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Evidence of anoxia-induced channel arrest in the brain of the goldfish (*Carassius auratus*)☆

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ABSTRACT

The common goldfish (*Carassius auratus*) is extremely anoxia tolerant and here we provide evidence that "channel arrest" in the brain of these fish contributes to ATP conservation during periods of anoxia. Whole-cell patch-clamp recordings of slices taken from the telencephalon indicated that the *N*-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamate receptor and Ca^{2+}-channel, underwent a 40–50% reduction in activity during 40 min of acute anoxia. This is the first direct evidence of channel arrest in an anoxia-tolerant fish. Because goldfish produce ethanol as a byproduct of anaerobic metabolism we then conducted experiments to determine if the observed reduction in NMDA receptor current amplitude was due to inhibition by ethanol. NMDA receptor currents were not inhibited by ethanol (10 mmol L^{-1}), suggesting that channel arrest of the receptor involved other mechanisms. Longer-term (48 h) *in vivo* exposure of goldfish to anoxic conditions (less than 1% dissolved O_2) provided indirect evidence that a reduction in Na^+/K^+-ATPase activity also contributed to ATP conservation in the brain but not the gills. Anoxia under these conditions was characterized by a decrease in brain Na^+/K^+-ATPase activity of 30–40% by 24 h. Despite 90% reductions in the rates of ventilation, no change was observed in gill Na^+/K^+-ATPase activity during the 48-h anoxia exposure, suggesting that branchial ion permeability was unaffected. We conclude that rapid "channel arrest" of NMDA receptors likely prevents excitotoxicity in the brain of the goldfish, and that a more slowly developing decrease in Na^+/K^+-ATPase activity also contributes to the profound metabolic depression seen in these animals during oxygen starvation.

1. Introduction

The freshwater turtles *Chrysemys picta* and *Trachemys scripta*, crucian carp (*Carassius carassius*), and goldfish (*Carassius auratus*) are four of the most anoxia-tolerant vertebrates known, and are capable of surviving days or weeks without oxygen at low temperatures (see Lutz and Nilsson, 1997; Nilsson, 2001; Bickler and Buck, 2007 for reviews). However, the strategies used by these animals to survive anoxia are distinct. The turtles become dormant as they overwinter for up to 4 months at the bottom of lakes, ponds or streams, without oxygen (Jackson et al., 1984). In contrast, the crucian carp and goldfish remain active during prolonged anoxia, albeit at reduced activity levels, which provides them with the opportunity to seek out oxygenated waters (Nilsson et al., 1993; Rausch et al., 2000).

During anoxia, these facultative anaerobes reduce their ATP demands to a degree greater than that predicted by temperature-dependent metabolic depression. Further, anoxic ATP production proceeds solely by glycolysis which leads to the generation of lactate and metabolic acid (Jackson et al., 1984; Lutz and Nilsson, 1997; Bickler and Buck, 2007). The turtle is capable of tolerating very high lactate and metabolic acid loads, much of which is buffered by the shell (Jackson et al., 1984). The crucian carp and goldfish, on the other hand, avoid such "self-pollution" (Lutz and Nilsson, 1997) by transporting the lactate to the muscle where it is converted into ethanol via alcohol dehydrogenase (Shoubridge and Hochachka, 1980; Nilsson, 1988) and excreted across the gills (Shoubridge and Hochachka, 1980; Johnston and Bernard, 1983; Nilsson, 1991).

In most vertebrate neurons, anoxia starves ion motive transporters, such as the Na^+/K^+-ATPase, of ATP. As a result transmembrane ion gradients breakdown, causing neurons to depolarize due to the loss of intracellular K^+, and gain of intracellular Na^+ and Ca^{2+}. The breakdown of ion gradients results in the excess release of neurotransmitters such as glutamate, which contributes to overactivation of NMDA receptors, further increasing intracellular Ca^{2+} concentration, and the activation of proteases which contribute to cytoskeletal breakdown. Further,

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increases in [Ca^{2+}] promote the generation of reactive oxygen species (ROS) during subsequent re-oxygenation/re-perfusion and further neuron damage. Together, NMDA receptor overactivation, loss of ion gradients, and ATP depletion result in cell swelling, membrane blebbing and finally necrosis. These events are collectively termed "excitotoxicity" (see Nilsson, 2001; Mishra et al., 2001; Sattler and Tymianski, 2000; Walsh et al., 2007 for reviews).

Under anoxic conditions, ATP may be conserved by reducing the permeability of the plasma membrane to ions through "channel arrest" (Hochachka, 1986), which would decrease the ATP requirements of ion motive pumps such as the Na^+/K^+-ATPase. In neurons, channel arrest of excitatory receptors permeable to Ca^{2+} would also minimize the risk of excitotoxic cell death caused by excess intracellular Ca^{2+} accumulation (Nilsson, 2001; Bickler and Buck, 2007). In the red-eared slider (*T. scripta*) anoxia-induced decreases in the abundance of voltage gated Na^+ channels (Pérez-Pinzón et al., 1992), reduced K^+ permeability in brain (Chih et al., 1989), and reduced Na^+/K^+-ATPase activity in brain (Hylland et al., 1997) and in hepatocytes (Buck and Hochachka, 1993) are indicative of channel arrest. The only direct measures of channel arrest are the reduction in NMDA receptor, and more recently AMPA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor, currents reported in anoxic turtle cortical sheets using single-channel and/or whole-cell patch-clamping (Buck and Bickler, 1998; Bickler et al., 2000; Shin and Buck, 2003; Shin et al., 2005; Pamenter et al., 2008).

There is little support for the channel arrest hypothesis in crucian carp or goldfish (Lutz and Nilsson, 1997). However, there is evidence of metabolic depression under anoxic conditions using a crucian carp brain slice model (Johansson et al., 1995), and respective reductions in the sensitivity of visual (Johansson et al., 1997) and auditory nerves (Suzue et al., 1987; Fay and Ream, 1992) have been reported during anoxia and hypoxia in crucian carp and goldfish. The goal of the present study was to examine channel arrest of the NMDA receptor during *in vitro* anoxia in goldfish telencephalon slices using the whole-cell patch-clamp technique. Because ethanol is known to interfere with mammalian NMDA receptor function (Lovinger et al., 1989), another objective was to test the hypothesis that the ethanol inhibits NMDA receptors during anoxia. In a second series of experiments, we also measured Na^+/K^+-ATPase activity in the brains and gills of goldfish exposed to anoxia *in vivo*, to determine if other mechanisms of ion channel arrest might be used during longer-term (48 h) oxygen starvation.

2. Methods and materials

2.1. Experimental animals and holding

Large goldfish (common comets; *C. auratus*; 50–100 g) used in patch-clamp studies were purchased from a commercial supplier (Aleong International, Mississauga, Ontario) in the summer–autumn of 2003 and 2004 and held in flowing dechlorinated City of Toronto tap water at 15–20 °C at the University of Toronto. Smaller goldfish (5–10 g) were purchased in late autumn 2006 and used for *in vivo* experiments investigating the effects of anoxia on Na^+/K^+-ATPase activity in the brain and gills. The smaller fish were held in flowing (flow rate ~ 1–2 L min^{-1}), aerated (dissolved oxygen > 90% saturation), well water [composition (in mmol L^{-1}) Na^+ ~ 0.8; Cl^- ~ 0.5; Ca^{2+} ~ 3; pH 8.0; temperature 12 ± 1 °C] in 110-L tanks at Wilfrid Laurier University. All fish were fed three times weekly with appropriately sized commercial pellets, but the smaller fish were starved for 1 week prior to *in vivo* anoxia exposure experiments to minimize the effects that build-ups of nitrogenous waste could have on their response to oxygen starvation.

2.2. Experimental protocols

2.2.1. Whole-cell patch-clamping of goldfish brain slices

The whole brain of the large goldfish was removed from the cranium following decapitation of the fish and immediately placed in a solution of oxygenated and chilled (4 °C) artificial cerebrospinal fluid [aCSF; Composition (in mmol L^{-1}): NaCl 107, KCl 2.6, NaH_2PO_4 2.0, $NaHCO_3$ 26.5, glucose 20, HEPES–Na 10.0, $MgCl_2$ 1.0, $CaCl_2$ 1.2, osmolality 280–290 mOsm; pH 7.6]. Both lobes of the telencephalon were then dissected away from the main body of the brain while in chilled aCSF solution, and temporarily stored in a vial (15 mL) of oxygenated aCSF on ice. Within 15 min, each telencephalon was affixed to a sectioning block using cyanoacrylate glue ("Krazy Glue"), and submerged in ice-cold aCSF contained in the reservoir of a Vibtratome 1000 tissue slicer (Vibratome, St. Louis, MO, USA). Tissue slices were then cut in the parasaggital plane (300 μm thick; 3–4 for each lobe). Slices were gently lifted out of the reservoir using a fine paint brush and transferred to a vial of oxygenated aCSF and stored for up to 48 h. Preliminary experiments revealed that the slices remained electrophysiological viable, and capable of generating action potentials and NMDA receptor currents, for up to 48 h following their preparation.

Individual telencephalon slices were placed on a coverslip contained in a flow-through perfusion chamber (RC-26, Warner Instruments, Hamden, CT, USA) and held in place by a horseshoe-shaped stainless steel slice hold-down anchor across which strands of lycra thread were stretched at 2-mm intervals (Warner Instruments). The chamber was gravity perfused with oxygenated or anoxic aCSF (at room temperature — 22–23 °C) from a 1.0-L glass bottle via an intravenous (IV) dripper. A fast-step drug perfusion system (VC-6 Perfusion System, Warner Instruments) was used to deliver tetrotodotoxin (TTX; 1 μmol L^{-1}), *N*-methyl-D-aspartate (NMDA; 300 μmol L^{-1}), or other drugs to the slice (e.g. MK801, ethanol; see below) during whole-cell patch-clamp recording experiments.

Whole-cell patch recordings were made using 2–5-MΩ pipettes, into which a Ag–AgCl electrode was connected to a CV-4 headstage and AxoPatch-1D amplifier. The electrolyte solution in the recording electrode was composed of (in mmol L^{-1}) NaCl 8, $CaCl_2$ 0.0001, HEPES–Na 10, KCl 20, potassium gluconate 110; $MgCl_2$ 1, NaGTP 0.3; NaATP 2 (pH 7.4). Gigaohm seals (5–10 GΩ) were established by advancing the recording electrode towards the slice in μm increments using a "stepper-motor". A whole-cell configuration was established by voltage-clamping the recording electrode potential to −60 mV, and applying a sharp pulse of suction. The resting membrane potential was then recorded by switching from the voltage clamp to zero-current setting of the amplifier. Resting membrane potentials typically ranged from −50 to −70 mV. Data were collected using a TL-1 DMA interface (Axon Instruments) connected to the amplifier, and digitized and stored on a personal computer with Clampex 6 software (Axon Instruments).

2.2.2. Measurement of NMDA receptor currents and effects of MK801 and Mg^{2+}

NMDA receptor currents were measured from slices pre-perfused with TTX for 10 min prior to NMDA application. TTX suppressed action potentials that could interfere with NMDA receptor current measurement. Neurons were voltage clamped at −70 mV and NMDA applied to the preparation until a current response was noted (usually 5–10 s). The current response was followed for 5 min, when the amplifier was switched back to zero-current mode from voltage-clamp mode.

The irreversible NMDA receptor antagonist — MK801, and high doses of Mg^{2+} (4 mmol L^{-1}) were also applied to block/inhibit NMDA receptor currents and confirm that we were measuring NMDA receptor-mediated currents. Because MK801 is an open channel NMDA receptor antagonist, the slice was pre-perfused with the blocker for 10 min and a second NMDA receptor current was initiated to allow the drug to penetrate and block the open channel. Additional NMDA receptor recordings were then made at 10 min intervals to confirm an effective blockade. High concentrations of Mg^{2+} reversibly abolish NMDA receptor currents. Accordingly, NMDA receptor currents were measured in slices bathed in aCSF containing 0.1 mmol L^{-1} Mg^{2+}, followed by 10 min of perfusion with 4 mmol L^{-1} Mg^{2+} to confirm blockade of the channel.