Comparative Biochemistry and Physiology, Part A 214 (2017) 50–57

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Purinergic and adenosine receptors contribute to hypoxic hyperventilation in zebrafish (*Danio rerio*)

Alisha J. Coe, Alexina J. Picard, Michael G. Jonz*

Department of Biology, University of Ottawa, 30 Marie Curie Pvt., Ottawa, ON K1N 6N5, Canada

ARTICLE INFO

Keywords:
ATP
P2X
Adenosine
Purinergic
Neurotransmitter
Zebrafish
Hypoxia
Chemical screen
NEC

ABSTRACT

The chemoreceptors involved in oxygen sensing in teleost fish are neuroepithelial cells (NECs) in the gills, and are analogous to glomus cells in the mammalian carotid body. Purinergic signalling mechanisms involving the neurotransmitters, ATP and adenosine, have been identified in mediating hypoxic signalling in the carotid body, but these pathways are not well understood in the fish gill. The present study used a behavioural assay to screen for the effects of drugs, that target purinergic and adenosine receptors, on the hyperventilatory response to hypoxia in larval zebrafish (*Danio rerio*) in order to determine if the receptors on which these drugs act may be involved in hypoxic signalling. The purinergic receptor antagonist, PPADS, targets purinergic P2X2/3 receptors and inhibited the hyperventilatory response to hypoxia (IC_{50} = 18.9 μM). The broad-spectrum purinergic agonist, ATPγS, elicited a hyperventilatory response (EC_{50} = 168 μM). The non-specific adenosine receptor antagonist, caffeine, inhibited the hyperventilatory response to hypoxia, as did the specific A2a receptor antagonist, SCH58261 (IC_{50} = 220 nM). These results suggest that P2X2/3 and A2a receptors are candidates for mediating hypoxic hyperventilation in zebrafish. This study highlights the potential of applying chemical screening to ventilatory behaviour in zebrafish to further our understanding of the pathways involved in signalling by gill NECs and oxygen sensing in vertebrates.

1. Introduction

Vertebrates monitor internal and external partial pressures of oxygen (P_{O2}) in order to ensure that there is adequate oxygen to carry out the processes necessary for survival. Low P_{O2}, or hypoxia, activates cardiorespiratory reflexes, which cause an increase in ventilatory rate in order to compensate for decreased oxygen uptake (reviewed Perry et al. 2009; Kumar and Prabhakar 2012). In mammals, the carotid body is the main peripheral organ that senses arterial P_{O2} (González et al. 1994; López-Barneo et al. 2008; Kumar and Prabhakar 2012; Nurse 2014), and neuroepithelial bodies in the lung epithelium sense external P_{O2} (Fu et al. 2004; Cutz et al. 2013). In teleost fish, both internal and external P_{O2} are sensed by chemoreceptors in the gills, called neuroepithelial cells (NECs; Burleson et al. 1992; Perry et al. 2009; Jonz 2014).

Many aspects of the process of chemoreception in the teleost gill are similar to those of the mammalian carotid body, and NECs are homologous or analogous to type I cells of the carotid body (Milsom and Burleson 2007; Zachar and Jonz 2012; Hockman et al. 2017). Within the carotid body are two main cell types: chemoreceptive type I (glomus) cells and glial-like type II cells (Gonzalez et al., 1994; Kumar and Prabhakar 2012). ATP released by type I cells mediates hypoxic signalling by acting on purinergic P2X and P2Y receptors. P2X2/3 receptors located on afferent nerve terminals play an excitatory role in hypoxic signalling (Zhang et al. 2000; Zhang and Nurse 2004), and P2Y2 receptors located on type II cells are thought to participate in the paracrine crosstalk between type I and II cells that amplifies the ATP signal onto afferent nerve terminals in the carotid body (Nurse 2014). In addition to ATP pathways, adenosine also participates in autocrine/paracrine signalling of hypoxia (Nurse 2014). Adenosine is generated via the breakdown of extracellular ATP by ecto-5′-nucleotidase and acts on adenosine A2a receptors to enhance the hypoxia response of type I cells (Conde et al. 2006; Piskuric and Nurse 2013). Thus, purinergic and adenosine signalling systems are crucial for hypoxia signalling in the carotid body.

The zebrafish (*Danio rerio*) is a powerful model organism in vertebrate biology. Oxygen-chemoreceptive NECs in zebrafish are located in the gill filaments and receive sensory innervation from intrabranchial neurons and fibres of the cranial nerves (Jonz and Nurse 2003; Jonz et al. 2004). NECs are characterized by immunoreactivity to serotonin and synaptic vesicle glycoprotein SV2 (Jonz and Nurse 2003), and NEC-like cells immunoreactive for vesicular acetylcholine transporters have

* Corresponding author.
E-mail address: mjonz@uottawa.ca (M.G. Jonz).

http://dx.doi.org/10.1016/j.cbpa.2017.09.013
Received 21 July 2017; Received in revised form 15 September 2017; Accepted 15 September 2017
Available online 21 September 2017

also been reported in the zebrafish gill (Shakarchi et al. 2013; Zachar et al. 2017). Studies on isolated gill arches in trout demonstrated that exogenous application of serotonin and acetylcholine increased spike frequency in afferent nerve fibres that innervate gill chemoreceptors (Burleson and Milsom 1995a, 1995b). Thus, while the importance of serotonin and acetylcholine in oxygen sensing in the gills has been established, the role of ATP and adenosine as signalling molecules are not well understood. Purinergic P2X3 receptors were found in gill cells, potentially neurons, in developing zebrafish and also co-localized with the serotonin-immunoreactive NECs; and purinergic receptor antagonists, suramin and A-317491, inhibited the hyperventilatory response in hypoxia in zebrafish larvae (Rahbar et al., 2016). These data support the stimulatory role of ATP acting on P2X3 or heteromeric P2X2/3 receptors in NECs, similar to the P2X2/3 receptors in the carotid body (Nurse 2014). A potential role for A2a receptors in the hypoxic hyperventilatory response in any fish has not been explored.

Given the importance of purinergic and adenosine signalling in the mammalian carotid body, we hypothesized that exogenous application of neurochemicals that target purinergic and adenosine receptors would accordingly modify the hyperventilatory response to hypoxia via chemosensory pathways in the gills. Zebrafish have conserved pharmacological targets comparable with those of mammals (Rinkwitz et al. 2011; Howe et al. 2013). We used procedures previously developed in our laboratory, with which we have screened and identified a number of neurochemicals that act rapidly upon the gills to modify ventilatory responses to hypoxia (Shakarchi et al. 2013; Jonz et al. 2015; Rahbar et al., 2016). The present results suggest the involvement of P2X2/3 and A2a receptors in mediating the hyperventilatory response to hypoxia in zebrafish, and will guide future studies aimed at confirming the sites and specific role(s) of these receptors in the gills.

2. Materials and methods

2.1. Animals

Animals were handled in accordance with the Canadian Council on Animal Care (CCAC), and protocols were approved by the University of Ottawa Animal Care Committee (protocol no. BL-1760). Experiments were performed under MS-222 (tricaine) to minimize stress to the animals.

Wild-type zebrafish (*Danio rerio*) adults bred to produce larvae used in this study were held in a closed re-circulated facility at the University of Ottawa. Animals were maintained at 28.5 °C on a 14:10 h light:dark cycle (Westerfield 2000). Breeding techniques were based on those used in Westerfield (2000). Once embryos were collected, they were placed in a 28.5 °C incubator in Petri dishes of E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2{\cdot}2H_2O$, 0.33 mM $MgSO_4{\cdot}7H_2O$, and 0.3 mg/l methylene blue at pH 7.8). After 1 day post-fertilization, embryo medium was replaced and dead embryos were removed. At 5 days post-fertilization, larvae were transferred to small tanks filled with system water and methylene blue at 0.3 mg/l.

2.2. Behavioural assays

The behavioural assays used in this experiment were adapted from Rahbar et al. (2016). Zebrafish larvae aged 15–16 days post-fertilization were used since the gills are fully developed at this time, and thus the larvae rely primarily on branchial, instead of cutaneous, respiration (Jonz and Nurse 2005). An opaque, flat-bottomed 96-well plate (Greiner Cellstar, Sigma-Aldrich, Oakville, ON, Canada) was pre-filled with Sylgard (Dow Corning Corporation, Midland, MI, USA) to reduce the well volume to ~100 µl in order to minimize the movement of the larvae out of the focal plane. Each well was filled with 50 µl of 1% methylcellulose with 0.04 mg/ml tricaine (Aqualife TMS, Syndel Laboratories, Vancouver, Canada). Methylcellulose was used to reduce the movement of larvae while minimizing the required concentration of tricaine. The remaining 50 µl of each well was then filled with the treatment drug plus 0.04 mg/ml tricaine or tricaine alone.

Larvae were not fed the day before experimentation since preliminary experiments showed a high ventilation frequency (f_v) if food was visibly present in their digestive systems. Larvae were captured from their tank with a Pasteur pipette, and were transferred directly into wells containing methylcellulose. Each well contained 1 larva, and up to 8 larvae were tested in a plate per trial. The transfer of tank water to the well was minimized as much as possible by placing the pipette tip to the methylcellulose surface, allowing the larva to swim out. The plate was then placed on the stage of a stereomicroscope (MZ6, Leica, Wetzlar, Germany). f_v measurements were taken 10 min after the transfer to ensure acclimation to the new environment. f_v was determined by recording a 10-s video using Leica Application Suite (Leica, Wetzlar, Germany), and counting the number of buccal or opercular movements of the larvae post hoc. To obtain f_v in min^{-1}, the rate was multiplied by 6. If the position of the larva was not optimal, fin or head movements (which are coordinated with ventilation; Jonz and Nurse 2005) were counted instead.

After the baseline f_v was determined, different concentrations of either stimulatory (agonist) or inhibitory (antagonist) drugs were applied in the absence or presence of hypoxia, respectively. For agonist drugs, the plate was left at room temperature (23–24 °C) and f_v was recorded after 5 min. For antagonist drugs, the plate was immediately placed in a 28.5 °C incubator (Forma 3130, ThermoFisher Scientific, Ottawa, ON, Canada) for 7 min. 100% N_2 was previously injected into the incubator to lower the O_2 level to 2% (P_{O2} = 14 mm Hg). Incubator P_{O2} was measured and stabilized with a thermal conductivity O_2 sensor, and a previous study determined that 7 min of hypoxia under these conditions produced a maximal f_v response (Rahbar et al., 2016). In addition, preliminary experiments had indicated that, at normal P_{O2}, the change from room temperature to 28.5 °C did not significantly affect f_v (data not shown). After hypoxic exposure, the plate was removed from the incubator and f_v measurements were made immediately. After treatment measurements were made, larvae were transferred to a new well containing 50 µl methylcellulose with tricaine. Recovery f_v was measured 5 min after transfer.

2.3. Drugs

Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS), adenosine 5′-(3-thiotriphosphate) (ATPγS), caffeine, and 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261) were obtained from Sigma Aldrich Corp. (Oakville, ON). All drugs were dissolved in water and 0.04 mg/ml tricaine. ATPγS and SCH58261 were first dissolved in dimethyl sulfoxide (DMSO) to give a maximum concentration of 0.5% in the wells, in order to improve drug solubility and delivery.

2.4. Data analysis

Treatment f_v was compared to baseline using a Wilcoxon matched-pairs signed rank test. Larvae that were not breathing or were hyperventilating (f_v > 120 min^{-1}) at baseline were excluded from data analysis. Furthermore, agonist and control group larvae that stopped breathing during treatment, and did not recover during the recovery phase of the experiments, were also excluded.

To compare the f_v response across concentrations of each drug, dose-response curves were plotted as normalized response ratio vs. drug concentration. This procedure was necessary because basal f_v varied between trials. The response ratio therefore presented a clear measure of the response within each trial relative to control. Mean *response ratios* were calculated by dividing treatment f_v by baseline f_v for each fish, and then taking the mean for each group. The response ratios for each concentration were normalized using the following equation: