The remarkable ability of the turtle brain to survive anoxia is based on its ability to match energy demand flexibly to energy production. Earlier studies indicate that reduced ion leakage is an important mechanism for energy conservation during anoxia. We tested the hypothesis that extracellular adenosine plays a role in the reduction of K⁺ flux (channel arrest) that occurs in the anoxic turtle brain. Changes in extracellular K⁺ concentration ([K⁺]₀) in the in situ brain of the turtle Trachemys scripta were monitored following inhibition of Na⁺/K⁺-ATPase with ouabain. The time to reach full depolarization ([K⁺]₀ plateau) was three times longer in the anoxic brain than in normoxic controls, and the initial rate of K⁺ leakage was reduced by approximately 70%. Superfusing the brain before and during anoxia with the general adenosine receptor blocker theophylline, or the specific adenosine A₁ receptor blocker 8-cyclopentyltheophylline, significantly shortened the time to full depolarization in the ouabain-challenged anoxic brain and increased the rate of K⁺ efflux. The results suggest that adenosine A₁ receptors are involved in the expression of anoxia-induced ion channel arrest in the turtle brain.

Key words: A₁ receptors, 8-cyclopentyltheophylline, depolarization, ouabain, [K⁺]₀, theophylline, ion flux, anoxia, turtle, Trachemys scripta.
(C. picta) cortical brain slice, adenosine causes a 64% reduction in the NMDA-mediated increase in intracellular Ca\textsuperscript{2+} concentration (Buck and Bickler, 1995).

The purpose of this research was to investigate whether extracellular adenosine is involved in channel arrest in the anoxic turtle brain. Specifically, the pattern of change in extracellular K\textsuperscript{+} concentration was monitored after inhibition of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase by ouabain. These studies were performed in the in situ brain, during normoxia, during prolonged anoxia and during prolonged anoxia after blockage of adenosine receptors.

Materials and methods

Experiments were conducted on freshwater turtles (Trachemys scripta, formerly Pseudemys scripta) weighing 300–500 g. Experimental procedures were approved by the FAU Institutional Animal Care and Use Committee and all applicable NIH guidelines were followed.

Turtles were ventilated with 4% AErrane (Isoflurane, USP, Anaquest) in ambient air (Shaw et al. 1992). The anesthesia was administered via a small-animal ventilator (Phipps and Bird Inc., Richmond, VA, USA) using an intratracheal cannula. Once a surgical plane had been reached, turtles were maintained on 1.7% isoflurane (Shaw et al. 1992) for the duration of surgery. The animals were ventilated at 1–1.5 breaths min\textsuperscript{-1} with a 5–7 s inspiration time. This method of delivery ensured a consistent induction time of the anesthesia.

The skin and muscle above the midline of the skull were retracted, and a portion of the skull bone was carefully removed by drilling over the telencephalon. Following opening of the dura and leptomeninges, a cortical surface was chosen for continuous monitoring of the extracellular K\textsuperscript{+} concentration. In selecting the optimal cortical area, the criteria were as follows: microscopic observation of normal blood flow and the absence of bleeding or cortical damage. The K\textsuperscript{+}-sensitive microelectrode was inserted to a depth of approximately 100–200 µm from the brain surface. Turtle artificial cerebrospinal fluid (ACSF) (Pérez-Pinzón et al. 1993) was administered continuously to the brain surface in every group.

The K\textsuperscript{+} concentration was measured using double-barrelled, ion-sensitive micropipette electrodes, as modified from Syková (1992). The electrodes were fabricated from Stoelting double-barrelled micropipettes, the tips were silanized with tributylchlorosilane (4%) in carbon tetrachloride, baked for 4 h at 200°C, and filled with K\textsuperscript{+} ion-exchanger from World Precision Instruments (IE-190). The barrel above the ion exchanger was filled with 100 mmol l\textsuperscript{-1} KCl, and the reference barrel was filled with 100 mmol l\textsuperscript{-1} NaCl. The voltage of the reference barrel was electronically subtracted from the voltage of the K\textsuperscript{+}-sensitive barrel, and the signal difference was amplified and displayed on a chart recorder. The K\textsuperscript{+} concentration was calculated by comparing potentials recorded in the brain with potentials recorded in the calibration solutions containing varying concentrations of K\textsuperscript{+} and varying concentrations of Na\textsuperscript{+}. The turtles were grounded with a AgCl wire implanted into the lateral skull muscles.

The turtles were divided into four groups. In the first group (N=5), the animals were ventilated with air for 30 min before the brain surface was superfused with ACSF containing 20 mmol l\textsuperscript{-1} ouabain. Because Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity differs in anoxic and normoxic turtle brains (M. Pék and P. L. Lutz, unpublished observations), it was necessary to use high concentrations of ouabain to ensure that all of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase was rapidly blocked (Chih et al. 1989). In the second group (N=5), after 30 min of air respiration, the animals were switched to 100% nitrogen ventilation for 2 h before the ouabain was administered. In the third group of animals (N=5), after an initial 30 min of air respiration, the brain was superfused with ACSF containing 100 µmol l\textsuperscript{-1} theophylline for a further hour of air respiration, and superfusion was continued for a subsequent 2 h of 100% N\textsubscript{2} respiration. Ouabain was administered at the end of this period. In the fourth group (N=5), the procedure for the third group was followed except that 5 µmol l\textsuperscript{-1} 8-cyclopentyltheophylline (CPT) instead of theophylline was dissolved in the brain superfusate. The adenosine receptor blockers were added before N\textsubscript{2} respiration commenced in order to prevent the activation of adenosine receptors during the initial hypoxic period, and the duration of exposure to receptor blocker was chosen to correspond with the time course of adenosine release in the anoxic turtle brain (Nilsson and Lutz, 1992). All experiments were conducted at room temperature (25°C).

Statistical analyses were performed using analysis of variance (ANOVA) and Dunnett’s tests; data are expressed as means ± s.e.m.

Results

Effects of ouabain on [K\textsuperscript{+}]\textsubscript{o} in the cortex of the anoxic and normoxic turtle

During normoxia, the brain extracellular K\textsuperscript{+} concentration [K\textsuperscript{+}]\textsubscript{o} averaged 1.9±0.17 mmol l\textsuperscript{-1} (N=20) and showed no increase over 2 h of anoxia. The pattern of change in [K\textsuperscript{+}]\textsubscript{o} in response to ouabain was similar under normoxic and anoxic conditions (Fig. 1) and could be divided into the same three phases described for mammals (Hansen, 1985): an initial slow increase in [K\textsuperscript{+}]\textsubscript{o} until an inflection or threshold level was reached (phase 1), followed by an abrupt elevation of [K\textsuperscript{+}]\textsubscript{o} (phase 2) to a plateau associated with anoxic depolarization (phase 3) (Fig. 1). However, the changes in [K\textsuperscript{+}]\textsubscript{o} were significantly slower in the anoxic brain (Figs 1, 2), which took almost three times longer to reach full depolarization following ouabain superfusion compared with the normoxic brain (Fig. 2). Both the phase 1 and phase 2 components of depolarization were significantly longer in the anoxic brain (Fig. 2).

Effect of blocking adenosine A\textsubscript{1} receptors on [K\textsuperscript{+}]\textsubscript{o} in anoxic turtle brain

To investigate the relationship between adenosine receptor activation and the reduction in the rate of K\textsuperscript{+} efflux in the
Role of adenosine in channel arrest

In anoxic turtle brain, anoxic brains were superfused with the general A₁ and A₂ receptor blocker theophylline and the specific A₁ receptor blocker CPT. Both theophylline and CPT superfusion resulted in significant decreases (approximately 35–40%) in the time for the anoxic brain to reach full depolarization (Fig. 2). Although the mean durations of phase 1 and phase 2 appeared to be reduced, the difference was only statistically significant for phase 1 in the theophylline-superfused brain (Fig. 2).

A K⁺ inflection value for the onset of phase 2 was determined as the point of intersection of linear extensions of the first and second phases. The value for the normoxic turtles (8.22±0.71 mmol l⁻¹, N=5) is similar to that found earlier for turtles (Sick et al. 1985) and for mammals (Hansen, 1985). However, the anoxic brain showed a substantial decrease in the K⁺ threshold value (4.1±0.64 mmol l⁻¹, N=5) or CPT (4.6±0.54 mmol l⁻¹, N=5) superfusion.

The rate of K⁺ leakage was calculated by measuring changes in [K⁺]₀ during ouabain superfusion. The rate of K⁺ leakage in phase 1 best represents the resting rate of K⁺ leakage in the brain because K⁺ conductance may be affected by other factors when the depolarization produced by changes in [K⁺]₀ is sufficiently large to open voltage-gated channels (phase 2) (Chih et al. 1989). The times for [K⁺]₀ to increase by 1 mmol l⁻¹ and 3 mmol l⁻¹ following ouabain superfusion are shown in Fig. 3. The initial rate of K⁺ leakage in normoxic brains was almost four times that in anoxic brains (air, 0.45±0.1 mmol l⁻¹ min⁻¹; N₂, 0.13±0.01 mmol l⁻¹ min⁻¹) (Fig. 3). Both theophylline and CPT superfusion resulted in an approximately twofold increase in the rate of K⁺ leakage in the anoxic brain (N₂+theophylline, 0.27±0.04 mmol l⁻¹ min⁻¹, N₂+CPT, 0.20±0.02 mmol l⁻¹ min⁻¹) (Fig. 3).
rates of K+ efflux between anoxic and normoxic crucian carp protocol, Johansson and Nilsson (1995) found no difference in species with extraordinary anoxia-tolerance. Using an identical anoxic survival since it is not seen in the crucian carp, the other However, ‘channel arrest’ does not appear to be essential for anoxic survival in K+ leakage rate does not appear to be involved. In the rat brain, anoxia produces a substantial depletion of intracellular K+ (Jiang and Haddad, 1991), which would presumably result in a lower [K+]o required to reach a fixed threshold transmembrane voltage. However, we have no information on tissue [K+] changes in the anoxic turtle brain. The increased resistance to K+ leakage in the anoxic turtle brain appears to be mediated, at least in part, by adenosine receptors. In both CPT- and theophylline-superfused anoxic brains, the time to full depolarization was significantly decreased compared with anoxic brains and the K+ leakage rate in phase 1 was increased. The observation that CPT and theophylline produced generally similar changes in the increase in [K+]o suggests that this response is primarily effected through the A1 receptor. However, adenosine receptor blockade did not fully disinhibit the anoxia-induced reduction in K+ leakage, indicating that other factors are also involved in producing channel arrest. There is evidence of the involvement of adenosine in ion channel activity. In the mammal, A1 receptor stimulation inhibits the brain’s electrical activity through K+ channel activation (direct coupling via G-proteins to ion channels) and/or by inhibiting the high-voltage-activated Ca2+ channels (Kasai and Aosaki, 1989; Trussel and Jackson, 1987). In the turtle, adenosine is involved in the down-regulation of NMDA-mediated Ca2+ influx (Buck and Bickler, 1995).

In contrast to the results described here, superfusion of the adenosine receptor antagonists in the isolated anoxic turtle cerebellum caused rapid full depolarization (Pérez-Pinzón et al. 1993). The reason for this difference is probably that the anoxic in vitro preparation is energy-limited, depending solely on the diffusion of glucose through the slice to meet its energy needs, whereas the anoxic brain in situ is probably much more robust, having a greatly enhanced glycolytic delivery through the maintained brain blood supply and a substantial hyperglycemia (Lutz and Nilsson, 1994).

[K+]o at the inflection point for ouabain-superfused normoxic turtle brains (8.22±0.7 mmol l⁻¹) is similar to that of adult rat brains (Erecinska and Silver, 1994; Hansen and Nordström, 1979). However, it is substantially (50 %) lower in the anoxic turtle brain (4.1±0.6 mmol l⁻¹). It is not at all clear how this change in the K+ inflection point comes about and what its significance is. In the rat, [K+]o at the inflection point is strongly influenced by maturity, being approximately 24 mmol l⁻¹ for the 4- to 12-day-old neonate and declining to approximately 10 mmol l⁻¹ in the adult (Hansen, 1977). Although lowering the body temperature of adult rats (to 34 °C) substantially reduces the initial (phase 1) rate of increase in [K+]o, [K+]o at the inflection point remains the same (Katsura et al. 1992). Since theophylline and CPT treatments had no effect on the inflection point in the turtle, an alteration in K+ leakage rate does not appear to be involved. In the rat brainstem, anoxia produces a substantial depletion of intracellular K+ (Jiang and Haddad, 1991), which would presumably result in a lower [K+]o required to reach a fixed threshold transmembrane voltage. However, we have no information on tissue [K+] changes in the anoxic turtle brain.

The rapid efflux during the second phase of the response to ouabain is thought to be due to an activation of Ca2+- and ATP-dependent non-selective K+-permeable cationic channels (Jiang and Haddad, 1991; Schaeffer and Lazdunski, 1991), which are abundant in the nervous tissue (Ashford et al. 1988; Jiang et al. 1992). This phase was also slowed down in the anoxic turtle brain, possibly because of a general decrease in membrane permeability. Because the anoxia-induced increase in [K+]o leads rapidly to depolarization and neuronal death in the mammalian brain, there is great interest in finding methods to reduce the rate of K+ efflux. However, the major pathways through which K+ exits the anoxic cell are unknown, and there are important regional differences. For example, in the rat substantia nigra during anoxia, K+ efflux is halted by sulfonylurea blockers of neuronal ATP-dependent K+ channels such as glibenclamide (Amoroso et al. 1990; Mourre et al. 1989), but in the rat brainstem glibenclamide only reduces the rate of K+ efflux by 43 % (Jiang and Haddad, 1991), and in the rat hippocampus during anoxia K+ efflux is insensitive to any known blockers of ATP-sensitive, Ca2+-sensitive or voltage-sensitive K+ channels (Schaeffer and Lazdunski, 1991). There may also be species differences: the turtle brain (unstated species) has a much lower density of ATP-sensitive K+ channels compared with the rat brain (Xia and Haddad, 1991).

In conclusion, the results indicate that extracellular adenosine plays a role, via A1 receptor-mediated processes, in promoting a reduction in the rate of K+ leakage in the anoxic turtle brain.

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References


