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SURVIVAL OF ENERGY FAILURE IN THE ANOXIC FROG BRAIN: DELAYED RELEASE OF GLUTAMATE

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Summary

This study investigated the relationship between energy failure and neurotransmitter release in the frog (*Rana pipiens*) brain during 1–3 h of anoxia. Unlike truly anoxia-tolerant species, the frog does not defend its brain energy charge. When exposed to anoxia at 25 °C, there is an immediate fall in brain ATP levels, which reach approximately 20 % of normoxic levels in approximately 60 min. The frog, nevertheless, survives another 1–2 h of anoxia. At 100 min of anoxia, there is an increase in extracellular adenosine concentration, probably originating from the increased intracellular adenosine concentration caused by the breakdown of intracellular ATP. Increases in the levels of extracellular glutamate and GABA do not occur until 1–2 h after ATP depletion. This response is quite unlike that recorded for other vertebrates, anoxia-tolerant or anoxia-intolerant, where energy failure quickly results in an uncontrolled and neurotoxic release of excitatory neurotransmitters. In the frog, the delay in excitotoxic neurotransmitter release may be one of the factors that allow a period of survival after energy failure. Clearly, energy failure by itself is not a fatal event in the frog brain.

Key words: anoxia, adenosine, GABA, glutamate, energy charge, ATP, brain death, frog, *Rana pipiens*.

Introduction

It is a widely accepted tenet in neurobiology that ATP levels are rigorously protected and that ATP depletion, caused by such events as ischemia or anoxia, invariably and rapidly results in cell death (Siesjö, 1978; Erecinska and Silver, 1989). Given its clinical importance, this is an intensively studied area, and there is a large amount of information on the physiological and biochemical consequences of energy failure in the brain (Siesjö *et al.* 1995; Katsura *et al.* 1994; Lutz and Nilsson, 1997*b*).

In essence, when oxygen supply is halted, membrane ion pumps fail, allowing ions to move across the cell membrane down concentration gradients (Ekholm *et al.* 1992). The resulting depolarization facilitates a massive release of the excitatory neurotransmitter amino acids glutamate and aspartate, believed to be an important cause of anoxic/ischemic brain damage (Rothman, 1992; Globus *et al.* 1988). The depolarization allows glutamate to activate the *N*-methly-D-asparate (NMDA) receptor, opening Ca^{2+}-permeable channels and resulting in a flood of Ca^{2+} moving into the cell from the extracellular fluid (Rothman, 1992; Siesjö *et al.* 1995). The uncontrolled and explosive rise in intracellular [Ca^{2+}] causes multiple dysfunctional effects including the stimulation of phospholipid hydrolysis, resulting in a rise in the concentration of harmful free fatty acids, particularly arachidonic acid, that leads to the formation of free radicals (Siesjö, 1985).

The importance of defending brain ATP levels is exemplified by the highly anoxia-tolerant (days to months) freshwater turtle *Trachemys scripta* and the crucian carp *Carassius carassius*. These species maintain normoxic levels of brain ATP throughout many hours of anoxia (Lutz and Nilsson, 1997*b*). When energy failure was induced in the turtle brain by ischemia or by inhibiting glycolysis during anoxia, ATP levels were depleted, ionic gradients were lost and electrical activity was irreversibly suppressed (Sick *et al.* 1993). The turtle survives long-term anoxia by substantially down-regulating brain energy demand to a level where its energy needs can be fully met by anaerobic glycolysis (Lutz and Nilsson, 1997*a*). This is achieved, in part, through the early release of adenosine, which has neuroprotective effects (Buck and Bickler, 1995; Pék and Lutz, 1997), and by the later sustained release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Nilsson and Lutz, 1991, 1993). In contrast to the situation in the anoxia-sensitive mammal, the excitatory neurotransmitter glutamate is not released by the turtle brain during anoxia (Nilsson and Lutz, 1991).

Some species of frog appear to show a moderate degree of anoxia-tolerance. For example, the common frog *Rana temporaria* can withstand 3 h of anoxia at 20 °C (Wegener *et al.* 1986) and can tolerate months of severe hypoxia in cold (3 °C) water (Boutilier *et al.* 1997). However, unlike the highly

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anoxia-tolerant species, brain [ATP] is not defended in *R. temporaria* and falls to approximately 30 % of control (normoxic) values after 1 h of anoxia, yet they survive for 1–2 h after ATP depletion (Wegener and Krause, 1993).

The North American leopard frog *R. pipiens* appears to have a similar capacity for anoxia-tolerance to that of *R. temporaria*. It can withstand in excess of 2 h of anoxia at 25 °C (Rose and Drotman, 1967) (in preliminary experiments for this study, we found that *R. pipiens* could survive 3 h of anoxia at 25 °C) and survives at least 30 h of anoxia at 5 °C (Hermes-Lima and Storey, 1996). The purpose of the present study was to determine whether [ATP] was defended during anoxia in *Rana pipiens* and to initiate an investigation into how these frogs manage to survive an energy failure that is quickly fatal to other vertebrates, anoxia-tolerant and anoxia-intolerant alike. As an initial step, we investigated the relationship between brain energy status and the release of adenosine, GABA and glutamate. A preliminary account of this work has been presented previously in abstract form (Lutz and Reiners, 1997).

Materials and methods

Animals

Leopard frogs (*Rana pipiens*) weighing 40–60 g were purchased from Lemberger Animals (OshKosh, WI, USA). The animals were housed in plastic pens at 25 °C and had constant access to fresh water; they were fed with crickets and kept on a 12 h:12 h light:dark cycle. Experimental procedures were approved by the FAU Institutional Animal Care and Use Committee and all applicable NIH guidelines were followed.

Animal preparation

Frogs were ventilated with air and Aerrane (isoflurane, ISP) using a small-animal respirator (SAR-830 from CME, Inc.). Animals were anesthetized with an initial concentration of 4 % isoflurane in air. The animal was secured on a stereotaxic instrument and the dura was exposed. A microdialysis probe (CMA/12, 0.5 mm diameter) was inserted into the striatum to a depth of 3 mm and perfused with unbuffered Ringer's solution (125 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1.18 mmol l^{-1} $MgCl_2$, 1.26 mmol l^{-1} $CaCl_2$) at a rate of 5 µl min^{-1}. The ventilated frogs were enclosed within an air-tight plastic chamber (at 25 °C) that was flushed with air (normoxia). After an initial 2 h period of air respiration to establish baseline values, experimental animals were switched to 100 % N_2 containing 0.5 % isoflurane and the chamber was continuously flushed with 100 % nitrogen (anoxia). Control animals respired air containing 0.5 % isoflurane throughout the experiment. Samples (100 µl) of dialysate were collected every 20 min and either analyzed immediately or frozen at −80 °C for analysis within 1 month.

Sample analysis

Adenosine was measured by high-performance liquid chromatography (HPLC) on an Adsorbosphere C18 column (Alltech) using an ultraviolet detector (Shimadzu) at a wavelength 254 nm (Nilsson and Lutz, 1991). Concentrations were determined by comparison with known standards. Glutamate and GABA concentrations were assayed using HPLC with fluorescence detection. Each dialysate sample was derivatized in complete *o*-phthaldialdehyde reagent solution. Experiments were not started until the elevated levels of metabolites found in the initial samples (due to tissue damage) were reduced to constant low levels. Recovery rates for adenosine and the amino acids were determined at the end of each experiment and ranged from 10 to 15 %. After an experimental run had been completed, the probe location was confirmed by adding Methylene Blue to the perfusate and examining the brain after its removal (Nilsson and Lutz, 1991).

Whole brain tissue was analyzed for nucleotides (ATP, ADP and AMP). For whole brain assays, brains were frozen at times determined by the parameters of individual experiments. Liquid nitrogen was poured over the head of the anaesthetized frog until the brain was frozen. The frog was then decapitated and the entire head dropped into liquid nitrogen. The brain was dissected out while continuously submerged in liquid nitrogen. The samples were stored at −80 °C in cryotubes until processing (stored for no longer than 1 week). For processing, frozen brains were weighed and homogenized in 1 ml of cold 4 % perchloric acid (PCA). The homogenate was then centrifuged at 14 000 ***g*** for 6 min. The supernatant was neutralized with 0.4 ml of cold 0.85 mol l^{-1} K_2CO_3. The solution was again centrifuged at 14 000 ***g*** for 6 min. A modification of the reverse-phase HPLC with ultraviolet detection system described above for adenosine analysis was used to measure ATP, ADP and AMP levels in the supernatant. The ultraviolet spectrophotometric detector was adjusted to 460 nm and the mobile phase consisted of 0.1 mol l^{-1} NaH_2PO_4, 5 mmol l^{-1} tetrabutyl ammonium hydrogen sulfate plus 10 % (v/v) MeCN. Concentrations of each nucleotide were determined by comparing peak height with the appropriate 1 mmol l^{-1} standard. Changes during anoxia were evaluated using analysis of variance (ANOVA) followed by a Dunnett's test for comparisons against the final aerobic value. Student's *t*-tests were used to compare times of increase for GABA, glutmate and adenosine.

Results

The normoxic ATP content of the *Rana pipiens* brain, 1.34 µmol g^{-1} (Fig. 1), corresponds to earlier estimations for this species (1.1–1.56 µmol g^{-1}; McDougal *et al.* 1968) and for *R. temporaria* (1.28 µmol g^{-1}; Wegener and Krause, 1993). As in anoxia-intolerant species, brain ATP levels are not defended during anoxia and fall rapidly, reaching approximately 20 % of normoxic values at approximately 60 min of anoxia, and are depleted after approximately 100 min of anoxia (Fig. 1A). Anoxic energy failure is further indicated by the immediate fall in the adenylate energy charge [Energy charge = $(ATP+0.5\,ADP)(AMP+ADP+ATP)^{-1}$], which at 60 min of anoxia is approximately 60 % of the normoxic value and after

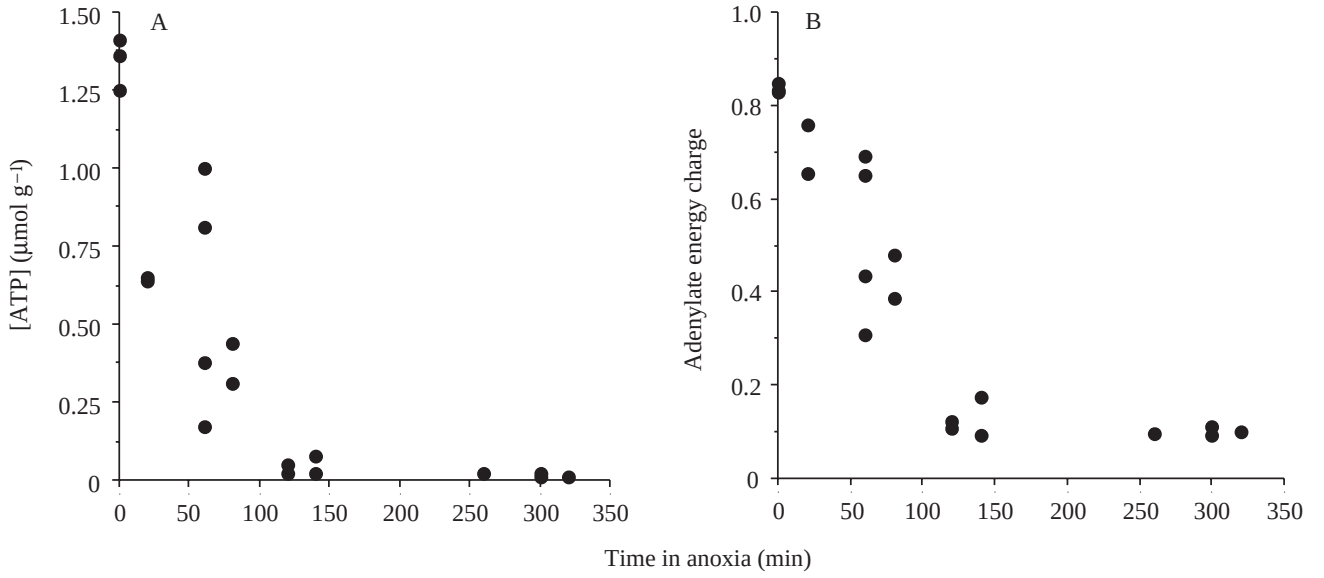

Fig. 1. Changes in frog brain ATP levels (A) and adenylate energy charge (B) during anoxia.

120 min of anoxia is only 14 % of the normoxic value (Fig. 1B).

The normoxic extracellular adenosine concentration the frog brain (0.301±0.221 μmol l^{-1}) (mean ± S.E.M., N=12) is maintained during early anoxia (Fig. 2). But after approximately 100 min of anoxia, a gradual increase in extracellular adenosine concentration occurs, reaching a maximum of approximately 7–10 μmol l^{-1} after 200 min of anoxia and declining towards normoxic levels after 260 min of anoxia (Fig. 2).

Changes in extracellular GABA concentration were not seen until 262±20.9 min of anoxia or in glutamate concentration until 273±20.5 min of anoxia, after which both increased rapidly (mean ± S.E.M., N=12) (Fig. 3A,B). There was no significant difference between the times of increase for GABA and glutamate, but the times at which their concentrations differed significantly from the normoxic value were both significantly different from that for adenosine (P<0.01).

A representative record of the concurrent changes in an individual animal during anoxia is shown in Fig. 4. After 100 min of anoxia, extracellular adenosine concentration begins to rise, peaks at approximately 200 min and then declines. During the period when extracellular adenosine level is falling, simultaneous increases in GABA and glutamate level occur.

Discussion

We have shown that, as in *R. temporaria* (Wegener and Krause, 1993), brain [ATP] immediately starts to fall when the leopard frog *R. pipiens* is exposed to anoxia and is 80 % depleted in 60 min at room temperature (25 °C). This collapse in ATP levels is similar to the more rapid response seen in anoxia-intolerant mammals (Siesjö, 1978), but it is quite unlike that shown by long-term anoxic survivors such as the turtle *T. scripta* and the crucian carp *C. carassius*, which maintain brain

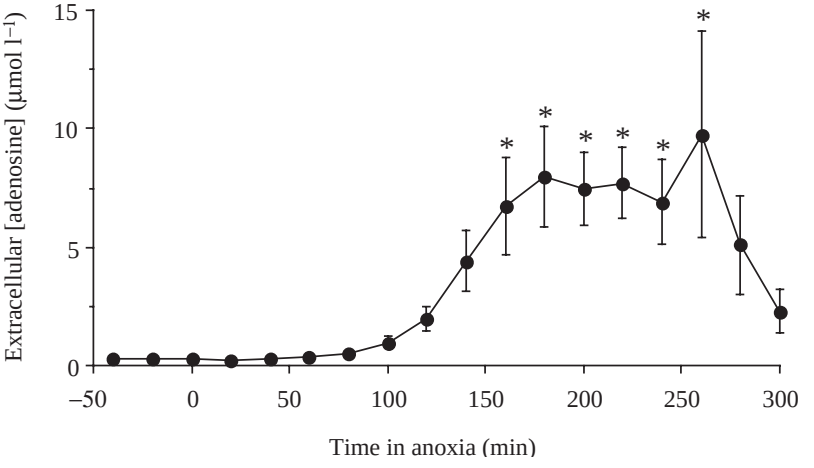

Fig. 2. Effect of anoxia on extracellular adenosine concentration in the frog striatum. Values are means ± S.E.M., N=12. Where not shown, error bars are smaller than the symbols. *Significant differences from the final aerobic value (P<0.05).

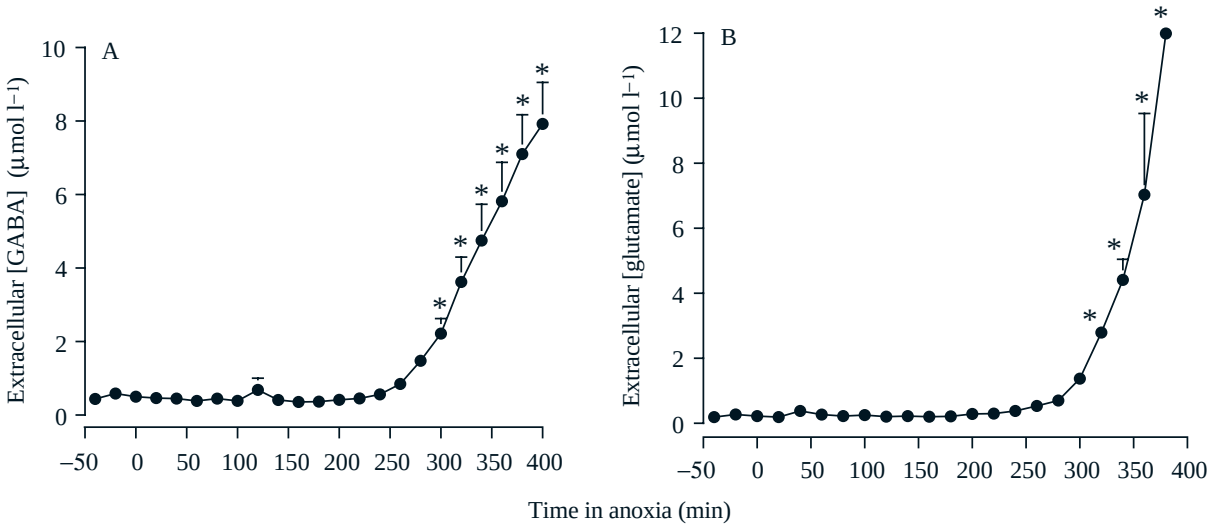

Fig. 3. Effect of anoxia on extracellular GABA (A) and glutamate (B) concentrations in the frog striatum. Values are means ± S.E.M., N=12. Where not shown, error bars are smaller than the symbols. *Significant differences from the final aerobic value (P<0.05).

[ATP] for many hours of anoxia (Lutz and Nilsson, 1997*b*). However, while the loss of ATP results in rapid degenerative changes in the mammalian brain, both frog species can withstand additional hours of anoxia. This remarkable phenomenon has also been seen in the isolated sciatic nerve of *R. pipiens*. ATP was lost rapidly from the sciatic nerve following exposure to anoxia, and this loss was accompanied by a gradual reduction in the amplitude of the action potential (Okada and McDougal, 1971). However, the sciatic nerve survived a further 150 min of anoxia and the action potential recovered rapidly when O_2 was reintroduced into the nerve chamber (Okada and McDougal, 1971).

How the frog brain survives the loss of ATP is an intriguing question since the defense of [ATP] is widely regarded as essential to neuronal survival. In the ischemic rat brain, there is a rapid increase in extracellular glutamate and GABA concentrations as ATP is depleted (Shimizu *et al.* 1993), and there is a precipitous efflux of K^+ when ATP levels fall below 50 % of normoxic values (Katsura *et al.* 1994). The uncontrolled release of excitatory neurotransmitters and the large increases in intracellular [Ca^{2+}] have pathological consequences that result in cell death (Siesjö, 1985). Energy failure produces similar effects in the anoxic turtle brain (Lutz and Nilsson, 1977*b*). In the frog, however, the sequence of events during and after energy failure is quite different.

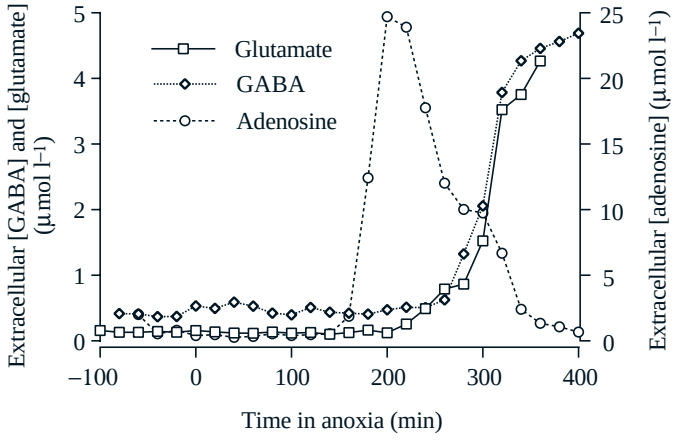

Fig. 4. Concurrent changes in extracellular adenosine, GABA and glutamate concentration in the striatum of an individual frog during anoxia.

The rise in extracellular adenosine concentration, starting at 100 min of anoxia, probably originates from the increased intracellular adenosine concentration caused by the breakdown of ATP. Extracellular adenosine has a protective role in the turtle (Lutz and Nilsson, 1997b) and mammalian (Sweeney, 1997) brain and may have a similar function in the frog. In the turtle and rat, adenosine is released early in the energy crisis and acts as a retaliatory metabolite to restore energy balance (Newby *et al.* 1990; Lutz and Nilsson, 1997*b*), while in the frog adenosine is not released until ATP is almost fully depleted. In the frog, extracellular glutamate and GABA concentrations start to increase much later, at approximately 260 min of anoxia. This contrasts with the situation in the turtle, where the inhibitory neurotransmitter GABA is released slowly during anoxia while release of the excitatory neurotransmitter glutamate is delayed (Nilsson and Lutz, 1991). However, in the mammal and turtle, both GABA and neurotoxic amounts of glutamate are released rapidly during the depolarization that follows energy failure (Lutz and Nilsson, 1997*b*). Thus, one of the mechanisms that account for the frogs' ability to endure brain energy failure may be the delay in the release of excitatory amino acids. It is possible that, in the frog brain, anoxic depolarization is either delayed for up to 2 h after ATP depletion or excitatory amino acid release occurs well after depolarization. Both intriguing questions are under investigation.

Clearly, energy failure by itself is not a fatal event in the frog brain. Indeed, the frog model indicates that ATP loss and neuronal death are not as tightly linked as is widely believed.

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