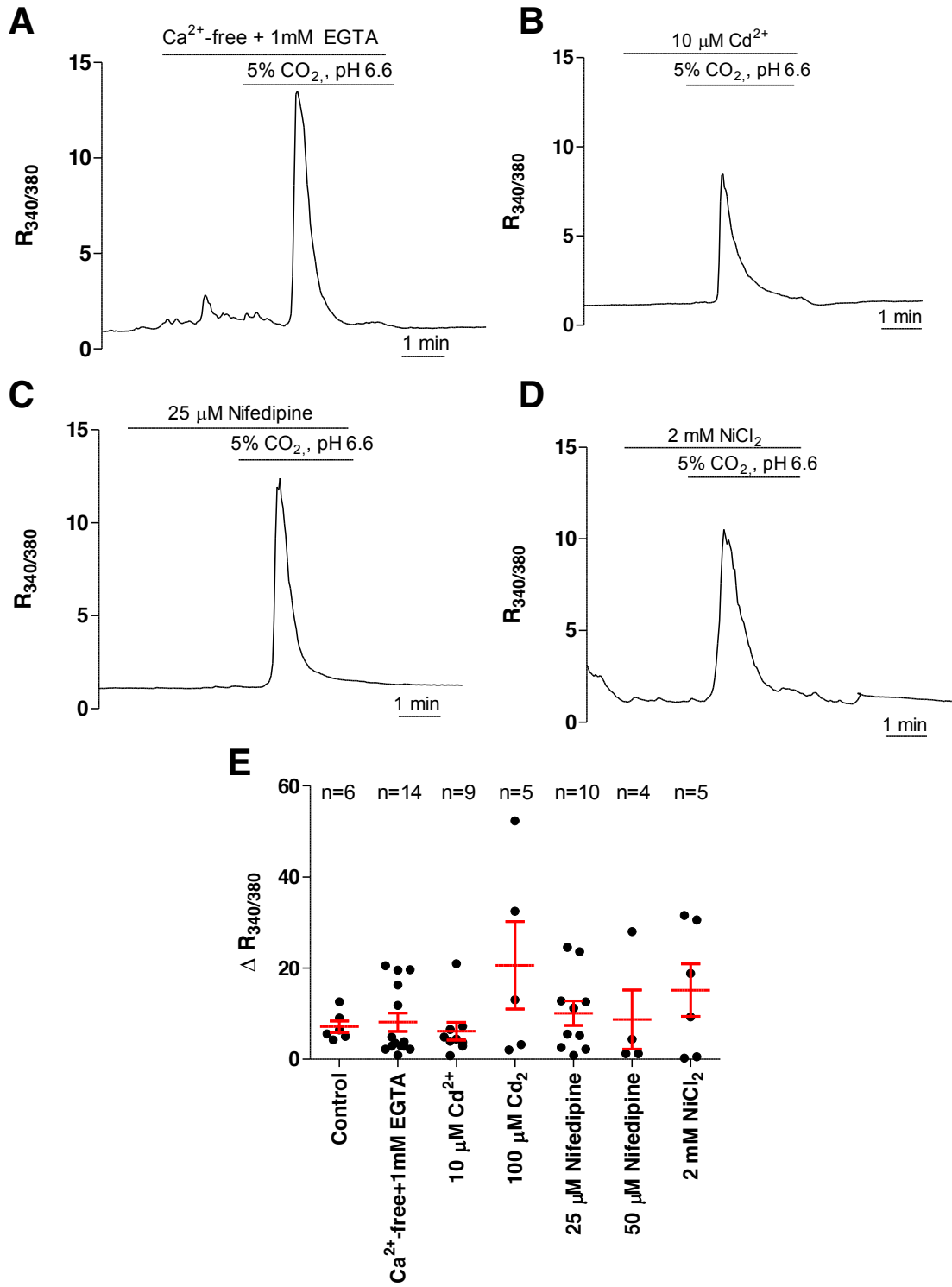


Table 2. Summary of type I and type II responsive neuroepithelial cells (NECs) of the zebrafish, *Danio rerio* gill filament to acidic hypercapnia in the presence of various Ca²⁺ channel blockers.

	Ca ²⁺ -free + 1mM EGTA	10 μM Cd ²⁺	100 μM Cd ²⁺	25 μM nifedipine	50 μM nifedipine	2mM Ni ²⁺
% Responsive Type I	31.12	52.94	50	66.67	42.85	33.33
% Responsive Type II	20	29.4	30	13.33	28.5	25.92
N (total)	45	17	10	15	14	27

Figure 7. The effect of isocapnic acidosis on the $[Ca^{2+}]_i$ response of isolated neuroepithelial cells (NECs) of zebrafish, *Danio rerio* in Ca^{2+} -free solution. (A) Representative trace of a $[Ca^{2+}]_i$ response (as indicated by changes in $R_{340/380}$) of a NEC during exposure (horizontal line) to acidosis (normocapnia, pH ~6.6) after the application of Ca^{2+} -free +1 mM EGTA solution. (B) Summary data of $\Delta[Ca^{2+}]_i$ responses to isocapnic acidosis in Ca^{2+} -free solution (N = 7). Means (horizontal lines) \pm S.E.M. (bars) are indicated. Responses to isocapnic acidosis under Ca^{2+} -free conditions did not differ from controls ($[Ca^{2+}]_i$ responses in normal Ringer's solution; two-tailed, unpaired Student's t-test). An increase in $R_{340/380}$ denotes an increase in $[Ca^{2+}]_i$.

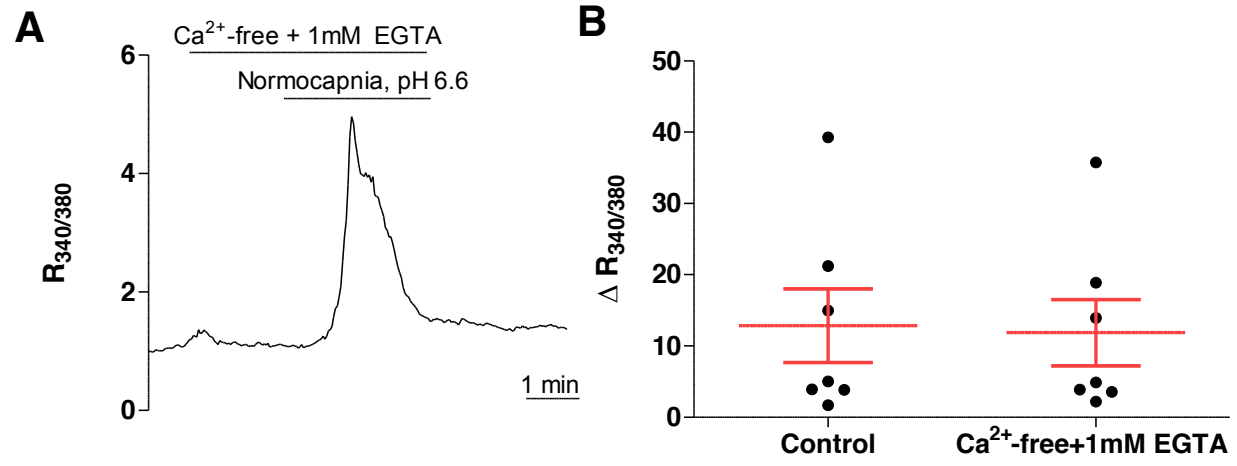


Figure 8. The effects of acetazolamide (ACTZ) on $[Ca^{2+}]_i$ and pH_i responses of isolated neuroepithelial cells (NECs) of zebrafish, *Danio rerio* to acidic hypercapnia. (A) Representative trace of the $[Ca^{2+}]_i$ response (as indicated by changes in $R_{340/380}$) to acidic hypercapnia (5% CO_2 , $pH \sim 6.6$) in a cell that was pre-exposed to 50 μM ACTZ for 5 min. (B) Summary data of $\Delta [Ca^{2+}]_i$ responses (top) and latency (s) to the maximal $[Ca^{2+}]_i$ response (bottom) after exposure to acidic hypercapnia; responses after ACTZ incubation ($N = 10$) were not significantly different from controls not treated with ACTZ ($N = 3$; two-tailed, unpaired Student's t-test). (C) Representative trace of pH_i response (as indicated by changes in $R_{495/440}$) to acidic hypercapnia before and after the application of 50 μM ACTZ (indicated by the horizontal bar). (D) Summary of the effect of ACTZ ($N = 8$) on ΔpH_i (top) and latency (s) to the half maximal drop in pH_i (bottom) after the application of acidic hypercapnia. Significant differences from control are indicated by an asterisk (two-tailed, paired Student's t-test, $P < 0.05$). (E, G) The intracellular acidification response (E) and recovery (G) of the NEC in panel C is shown again in expanded time scale. Solid traces indicate recording before the application of ACTZ and dashed traces indicate recordings after ACTZ application. Note that the traces are superimposed to clearly illustrate the effects of ACTZ on the rate of response and recovery. Summary data comparing the slope of pH_i during acidic hypercapnia (F) and recovery (H) before (control) and after ACTZ treatment. See text for detailed explanation of analysis. Note that intracellular acidification in the presence of ACTZ appeared to occur at a slower rate. Asterisks indicate a significant difference between control and ACTZ treated NECs (two-tailed, paired Student's t-test, $P < 0.05$).

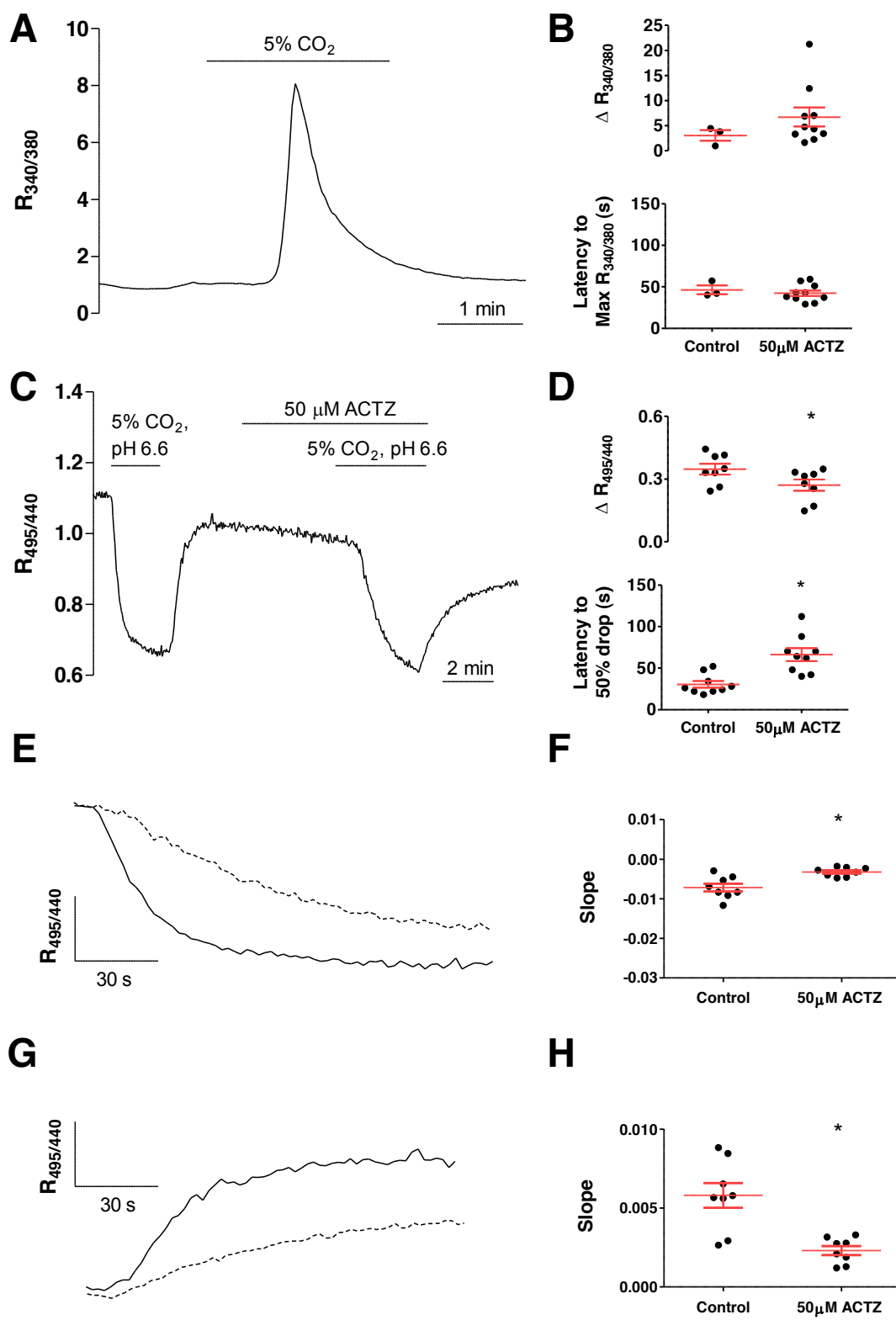
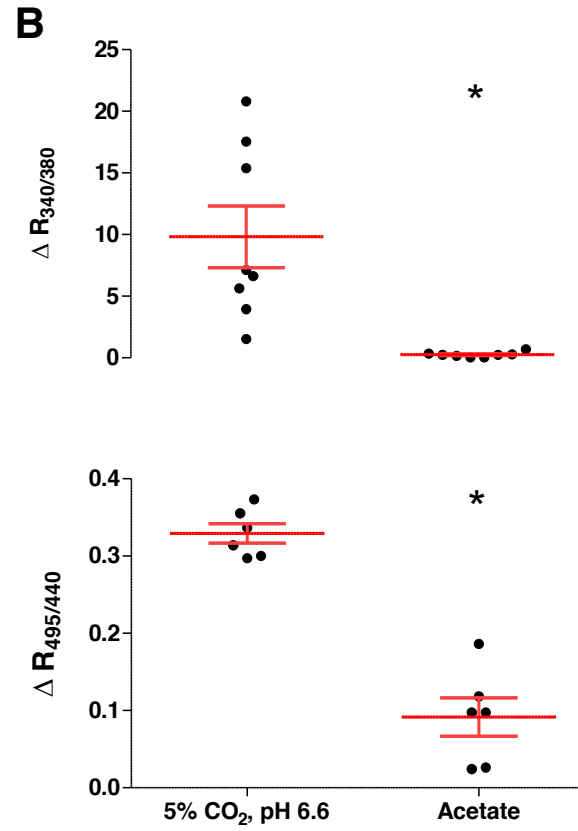
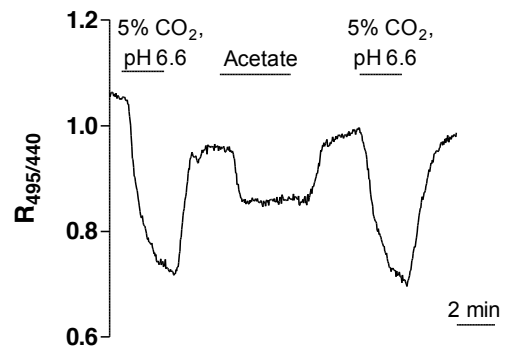
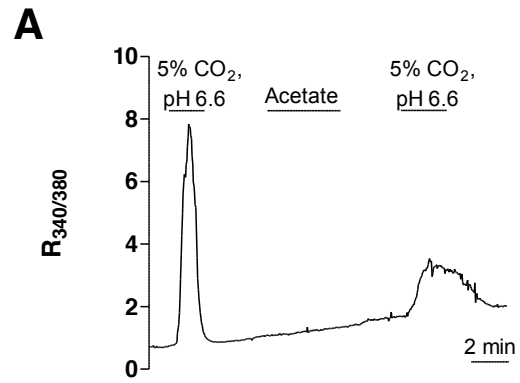


Figure 9. The effects of acetate on $[Ca^{2+}]_i$ and pH_i responses of isolated neuroepithelial cells (NECs) of zebrafish, *Danio rerio*. Representative traces of $[Ca^{2+}]_i$ (as indicated by changes in $R_{340/380}$; top panel) and pH_i (as indicated by changes in $R_{495/450}$; bottom panel) responses of NECs during exposure (horizontal lines) to acidic hypercapnia (5% CO_2 , pH ~6.6) and 20mM acetate. NECs were first exposed to acidic hypercapnia and allowed sufficient time to recover before the 4 min application of acetate. Note that acetate induced intracellular acidification but did not stimulate a change in $[Ca^{2+}]_i$. After the acetate exposure, NECs were exposed to acidic hypercapnia (5% CO_2 , pH ~6.6) again to determine their viability. (B) Summary of data from all responsive cells showing $\Delta[Ca^{2+}]_i$ (N=8; top panel) and $\Delta[pH_i]$ (N=6; bottom panel) responses to acidic hypercapnia and acetate. Asterisks indicate a significant difference (two-tailed, paired Student's t-test, $P < 0.05$) between acidic hypercapnia and acetate treatments. An increase in $R_{340/380}$ denotes an increase in $[Ca^{2+}]_i$ and a decrease in $R_{495/440}$ denotes a drop in pH_i .



4. Discussion

The present study is the first to directly evaluate the involvement of changes in $[Ca^{2+}]_i$ and pH_i to the hypercapnic chemotransduction cascade of the fish gill CO_2 chemoreceptors, the neuroepithelial cell (NEC). Because of their previous identification as bimodal O_2/CO_2 chemoreceptors (Jonz *et al.*, 2004; Qin *et al.*, 2010), adult zebrafish gill filament NECs were selected as the model system. The results of these experiments provide significant new information on the interrelationship among CO_2 , $[Ca^{2+}]_i$, pH_o and pH_i in CO_2 chemotransduction. The following discussion focuses on the similarities as well as the profound differences in the CO_2 chemotransduction cascade between zebrafish NECs and type I cells of the mammalian carotid body.

4.1 Zebrafish hyperventilation

Vulesevic *et al.* (2006) previously established that zebrafish respond to hypercapnia ($PCO_2=3.5$ mmHg) by increasing V_{amp} , and to hypoxia by increasing V_f . In the present study, I show for the first time, that at higher levels of hypercapnia (3% CO_2 , $PCO_2=22.5$ mmHg; Fig 1A), zebrafish display an increase in V_f . Because V_{amp} was not measured in parallel with V_f , it is difficult to determine whether or not the increase in V_f was accompanied by changes in V_{amp} , as has been documented for other species (Gilmour, 2001). As in previous studies (see review by Gilmour, 2001), zebrafish lacked ventilatory responses to changes in water pH. These findings reinforce the prevailing view that cardiorespiratory responses of fish are mediated by changes in P_wCO_2 and not water pH (Burlison and Smatrestk, 2000; McKendry *et al.*, 2001; McKendry and Perry, 2001; Perry and Reid, 2002; Gilmour *et al.*, 2005; Vulesevic *et al.*, 2006).

Vulesevic *et al.* (2006) previously described episodic breathing under resting conditions in the zebrafish, and found that episodic breathing was unaffected by hypercapnia, eliminated by hypoxia and increased by hyperoxia. Although not studied in detail, the findings of the present study show that high levels ($> 1\% \text{ CO}_2$) of hypercapnia eliminated episodic breathing, suggesting that the lower levels of hypercapnia used by Vulesevic *et al.* (2006) did not maximally alter respiratory drive. Indeed, these findings are consistent with the view that breathing patterns (episodic versus continuous) are determined, at least in part, by sensory afferent input from peripheral chemoreceptors (see review by Smatresk, 1990). As suggested by Reid *et al.* (2003), episodic breathing may be eliminated at high levels of respiratory drive owing to enhanced peripheral feedback, a view that is supported by the enhanced sensitivity of NECs to higher levels of hypercapnia.

NEC stimulation is believed to trigger a transduction pathway that ultimately results in ventilatory responses to hypoxia and hypercapnia. Consequently, the variability in the pattern of hyperventilation [increase in V_{amp} to $1\% \text{ CO}_2$ (Vulesevic *et al.* (2006), and increase in V_f to $5\% \text{ CO}_2$] may reflect the dose-dependent increase in $[\text{Ca}^{2+}]_i$ in NECs in response to hypercapnia (Fig 3). In fact, $5\% \text{ CO}_2$ may be stimulating a higher percentage of NECs, as 2 of the 7 NECs that responded to $5\% \text{ CO}_2$ with an increase in $[\text{Ca}^{2+}]_i$ did not respond to $1\% \text{ CO}_2$. Therefore, the augmented increase in $[\text{Ca}^{2+}]_i$ and the increased number of NECs responsive to $5\% \text{ CO}_2$, relative to $1\% \text{ CO}_2$, may lead to enhanced sensory afferent input resulting in the elimination of episodic breathing and the modulation of V_f .

4.2 Effect of hypercapnia on $[Ca^{2+}]_i$

Acidic hypercapnia and isocapnic acidosis elicited transient increases in $[Ca^{2+}]_i$ in NECs of the zebrafish gill filament, whereas NECs exposed to isohydric hypercapnia were unresponsive. In this study and as previously described by Qin *et al.* (2010), only a subset of NECs exposed to acidic hypercapnia stimuli were responsive. The process of cell isolation and culturing may have rendered some cells unresponsive. Alternatively, the number of responsive NECs may simply reflect the variability in sensitivity to stimuli, such that a higher proportion of cells respond to a greater acidic challenge. Regardless of the explanation, it is apparent that the proportion of NECs responding to an isocapnic acidosis challenge is greater than that responding to an acidic hypercapnia challenge (Table 1).

In the present study, NECs exhibited two distinct $[Ca^{2+}]_i$ profiles to acidic hypercapnia and isocapnic acidosis: a response to an acidic challenge with an abrupt transient increase in $[Ca^{2+}]_i$, was termed a type I response, whereas a type II response was characterized by a slow gradual increase in $[Ca^{2+}]_i$. Similar distinct Ca^{2+} profiles were recently reported in response to elevated ammonia in NECs of the trout gill filament (Zhang *et al.*, 2011). Zhang *et al.* (2011) suggested that type I responses (the “fast” component of type B responses in their study) may be a consequence of NH_4^+ mediated depolarization and that type II responses (referred to as type A responses in their study) could be attributed, at least in part, to NH_4^+ induced pH_i changes. In the present study, the two responses cannot be explained on the basis of pH_i changes (see below). However, the mechanism(s) mediating the type I response to acidic hypercapnia in the zebrafish, and the “fast” component of the type B responses to NH_4^+ in the trout may be similar

given the remarkably similar $[Ca^{2+}]_i$ profiles. The occurrence of two distinct responses to chemostimuli within isolated NECs may reflect different NEC populations, or more simply, cells of a single NEC population examined at varying stages of viability during the experiment. Type I responses were the focus of the present study, as they were typically found in higher proportions in response to acidic stimuli.

The increase $[Ca^{2+}]_i$ in response to acidic hypercapnia and isocapnic acidosis in NECs is consistent with data reported for mammalian type I cells (Buckler and Vaughan-Jones, 1993; Buckler and Vaughan-Jones, 1994). The profile of the $[Ca^{2+}]_i$ response to acidic hypercapnia and isocapnic acidosis in the NECs, however, differed from those exhibited by type I cells. Acidic hypercapnia and isocapnic acidosis induced an almost identical $[Ca^{2+}]_i$ response in NECs; an abrupt transient increase in $[Ca^{2+}]_i$ that was typically recovered well before the end of the stimulus period. Isolated type I cells of the carotid body, on the other hand, respond to an acidic challenge with an increase in $[Ca^{2+}]_i$ fluctuations that is sustained throughout the stimulus period (Buckler and Vaughan-Jones, 1993; Buckler and Vaughan-Jones, 1994). The differences in the $[Ca^{2+}]_i$ profiles between NECs and type I cells may reflect differences in the source of Ca^{2+} mediating the increase in $[Ca^{2+}]_i$ (see below).

Unlike in type I cells of the carotid body (Buckler and Vaughan-Jones, 1993), isohydric hypercapnia did not elicit a transient increase in $[Ca^{2+}]_i$ in NECs. In type I cells, the decrease in pH_i was suggested by Peers and Buckler (1995) to be the single factor that consistently changed in response to all 3 acidic challenges. Thus, it was proposed that the decrease in pH_i caused an inhibition of K^+ channels resulting in membrane depolarization and the influx of Ca^{2+} via voltage-gated Ca^{2+} channels of the plasma membrane (Buckler

et al., 1991; Iturriaga *et al.*, 1992; Buckler and Vaughan-Jones, 1993; Buckler and Vaughan-Jones, 1994). The results of the present study show that the common factor associated with increasing $[Ca^{2+}]_i$ in response to acidic challenges in NECs is a change in pH_o and not pH_i (see below) or extracellular PCO_2 . Based on these findings, it would appear that NECs of the zebrafish gill filament transduce the hypercapnic signal in a manner that is different than in type I cells of the carotid body.

The increase in $[Ca^{2+}]_i$ in response to changes in pH_o and not extracellular PCO_2 may be reflective of the overall orientation of the NECs. The precise orientation of NECs within the gill filament has been a subject of debate in recent years. To date, the bulk of available evidence supports the view that NECs are externally oriented sensing changes in P_wCO_2 (see review by Gilmour and Perry, 2006; Milsom, 2012), although there are also data supporting their internal orientation (Wood *et al.*, 1990; Wood and Munger, 1994). The results of the present study favors the premise that NECs elicit a Ca^{2+} response if their extracellular environment is acidified, and a definitive conclusion regarding their orientation is difficult to draw, especially given the ability of NECs to depolarize in response to acidic hypercapnia, isocapnic acidosis and isohydric hypercapnia (Qin *et al.*, 2010).

An increase in $[Ca^{2+}]_i$ was expected to be elicited in NECs in response to all 3 acidic stimuli, primarily owing to the observed depolarization under similar conditions (Qin *et al.*, 2010). Clearly, the results of this study suggest that there may be a dissociation between NEC membrane depolarization and the increase in $[Ca^{2+}]_i$, especially in response to isohydric hypercapnia. A critical role of type I cells in chemoreception is the release of neurotransmitters onto the CSN, triggering

cardiorespiratory responses to chemostimuli (see review by Gonzalez *et al.*, 1994; Iturriaga and Alcaayaga 2004; Nurse 2010). The acid induced rise in $[Ca^{2+}]_i$ in type I cells is a consequence of membrane depolarization (Buckler and Vaughan-Jones, 1994), and both components (membrane depolarization and increase in $[Ca^{2+}]_i$) of the chemotransduction cascade are thought to be critical for neurotransmitter release to the CSN (Buckler *et al.*, 1991b; Gonzalez *et al.*, 1992; Nurse, 2010). In NECs, it is not clear if membrane depolarization and an increase in $[Ca^{2+}]_i$ are potentially inclusive components to neurotransmitter release. It is important to remember that NEC depolarization and $[Ca^{2+}]_i$ regulation in response to acidic chemostimuli only represent individual components of the overall chemotransduction cascade.

4.3 Effect of hypercapnia on pH_i

The findings of the present study show, that as in type I cells, NECs exhibit intracellular acidification in response to all 3 acidic challenges. In response to acidic hypercapnia, NECs exhibited a rapid drop in pH_i that was sustained throughout the stimulus period, a response that is similar to that exhibited in type I cells of the carotid body (Buckler *et al.*, 1991a) and neurons from various chemosensitive brainstem regions in mammals (Kersh *et al.*, 2009; see review by Putnam *et al.*, 2004). The drop in pH_i with hypercapnia results from CA-mediated catalyzed hydration of CO_2 because treatment with the CA inhibitor acetazolamide reduced the rate of intracellular acidification (see below), as in type I cells (Buckler *et al.*, 1991b).

In the present study, NECs exhibited a sustained reduction in pH_i over the entire duration of the hypercapnic challenge. The maintained drop in pH_i to acidic hypercapnia may reflect a lack of pH-regulating transporters in the NECs as in some central

chemoreceptive neurons of the medulla (Rittucci *et al.*, 1997; Rittucci *et al.*, 1998). Alternatively, as in type I cells (Buckler *et al.*, 1991b), pH_i in NECs may be dependent on pH_o even in the presence of pH-regulating transporters. The sustained drop in pH_i to acidic hypercapnic exposure is not a unique property of chemoreceptors (Rittucci *et al.*, 1997; review by Putnam *et al.*, 2004). In fact, it has recently been demonstrated that a drop in pH_i is maintained in the trout hepatoma cell line, RTH 149 throughout the acidic exposure despite the presence of transmembrane acid-equivalent transport mechanisms (Huynh *et al.*, 2011). Although not yet identified, it is likely that NECs contain pH-regulating transporters.

Isocapnic acidosis caused a slower change in pH_i that never reached a new steady state, nor did it drop to the same $R_{495/440}$ elicited in response to acidic hypercapnia. However, given the gradual decrease in pH_i over the isocapnic acidosis exposure, I suspect that pH_i would have continued to decrease, perhaps reaching a drop in pH_i that was similar to that elicited in response to acidic hypercapnia. Isohydric hypercapnia reduced pH_i to similar levels observed in cells exposed to acidic hypercapnia. A subset of NECs exhibited partial recovery in pH_i during the isohydric hypercapnia exposure, a response that is similar to that reported in type I cells (Buckler *et al.*, 1991a). The variability in pH_i regulation during isohydric hypercapnia may reflect two different NEC populations; NECs with and without pH-regulating mechanisms. Overall, these results suggest, that changes in pH_o induce changes in pH_i , although not to the same extent as that exhibited by CO_2 . Consequently, pH_o is likely contributing to the sustained drop in pH_i during the hypercapnic exposure. Unlike type I cells, however, a steep linear relationship between pH_i and pH_o in NECs was not prominent.

4.4 Source of the increase in $[Ca^{2+}]_i$ in CO_2 chemoreception in NECs

The results of this study demonstrate that the acid-induced increase in $[Ca^{2+}]_i$ is not strictly dependent on the presence of Ca^{2+} in the extracellular solution. The data therefore implicate intracellular Ca^{2+} stores (e.g. the endoplasmic reticulum or mitochondrion) as the source of Ca^{2+} stores, and not influx via plasma membrane voltage-gated Ca^{2+} channels. These results differ markedly with those from type I cells and central chemoreceptors in mammals, in which the influx of Ca^{2+} via voltage-gated Ca^{2+} channels is accepted as the principal source of the hypercapnia-induced increase in $[Ca^{2+}]_i$ (Buckler and Vaughan-Jones, 1994; Peers and Buckler, 1995). However, internal Ca^{2+} stores may play a minor role in the elevation of $[Ca^{2+}]_i$ in type I cells. Indeed, in Ca^{2+} -free solutions, the increase in $[Ca^{2+}]_i$ during hypercapnic stimuli is not completely abolished, as a small increase in the $[Ca^{2+}]_i$ was still noted under these conditions (Buckler and Vaughan-Jones, 1993). Furthermore, Biscoe and Duchon (1990) demonstrated that the rise in $[Ca^{2+}]_i$ to a hypoxic stimulus originated largely from a mitochondrial Ca^{2+} pool. The contribution of intracellular Ca^{2+} stores to high a P_{CO} (>300 Torr) induced increase in $[Ca^{2+}]_i$ has also been established (Mokashi *et al.*, 1998). Rozanov *et al.* (1999) expanded on the findings by Mokashi *et al.* (1998) and proposed a capacitative Ca^{2+} entry model for CO sensing whereby the depletion of Ca^{2+} from intracellular stores stimulates the influx of extracellular Ca^{2+} via voltage gated calcium channels on the plasma membrane.

The precise intracellular pool contributing to the hypercapnia-induced increase in $[Ca^{2+}]_i$ in zebrafish NECs remains to be elucidated, but the results of the present study suggest that they are stimulated primarily by a decrease in pH_o . A lowering of

extracellular pH was shown to induce the release of Ca^{2+} from intracellular pools in rat brain synaptosomes (Saadoun *et al.*, 1997), parietal cells of intact rabbit gastric glands (Negulescu and Machen, 1990), and human fibroblasts (Smith *et al.*, 1989). The precise transduction cascade that is activated by a fall in pH_o remains to be elucidated, although phospholipase C, and consequently IP_3 -release Ca^{2+} pools, have been implicated (Smith *et al.*, 1989, Saadoun *et al.*, 1997). The contribution of IP_3 stores to the acid-induced rise in $[\text{Ca}^{2+}]_i$ of NECs remains largely speculative. In fact, the contribution of extracellular Ca^{2+} cannot be completely excluded, as evidence for capacitative Ca^{2+} entry was not investigated in this study.

4.5 Carbonic anhydrase and CO_2 chemoreception in NECs

The reduced rate of intracellular acidification in NECs after ACTZ treatment is consistent with observations in type I cells of the carotid body (Buckler *et al.*, 1991), and other non-chemoreceptive cells in which CA is found, such as Müller cells of the retina (Newman, 1994) and cultured human chondrocytes (Swietach *et al.*, 2002). These results suggest that, as in type I cells, pH_i likely contributes to membrane depolarization because ACTZ treated NECs exhibit a reduced rate and magnitude of depolarization (Qin *et al.*, 2010). Indeed, several pH_i -sensitive K^+ channels have been identified in type I cells and central chemoreceptors, including inwardly rectifying K^+ channels (K_{ir}), Ca^{2+} activated K^+ channels (K_{Ca}) (see Table 3 in Putnam, 2004) and transient receptor potential (TRP) channels (Hirata and Oku, 2010). Qin *et al.* (2010) implicated K^+ channels in the CO_2 sensing mechanism in NECs. The results of this study (reduced rate of intracellular acidification in response to ACTZ treatment) combined with the observations presented by Qin *et al.* (2010) suggest that pH_i -sensitive K^+ channels are likely found in NECs.

However, because NECs depolarize in response to isocapnic acidosis (Qin *et al.*, 2010) with little change in pH_i , it is likely that pH_o -sensitive K^+ channels are also contained within NECs. Candidate target channels include, voltage-sensitive K^+ channels (K_v), voltage-insensitive (or background) such as TWIK-related acid-sensitive K^+ channel (TASK) (see Table 3 in Putnam, 2004), and acid sensing ion channels (ASIC) (Tan *et al.*, 2007), all of which have been identified in type I cells (Buckler, 1999; Tan *et al.*, 2007; Buckler, 2007).

The lack of an effect of ACTZ on the magnitude of the increase in $[\text{Ca}^{2+}]_i$ or the latency to peak $\text{R}_{340/380}$, suggests a dissociation between pH_i and $[\text{Ca}^{2+}]_i$ regulation in response to acidic hypercapnia. Within central chemoreceptors and type I cells, ACTZ treatment delays and reduces the magnitude of neurosecretion onto afferent nerves (Hanson *et al.*, 1981; Iturriaga *et al.*, 1991); the effects on $[\text{Ca}^{2+}]_i$ have rarely been measured. Presumably, given the marked relationship between membrane depolarization, $[\text{Ca}^{2+}]_i$ and neurosecretion (Buckler and Vaughan-Jones, 1994b), ACTZ treatment within these mammalian chemoreceptors likely decreases the magnitude of the hypercapnia induced $[\text{Ca}^{2+}]_i$ increase. Indeed, ACTZ treatment of the glia-rich medullary cultures abolishes the hypercapnia induced $[\text{Ca}^{2+}]_i$ increase and decrease in pH_i (Hirata and Oku, 2010). However, Hirata and Oku (2010) pre-incubated the cells for 10 min in 10 mM ACTZ, a concentration that is substantially higher than that used in this study. Even though ACTZ mediates its effects on the mammalian chemotransduction cascade, its effects may not be strictly attributed to changes in pH_i and membrane depolarization. For example, the hypoxia induced rise in $[\text{Ca}^{2+}]_i$ in pulmonary arterial smooth muscle cells (PASMC) was decreased by ACTZ treatment independent of membrane potential, CA

inhibition or changes in pH_i (Shimoda *et al.*, 2006). Indeed, the effects of ACTZ on ion channels are widespread and it may be mediating its effects via multiple mechanisms (Chipperfield and Harper, 1993; Tricarico *et al.*, 2000; Pickkers *et al.*, 2001; McNaughton *et al.*, 2004; Tricarico *et al.*, 2004). Regardless, the results of this study suggest that the increase in the $[\text{Ca}^{2+}]_i$ to hypercapnia in NECs is independent of a CA mediated mechanism.

4.6 Relationship between pH_i and $[\text{Ca}^{2+}]_i$

Intracellular acidification preceded the acid-induced increase in $[\text{Ca}^{2+}]_i$, results that are similar to observations in the carotid body (Buckler *et al.*, 1991; Buckler and Vaughan-Jones, 1993) and glia-rich medullary cultures (Hirata and Oku, 2010). The drop in pH_i in NECs was 50% complete in 31.5 ± 4.4 s, which is comparable to the 20-50 s required for the change in pH_i to be 50% complete in the type I cells (Buckler *et al.*, 1991). However, the time to reach peak $\text{R}_{340/380}$ in NECs in response to acidic hypercapnia and isocapnic acidosis (52.3 ± 3.8 s and 59.7 ± 7.3 s, respectively) was substantially longer than that exhibited in type I cells (Buckler and Vaughan-Jones, 1993). Indeed, it seems unlikely that such a sensitive cellular process would be delayed by almost a minute after its stimulation. These exaggerated results may simply reflect a poor solution exchange time within our chamber. In fact, Buckler *et al.* (1991) noted that the rate of intracellular acidification in type I cells is rate limited by the solution exchange time. By improving their bath-exchange time, the time to reach 50% of CO_2 induced acidification in type I cells was reduced from 20-50 s to ~ 4 s. Despite these exaggerated times, it is interesting to note that the time to reach peak $\text{R}_{340/380}$ in response to acidic hypercapnia and isocapnic acidosis in NECs was almost identical, whereas the

time to reach peak $[Ca^{2+}]_i$ in type I cells is 1.5 times slower in response to isocapnic acidosis than to acidic hypercapnia (Buckler and Vaughan-Jones, 1993). Although the poor bath-exchange time may be masking a difference in the latency to reach maximum $R_{340/380}$, the results at present (latency to peak $R_{340/380}$), coupled with the remarkably similar peak $R_{340/380}$ responses suggest that acidic hypercapnia and isocapnic acidosis act via a similar, indeed, almost identical mechanism to stimulate an increase in the $[Ca^{2+}]_i$. As discussed above, pH_i appears to be regulated differently in response to these two chemostimuli, as such, it seems unlikely that the increase in $[Ca^{2+}]_i$ is a consequence of the hypercapnia induced intracellular acidification. Furthermore, isohydric hypercapnia induces intracellular acidification in the absence of a marked increase in $[Ca^{2+}]_i$, supporting the argument that pH_i is independent of the $[Ca^{2+}]_i$ response to hypercapnia.

In order to further elucidate the relationship between the pH_i and $[Ca^{2+}]_i$ in the hypercapnic chemotransduction cascade, acetate was used to induce intracellular acidification in the absence of CO_2 or changes in pH_o . The results of this experiment further support the ACTZ data, and show that changes in pH_i are independent of changes in $[Ca^{2+}]_i$. Although the magnitude of the acetate induced acidosis was smaller than that elicited by 5% hypercapnia, it was comparable to the acidification induced by 1% hypercapnia and isocapnic acidosis. Therefore, the acidosis induced by acetate, especially when compared with the pH_i -induced drop by isocapnic acidosis, should have been sufficient to evoke an increase in $[Ca^{2+}]_i$ if the hypercapnia induced changes in pH_i and $[Ca^{2+}]_i$ were coupled. Because NECs did not augment their $[Ca^{2+}]_i$ to acetate, but were still capable of responding so subsequent hypercapnic stimuli, it would suggest that a drop in pH_i does not stimulate an increase in $[Ca^{2+}]_i$. These findings are at odds with

those reported by Sato (1994), in which it was demonstrated that acetate induced intracellular acidification and an increase in $[Ca^{2+}]_i$ in type I cells of the newborn rabbit carotid body. However, more recently, Summers *et al.* (2002) demonstrated that the effects of hypercapnia on the L-type Ca^{2+} current in the rabbit carotid body are not secondary to changes in pH_i , but may be a consequence of a protein kinase A-dependent mechanism. Clearly, the relationship between pH_i and $[Ca^{2+}]_i$ in type I cells is complex, and remains to be fully elucidated.

4.7 Summary and perspectives

Hypercapnia is associated with changes in P_wCO_2 , P_aCO_2 , water pH and blood pH, and therefore, it may be mediating its effect by any combination of these factors. The results of this thesis suggest that the principal factor promoting the increase in $[Ca^{2+}]_i$ in zebrafish NECs during hypercapnic stimuli is pH_o . Extracellular acidification induces membrane depolarization (Qin *et al.*, 2010), a decrease in pH_i and an increase in $[Ca^{2+}]_i$. Intracellular acidification may contribute to membrane depolarization, but its effects are independent of the increase in $[Ca^{2+}]_i$ since extracellular Ca^{2+} does not appear to be involved. pH_o and not CO_2 or pH_i appears to trigger the release of Ca^{2+} from an unidentified intracellular pool. These results demonstrate several similarities and difference with mammalian chemoreceptors (specifically with type I cells), and provide further support for an evolutionary link between gill NECs and peripheral chemoreceptors of the carotid body.

The cardiorespiratory reflexes initiated by fish to a hypercapnic stimulus originate, presumably, from the activation of the NECs. It has been proposed that NEC activation triggers neurotransmitter release onto the afferent nerves leading to

cardiorespiratory compensation. Within this study, it has been demonstrated that there may be a disconnect between membrane depolarization and an increase in the $[Ca^{2+}]_i$ in response to an acidic challenge. Moving forward, it would be of interest to identify the neurotransmitters released in response to hypercapnia, and the relative contribution of membrane depolarization and $[Ca^{2+}]_i$ to this response. Additionally, it would be of interest to identify the calcium pools contributing to the increase in $[Ca^{2+}]_i$ during hypercapnia.

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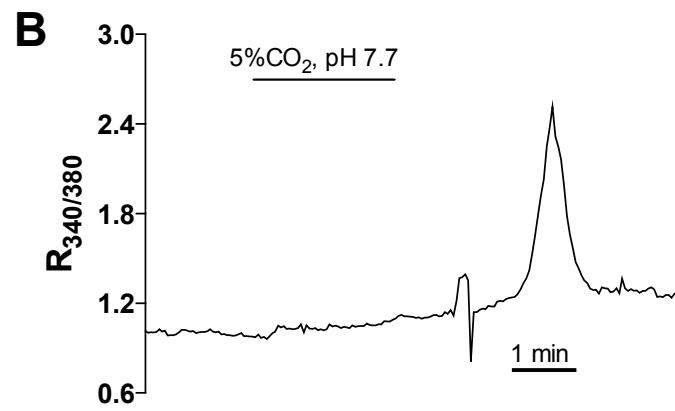
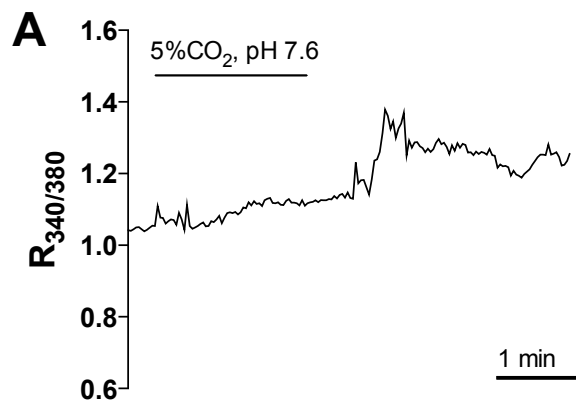


Fig S1. $[Ca^{2+}]_i$ responses of isolated neuroepithelial cells (NECs) of zebrafish, *Danio rerio* to isohydric hypercapnia (5% CO₂, pH ~7.8). (A) Representative trace of NEC that increased its baseline R_{340/380} and (B) exhibited a transient increase in R_{340/380} upon reperfusion with normocapnic solution after exposure to isohydric hypercapnia.

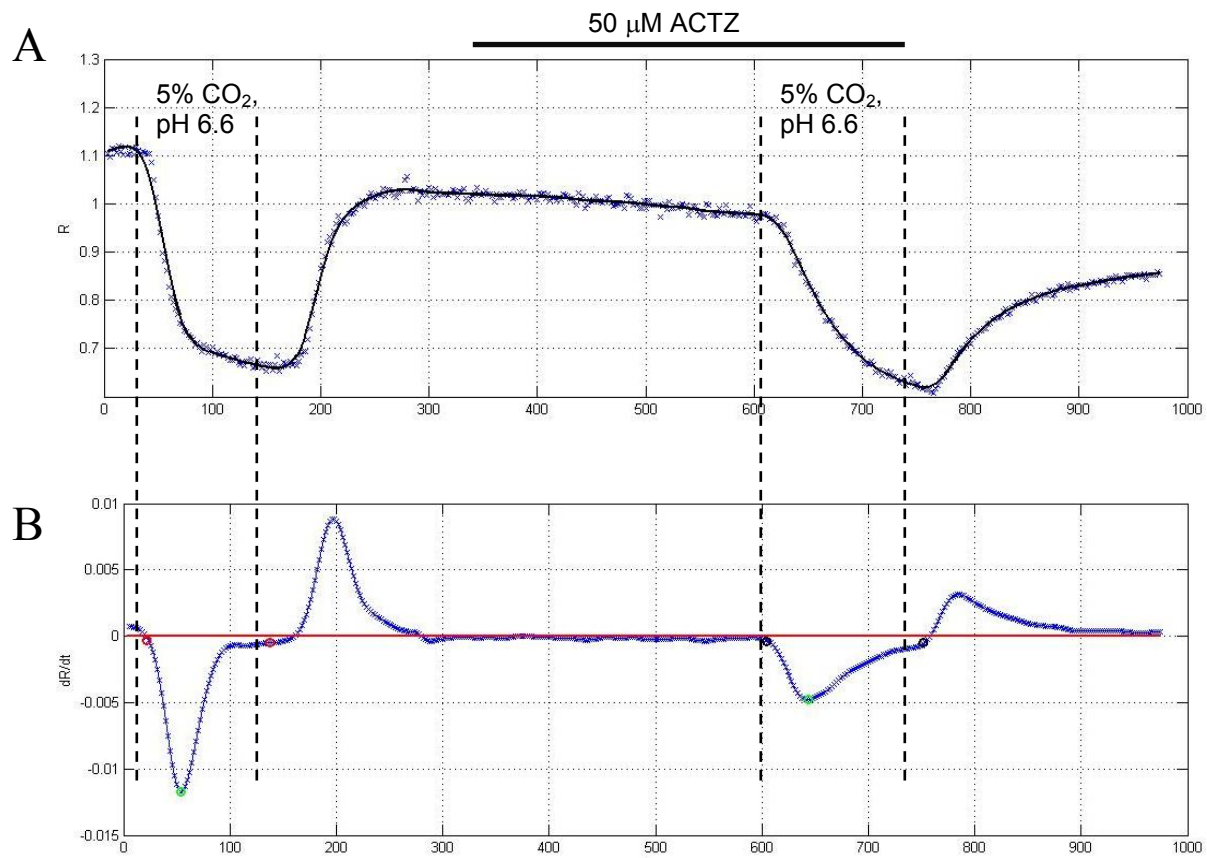


Fig S2. pH_i response of isolated neuroepithelial cell (NEC) of zebrafish, *Danio rerio* to acidic hypercapnia (5% CO_2 , $\text{pH} \sim 7.8$) before and after ACTZ treatment. (A) Representative trace of NECs responding to acidic hypercapnia before (first bout) and after 5 min of ACTZ incubation (second bout). The solid black line through the blue data points represents the smoothed data. (B) The derivative of (A). Note that the green circle denotes the point at which the maximal rate of change occurred.