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Effect of anoxia on the electroretinogram of three anoxia-tolerant vertebrates

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ABSTRACT

To survive anoxia, neural ATP levels have to be defended. Reducing electrical activity, which accounts for 50% or more of neural energy consumption, should be beneficial for anoxic survival. The retina is a hypoxia sensitive part of the central nervous system. Here, we quantify the *in vivo* retinal light response (electroretinogram; ERG) in three vertebrates that exhibit varying degrees of anoxia tolerance: freshwater turtle (*Trachemys scripta*), epaulette shark (*Hemiscyllium ocellatum*) and leopard frog (*Rana pipiens*). A virtually total suppression of ERG in anoxia, probably resulting in functional blindness, has previously been seen in the extremely anoxia-tolerant crucian carp (*Carassius carassius*). Surprisingly, the equally anoxia-tolerant turtle, which strongly depresses brain and whole-body metabolism during anoxia, exhibited a relatively modest anoxic reduction in ERG: the combined amplitude of turtle ERG waves was reduced by ~50% after 2 h. In contrast, the shark b-wave amplitude practically disappeared after 30 min of severe hypoxia, and the frog b-wave was decreased by ~75% after 40 min in anoxia. The specific A_1 adenosine receptor antagonist CPT significantly delayed the suppression of turtle ERG, while the hypoxic shark ERG was unaffected by the non-specific adenosine receptor antagonist aminophylline, suggesting adenosinergic involvement in turtle but not in shark.

1. Introduction

Prolonged anoxia tolerance is limited to a few vertebrate species. Most spectacularly, the crucian carp (*Carassius carassius*) and the freshwater turtles (genera *Trachemys* and *Chrysemys*) can survive without oxygen for 24–48 h at room temperature and for months when acclimated to temperatures near 0 °C (Nilsson and Lutz, 2004). More moderately, the epaulette shark (*Hemiscyllium ocellatum*), which lives on tropical coral reef platforms that become severely hypoxic with nocturnal low tide, can survive approximately 1 h of anoxia at 28 °C (Renshaw et al., 2002; Nilsson and Renshaw, 2004). Additionally, the leopard frog (*Rana pipiens*) can survive 4 h of anoxia at room temperature. The anoxic frog exhibits a "slow death" via a down-regulation of metabolism and is thus not considered to be truly anoxia-tolerant (Donohoe and Boutilier, 1998).

Successful survival of prolonged anoxia requires ATP demand to be matched to the limited ATP production from anaerobic glycolysis; a feat the anoxia-tolerant vertebrates appear to have evolved different strategies to accomplish (Lutz and Nilsson, 1997; Farrell and Stecyk, 2007). Anoxic turtles drastically reduce their activity level, cardiovascular status and metabolic rate such that ATP demand is depressed to a level that precludes an up-regulation of glycolysis. In contrast, anoxic crucian carp remain active in anoxia, albeit at a reduced level compared to normoxia (Nilsson et al., 1993), and maintain cardiovascular status at normoxic levels (Stecyk et al., 2004). *Carassius* do exhibit metabolic depression with anoxia exposure, but not nearly to the extent of the anoxic turtle (Van Waversveld et al., 1989). Therefore, anoxic crucian carp also require an up-regulation of glycolysis to match ATP supply and demand (Lutz et al., 2003). The epaulette shark becomes unresponsive within 40 min of anoxia and may rely on hypometabolism (Mulvey and Renshaw, 2000; Routley et al., 2002) combined with glycolytic activation for survival (Nilsson and Renshaw, 2004).

Electrical activity accounts for 50% or more of the neural energy consumption in vertebrates (Lutz et al., 2003). Therefore, any reduction in excitatory electrical activity should be beneficial for anoxic survival. Indeed, sodium (Perez-Pinzon et al., 1992) and potassium (Pek-Scott and Lutz, 1998) channels are down-regulated in the anoxic turtle brain, and calcium channels show a reduced ion conductance (Bickler, 1992; Bickler et al., 2000), a phenomenon termed channel arrest (Lutz et al., 1985; Hochachka, 1986). Similarly, evidence for a down-regulation of potassium leakage channels has been found in the anoxic leopard frog brain (Knickerbocker and Lutz, 2001). Conversely, available data do not support the occurrence of channel arrest in the brain of anoxic crucian

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carp (Johansson and Nilsson, 1995; Nilsson, 2001). It is not known whether channel arrest occurs during anoxia in the brain of epaulette shark.

The retina is part of the central nervous system and can be expected to have an equally high rate of energy utilization, making it sensitive to oxygen deprivation (Derwent and Linsenmeier, 2000). An electroretinogram (ERG) is a transretinal field potential representing the sum of all electrical activity of retinal receptor cells and neurons following exposure to a light stimulus. Previously, we have shown that the anoxic crucian carp has a strongly suppressed ERG and is most likely blind during anoxia (Johansson et al., 1997). Apparently, vision is not a necessity for the anoxic crucian carp because of a lack of predators in its anoxic waters. Similarly, *in vitro* investigations of turtle neural tissue and visual system have revealed that hypoxia suppresses spontaneous activity of the optic system and that adenosine plays an important role in the response (Gans and Ulinski, 1992; Ariel, 2006). Fittingly, adenosine is believed to be an important signaling molecule during the transition phase of anoxia in the turtle (Nilsson and Lutz, 1992), although it seems less important during prolonged oxygen deprivation (Stecyk et al., 2007). However, little is known of the *in vivo* responses of the visual system of turtles, as well as other anoxia-tolerant vertebrates, to anoxia.

Here, we report a comparative *in vivo* study on the effect of anoxia or severe hypoxia on the ERG of the turtle (*Trachemys scripta*), the leopard frog and the epaulette shark. Because these anoxia-tolerant organisms exhibit reduced physical and/or brain activity during anoxia, we hypothesized that like the crucian carp, their ERG would be substantially reduced during severe oxygen deprivation, resulting in functional blindness. Further, for the turtle and shark, we examined if adenosinergic mechanisms are involved in the hypoxia/anoxia-induced modulation of the ERG.

2. Materials and methods

2.1. Animals

All experiments were approved by the national and institutional animal care committees in the countries they were performed (USA and Australia). Turtles (*T. scripta*) (body mass ranging between 1000 and 2000 g) and frogs (*R. pipiens*) (50–70 g) were purchased from commercial suppliers (turtles, Lemberger, Oskosh, WI, USA; frogs, Charles D. Sullivan Co., Nashville TN, USA) and experiments were conducted at Florida Atlantic University, Boca Raton in December–January. Turtles and frogs were maintained at 22 °C, subjected to a 12 h:12 h light:dark cycle, and had continuous access to freshwater. Epaulette shark experiments were performed at Heron Island Research Station, Great Barrier Reef, Australia in April–May. Epaulette sharks (*H. ocellatum*) weighing 500–1000 g were captured by hand at low tide on the reef platform and kept in a 10 000 L tank continuously supplied with seawater (50 L min^{-1}; 25–28 °C). Sharks were kept in the tank for at least five days before any experiments were conducted.

2.2. ERG recording

ERG recordings were conducted at 22 °C (turtle and frog,) or 25 °C (Epaulette shark) within a dark Faraday cage. For all animals, recordings were made throughout a period of normoxic dark adaptation, oxygen deprivation and subsequent reoxygenation. All equipments were connected to a common ground to prevent electrical noise. The light source was a tungsten lamp (Intralux 150 H Volpi, Switzerland) and light was directed to the eye through a fibreoptic cable. Light was turned on/off with an iris shutter and the lamp voltage was adjusted to give maximal b-wave amplitude. The ERG was detected using a chloridated silver electrode lowered on to the right cornea with a micromanipulator. All signals were fed through an AC preamplifier (P55, Grass Instruments) with low and high cut-offs at 1 Hz and 1 kHz respectively. The reference electrode was placed under the skin, behind the eye ~1 cm from the recording electrode. All recordings were acquired digitally using a Powerlab 4/20 (AD Instruments) connected to a lap top computer running the program Chart 4.2 (AD Instruments). The sampling frequency was 1 kHz. Unless otherwise stated, all chemicals were purchased from Sigma.

2.3. Experimental protocol

2.3.1. Turtle

As previously described (Milton et al., 2002), turtles were anesthetized with 4% Aerrane (Isoflurane USP, Anaquest) in air until comatose and then artificially ventilated with a 1.7% isoflurane in air mixture using a rebreathing bag. After 30 min of normoxic dark adaptation, turtles were made anoxic for 2 h by changing the air in the rebreathing bag to a mixture of 1.7% isoflurane in 99.99% N_2 (County Welding, Pompano Beach, FL, USA). Following anoxia, turtles were reoxygenated and ERGs recorded for 70 min. 2 h was selected as the anoxia exposure period because in a pilot experiment, two turtles exposed to 4 h of anoxia did not exhibit further reduction of the ERG beyond 2 h. Normoxic and anoxic conditions of turtles were confirmed by measurements of blood P_{O2}. The heart was accessed by excision of a 2×2 cm piece of the plastron using a bone saw and an occlusive catheter (PE-50 containing saline with 100 IU mL^{-1} heparin) was placed in the left subclavian artery of six individuals. 100 μL blood samples were drawn at normoxia, 10 min intervals of the first 30 min of anoxia and after 20 min of reoxygenation. Blood P_{O2} was analyzed on a Cameron Instruments blood gas analyzer (Port Aransas, TX, USA). The anoxic ERG from the cannulated turtles (n=7) did not differ from uncannulated animals (n=5).

To examine if adenosinergic mechanisms modulate turtle ERG, seven individuals were injected intraperitoneally (i.p.) twice with the specific A_1 receptor antagonist 8-cyclopentyl-1,3 dimethylxanthine (CPT) at a dose of 100 μg kg^{-1}. Injections occurred 30 min prior to anoxia exposure and at 1 h of anoxia. CPT was dissolved in distilled water using a sonicator and given at a concentration of 100 μM (injection volume was ~2 mL). CPT was also injected into 4 normoxic turtles not exposed to anoxia (CPT control). CPT was used because in pilot studies, i.p. injection of the non-specific adenosine receptor antagonist aminophyline (5 mg kg^{-1}, dissolved in heparinised saline) had no visible effect on the anoxic suppression of ERG (n=2), whereas 50 mg kg^{-1} aminophylline decreased the ERG amplitude during normoxia for up to 2 h (n=3). It is known that aminophylline in high concentrations can inhibit phosphodiesterase (PDE) (Manallack et al., 2005) and that PDE inhibitors reduce ERG in cats (Schneider and Zrenner, 1986). To our knowledge, there are no reports suggesting that CPT interferes with PDE.

In another pilot experiment, two turtles were i.p injected with the $GABA_A$ receptor antagonist bicuculline (1 μmol kg^{-1}) 30 min prior to anoxia exposure. Bicuculline was dissolved in DMSO (to a concentration of 100 mM) and further diluted in distilled water to a final concentration of 1 mM.

2.3.2. Epaulette shark

Sharks were anesthetized in a 50 L tank by adding benzocaine dissolved in 96% ethanol (50 g L^{-1}) to seawater, achieving a final concentration of 60 mg L^{-1}. After weighing, the fish was placed in a plastic chamber and continuously ventilated with aerated seawater via a tube inserted in the mouth. This seawater (60 L containing 30 mg L^{-1} benzocaine) was recirculated in a system held at 25 °C and the water height adjusted with a standpipe to the level that covered half the shark. The remaining exposed areas, except those around the eye, were kept moist with paper towels. During experimentation, the eye was intermittently moistened manually with seawater using a 2 mL syringe. The animal was left to recover for 1 h before any experiments were conducted. A plexiglas column (1500 mm high, 80 mm in