The Journal of Experimental Biology 208, 681-686
Published by The Company of Biologists 2005
doi:10.1242/jeb.01436

Intracellular calcium and survival of tadpole forebrain cells in anoxia

Michael S. Hedrick[1,2,*], Christian S. Fahlman[1] and Philip E. Bickler[1]

[1]*Department of Anesthesia, University of California, San Francisco, CA 94143-0542, USA* and [2]*Department of Biological Sciences, California State University, Hayward, Hayward, CA 94542-3083, USA*

*Author for correspondence at address 2 (e-mail: mhedrick@csuhayward.edu)

Accepted 7 December 2004

Summary

The frog brain survives hypoxia with a slow loss of energy charge and ion homeostasis. Because hypoxic death in most neurons is associated with increases in intracellular calcium ($[Ca^{2+}]_i$), we examined the relationship between $[Ca^{2+}]_i$ and survival of a mixed population of isolated cells from the forebrain of North American bullfrog *Rana catesbeiana* tadpoles. Forebrain cells from stage V–XV tadpoles were isolated by enzymatic digestion and loaded with one of three different calcium indicators (Fura-2, Fura 2-FF and BTC) to provide estimates of $[Ca^{2+}]_i$ accurate at low and high $[Ca^{2+}]_i$. Propidium iodide (PI) fluorescence was used as an indicator of cell viability. Cells were exposed to anoxia (100% N_2) and measurements of $[Ca^{2+}]_i$ and cell survival made from 1 h to 18 h. Intracellular $[Ca^{2+}]$ increased significantly after 3–6 h anoxia (*P*<0.05), regardless of the type of Ca^{2+} indicator used; however, there were substantial differences in the measurements of $[Ca^{2+}]_i$ with the different indicators, reflecting their varying affinities for Ca^{2+}. Resting $[Ca^{2+}]_i$ was approximately 50 nmol l^{-1} and increased to about 9–30 μmol l^{-1} after 4–6 h anoxia. The significant increase in $[Ca^{2+}]_i$ during anoxia was not associated with significant increases in cell death, with 85–95% survival over this time period. Cells exposed to anoxia for 18 h, or those made anoxic for 4-6 and re-oxygenated for 12 h to 16 h, had survival rates greater than 70%, but survival was significantly less than normoxic controls. These results indicate that large increases in $[Ca^{2+}]_i$ are not necessarily associated with hypoxic cell death in vertebrate brain cells.

Key words: cell calcium, amphibian, *Rana catesbeiana*, neuroprotection, anoxia, Fura 2-FF, BTC, Fura-AM, propidium iodide.

Introduction

Mammalian neurons are typically intolerant of anoxic conditions, and short periods of anoxia or ischemia result in neuronal death, which is often associated with large increases in intracellular calcium concentration ($[Ca^{2+}]_i$) (Kristian and Siesjö, 1998; Lipton, 1999). A substantial body of evidence in mammals indicates that most of the Ca^{2+} influx occurs through the *N*-methyl-D-aspartate (NMDA) glutamate receptor channel (Choi, 1995) and the TRPM7 channel (Aarts et al., 2003). Although large increases in $[Ca^{2+}]_i$ result in acute and delayed cell death (Yu et al., 2001), recent evidence from mammalian neurons suggests that moderate increases of $[Ca^{2+}]_i$ (50–200 nmol l^{-1}) play a neuroprotective role in hypoxia and glucose deprivation (Bickler and Fahlman, 2004).

In contrast to the situation in mammals, some species, such as the freshwater turtle (*Trachemys scripta* and *Chrysemys picta*), are able to tolerate extended periods of anoxia (Hochachka and Lutz, 2001; Bickler and Donohoe, 2002; Bickler, 2004; Lutz and Nilsson, 2004). One adaptation of turtle neurons to survive anoxia is the ability to maintain ionic homeostasis and limit increases in $[Ca^{2+}]_i$ during prolonged anoxia (Bickler, 1998). Turtles subjected to anoxia for several weeks exhibit moderate and sustained increases in neuronal $[Ca^{2+}]_i$ (Bickler, 1998), and this appears to be linked to anoxia-induced suppression of NMDA receptors (Bickler et al., 2000). This pattern is also seen in hypoxia-tolerant neonatal mammalian neurons (Bickler and Hansen, 1998). Thus, studies in mammals and turtles suggest there may be common mechanisms of neuroprotection in vertebrate neurons that involve moderate increases in cell calcium.

There is much less known about the cellular mechanisms that allow other vertebrates to survive periods of severe hypoxia and anoxia. A number of studies indicate that frogs are intermediate in anoxia tolerance relative to anoxia-intolerant mammals and anoxia-tolerant turtles. For example, Ranid frogs survive only a few days of anoxia at low temperatures (Hutchison and Dady, 1964; Christiansen and Penney, 1973; Lillo, 1980; Stewart et al., 2004) and about 3 h of anoxia at room temperature (Lutz and Reiners, 1997; Knickerbocker and Lutz, 2001). During anoxia at room temperature, brain ATP levels slowly decrease and when ATP falls to about 35% of normoxic levels, ionic homeostasis is no longer maintained and there is a slow increase in extracellular K^+ ($[K^+]_o$) (Knickerbocker and Lutz, 2001). If anoxia is continued, this slow increase of $[K^+]_o$ is followed by a

sharp increase in $[K^+]_o$ and release of the amino acid neurotransmitters GABA and glutamate. Thus, the 'slow death' of the anoxic frog brain exhibits all the hallmarks of acute energy failure, similar to that seen in mammals, but on a significantly extended time scale (Wegner and Krause, 1993; Lutz and Reiners, 1997; Knickerbocker and Lutz, 2001; Lutz and Nilsson, 2004). Although it is not known whether large increases in cell calcium are correlated with the limits of survival in the anoxic frog brain, total plasma calcium concentration triples after 4 days of anoxic submergence in the frogs *Rana catesbeiana* and *Rana pipiens* (Stewart et al., 2004). An additional observation on the hypoxia tolerance of amphibians is that tadpoles have a greater hypoxia tolerance than do adult frogs (Bradford, 1983; Crowder et al., 1998). This is consistent with the general observation that developing animals are more hypoxia tolerant than their adult counterparts (e.g. Duffy et al., 1975).

There is virtually nothing known about the cellular mechanisms underlying the increased hypoxia tolerance of larval amphibians, nor have any studies examined the effects of hypoxia on $[Ca^{2+}]_i$ in amphibian neurons. Given that survival of anoxic neurons appears to be enhanced with moderate increases of $[Ca^{2+}]_i$, we sought to examine the relationship between $[Ca^{2+}]_i$ and survival of tadpole forebrain cells during anoxia. Specifically, we wished to test the hypothesis that moderately large increases in $[Ca^{2+}]_i$ may be associated with prolonged, but survivable, hypoxia in tadpole brain cells. To test this hypothesis, we measured cell survival and $[Ca^{2+}]_i$ during anoxic exposure (up to 18 h) on a mixed population of cells (neurons and glia) acutely dissociated from tadpole forebrain tissue.

Materials and methods

Animals

Experiments were carried out on pre-metamorphic *Rana catesbeiana* Shaw tadpoles [5–10 g; Taylor-Kollros (T-K) stages V–XV (Taylor and Kollros, 1946)]. Tadpoles were purchased from a commercial supplier (Charles Sullivan, Nashville, TN, USA) and kept in a 40 l aquarium in aged tapwater at room temperature (23–25°C). Tadpoles were fed regularly with boiled spinach. All procedures were approved by the UCSF Committee on Animal Research.

Isolation of tadpole forebrain cells

In each experiment, the forebrain tissue from 2–3 tadpoles was used to yield a sufficient number of cells for study. Isolation of forebrain cells followed a modified procedure described previously for isolation of sympathetic ganglion neurons in adult frogs (Selyanko et al., 1990). Briefly, tadpoles were anesthetized in ice-cold tapwater (5–10°C) that had been bubbled with 2-2.5% isoflurane (balance O_2) for 15–20 min (cf. Firestone et al., 1993). Tadpoles were placed in the cold tapwater until all movements ceased (ca. 10 min). The animal was removed and decapitated. The forebrain was exposed by opening a hole in the skull with a pair of sharp iris scissors, removed and placed in cold artificial cerebrospinal fluid (aCSF). The composition of aCSF was (in mmol l^{-1}): NaCl, 104.0; KCl 4.0; $MgCl_2$, 1.4; $NaHCO_3$, 25.0; $CaCl_2$, 2.4; glucose, 10.0 (Winmill and Hedrick, 2003). Removal of the forebrain usually required less than 2 min to complete. Forebrain tissue was minced finely with sharp scissors in cold aCSF and then placed in trypsin (1 mg ml^{-1}) for enzymatic digestion at room temperature for 1 h. Following this, brain tissue was triturated 2–3 times with a fire-polished glass pipette in a neuroprotective external solution containing no Mg^{2+} or Ca^{2+} (Selyanko et al., 1990). Tissue was centrifuged for 1 min at approximately 3000 ***g*** and the tissue pellet resuspended in aCSF. Aliquot samples of the triturated tissue suspension (150–200 µl) in aCSF were placed onto 10 mm coverslips pre-coated with Cell-Tak (Collaborative Research, Bedford, MA, USA) and left undisturbed for 1 h at room temperature to allow cells to adhere to the substrate.

Assessment of cell death

Cell viability was assessed by propidium iodide (PI; Molecular Probes, Eugene, OR, USA) fluorescence. Propidium iodide, a highly polar fluorescent molecule, penetrates damaged plasma membranes and binds irreversibly to DNA. The bound PI fluoresces while the unbound PI does not fluoresce. Excitation light was 490 nm and emission was at 590 nm. Prior to viability determinations, cells were incubated with 1–5 µmol l^{-1} PI for 15–30 min. Determination of the percentage of living and dead cells was made by counting the total number of cells exhibiting PI fluorescence. For cell viability measurements with PI, the percentage of live cells relative to the total number of cells was calculated.

In one experiment with forebrain tissue from 2 tadpoles, we used immunohistochemistry for a neuron-specific nuclear protein (neuN) to determine the relative number of neurons in the acutely dissociated cell population. We found that greater than 90% of the cells that adhered to coverslips were immunopositive for neuN, indicating that the vast majority of cells studied were neurons rather than glia.

Measurements of intracellular calcium levels

We estimated intracellular calcium concentrations in isolated tadpole forebrain cells using three different calcium-sensitive indicators. Several different indicators were employed because we anticipated that anoxia-induced $[Ca^{2+}]_i$ changes could be quite large, and no single indicator is appropriate for estimating $[Ca^{2+}]_i$ under both basal conditions and after hypoxic stress (Hyrc et al., 2000). Therefore, we chose indicators with low calcium affinity (fura-2FF and benzothiazole coumarin; BTC) and higher calcium affinity (Fura-2), in order to encompass the possible range of calcium concentrations that might occur in cells. Further, with calcium indicators such as fura-2 and fura-2FF, the excitation $[Ca^{2+}]_i$ and emission wavelengths of the dyes overlap with fluorescent compounds such as NADH, which are labile during hypoxic stress, making interpretation difficult. The indicator BTC involves the use of longer wavelengths and avoids these

pitfalls. Isolated cells were incubated with 5–10 μmol l^{-1} of the indicators for 60 min before measurements.

Calibration of calcium estimates were done as follows. First, the dissociation constant (K_D) of all three dyes was determined with calcium buffer calibration kit supplied by Molecular Probes using the same light source, optical path and filters on the microscope stage. Measured K_D values were 0.26 μmol l^{-1} (fura-2), 8.1 μmol l^{-1} (fura 2-FF) and 9.1 μmol l^{-1} (BTC). Measurements and calibration of $[Ca^{2+}]_i$ were made in dissociated cells using a dual excitation fluorescence spectrometer (Photon Technology International, South Brunswick, NJ, USA) and a Nikon Diaphot inverted microscope. Intracellular calcium concentration in studies involving fura-2 and fura-2FF was calculated from the 340/380 nm fluorescence intensity at an emission wavelength of 510 nm, using the equations of Buck and Bickler (1995). With BTC, excitation wavelengths were 400 nm and 480 nm and emission intensity was measured at 540 nm.

Experimental protocol

Following dissociation and isolation of forebrain cells, coverslips containing forebrain cells suspended in aCSF were placed in a 2 l Billups-Rothenberg chamber (Del Mar, CA, USA). Cells were exposed to an anoxic atmosphere by allowing 100% N_2 to flow through the chamber (ca. 1 l min^{-1}) for 15–20 min with the outflow tube placed in a beaker of water to create a slight positive pressure in the chamber. A moist paper towel was placed inside the chamber to create a humidified atmosphere and prevent desiccation of the cells. After 15–20 min, the flow was stopped and the chamber sealed until a coverslip was removed for measurement of $[Ca^{2+}]_i$ or PI. Each time a coverslip was removed, the remaining cells were briefly exposed to room air for approximately 30 s. The chamber was resealed and N_2 gas was allowed to flow through again as above and the chamber sealed after 15–20 min of N_2 exposure. In most experiments, coverslips were removed at 1, 2, 3, 4, 6 and 18 h after the start of anoxia for measurement of $[Ca^{2+}]_i$ or PI. Individual coverslips containing normoxic control cells were placed into a 24-well plate on the bench beside the chamber containing anoxic cells. The control coverslips with forebrain cells in aCSF were exposed to the same temperature as the anoxic cells and to room air for the same duration of time as the anoxic cells.

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) followed by the Neuman–Keuls *post hoc* test after determining homogeneity of variances using Bartlett's test (Zar, 1974). If significant differences were found among variances, a non-parametric analysis by ranks (Kruskal–Wallis test) was used instead of ANOVA. Percentage data were arcsine transformed prior to statistical analysis (Zar, 1974). Statistical analyses were done using commercially available software (GraphPad Prism, v. 4.0, San Diego, CA, USA or StatistiXL v.1.3).

Results

Survival during anoxia

Isolated tadpole forebrain cells tolerated anoxia for many hours without noticeable swelling, blebbing or decrease in phase-contrast brightness. The percentage of live cells after isolation, determined by PI uptake, was 94.6±2.3% (mean ± S.E.M., N=15) and did not change after 4–6 h anoxia (93.4±2.0%; N=13). Longer exposure to anoxia (18 h) produced a significant reduction in the percentage of live cells to 72.5±5.2% (N=14; P<0.05) in comparison with control cells maintained for an equivalent time period (94.8±1.3%; N=8). Cells that were exposed to anoxia for 4–6 h, reoxygenated overnight and examined after 18 h also showed a significant reduction in the percentage of live cells to 77.0±2.7% (N=15; P<0.001). This value was not significantly different from cells that were exposed to anoxia for 18 h (Fig. 1).

Anoxia and $[Ca^{2+}]_i$

We used three ratiometric calcium indicators with varying calcium affinities to determine $[Ca^{2+}]_i$ since we were unsure to what extent calcium levels might change during anoxic exposure. Fura-2, which has a relatively high calcium affinity, gave a resting (normoxic) $[Ca^{2+}]_i$ of 47±3 nmol l^{-1} (Table 1; Fig. 2). Fura-2 is a more appropriate indicator for measurement of resting calcium levels than either BTC or fura 2-FF, both of which have relatively low calcium affinities. Owing to the low calcium affinity of fura 2-FF and BTC, these indicators would overestimate resting cell calcium, as indicated by normoxic calcium values of 0.8 μmol l^{-1} for fura 2-FF and 3.1 μmol l^{-1} for BTC (Table 1).

Regardless of the type of calcium indicator used, $[Ca^{2+}]_i$ increased significantly after 3–6 h anoxia, but the maximal levels of $[Ca^{2+}]_i$ varied by nearly 30-fold, owing to the different

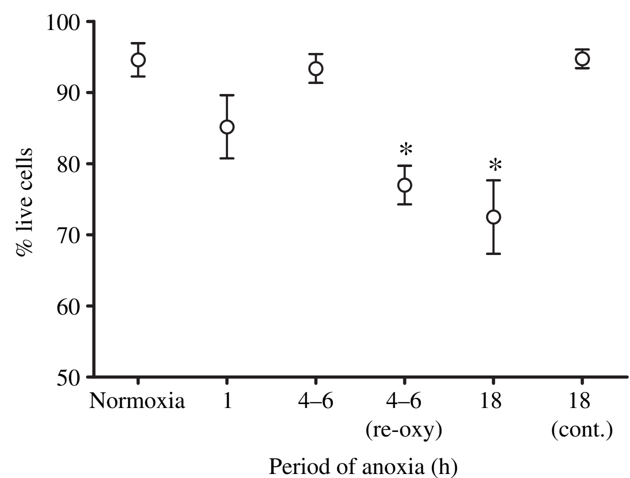

Fig. 1. Percentage of live cells exposed to normoxic and anoxic conditions. Cell death was determined by PI uptake following exposure to normoxia, 1 h anoxia, 4–6 h anoxia, 4–6 h anoxia followed by reoxygenation for 18 h (re-oxy), 18 h anoxia and 18 h normoxic control (cont). Values are means ± S.E.M. *P<0.05 compared with control.

Table 1. *Minimum and maximum $[Ca^{2+}]_i$ (μmol l^{-1}) measured with three different calcium ratiometric indicators*

Calcium indicator	$[Ca^{2+}]_i$ (μmol l^{-1}) Minimum	Maximum
Fura-2	0.05±0.003 (65)	0.83±0.56 (13)
Fura-2FF	0.80±0.05 (93)	9.18±5.47 (172)
BTC	3.12±0.98 (12)	31.8±8.0 (36)

Values are mean ± S.E.M. (*N*=number of cells).
Minimum values are normoxic controls.

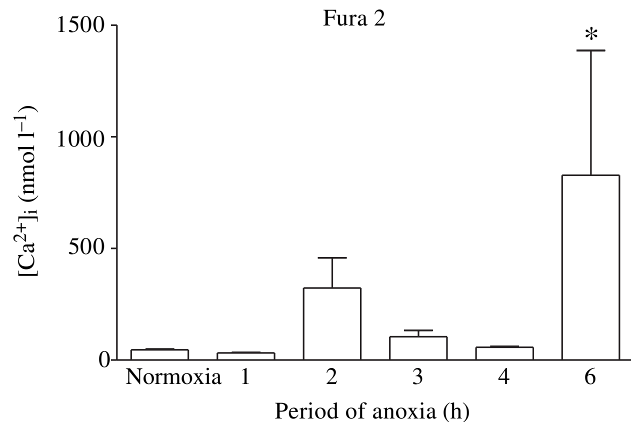

Fig. 2. Intracellular calcium ($[Ca^{2+}]_i$) (nmol l^{-1}) measured with fura-2. Measurements were taken from tadpole forebrain cells after exposure to normoxia and 1–6 h anoxia. Values are means ± S.E.M., *N*=13–65 cells; *$P<0.05$ from normoxia.

calcium affinities of the indicators. For example, there was no significant increase in $[Ca^{2+}]_i$ measured by fura-2 until 6 h anoxia (Fig. 2). Intracellular calcium levels measured by this method produced a maximum $[Ca^{2+}]_i$ of 0.83±0.56 µmol l^{-1} (Table 1). By contrast, $[Ca^{2+}]_i$, determined with fura 2-FF, increased significantly after 3 h anoxia ($P<0.05$) and increased to a maximal value of 9.2±5.5 µmol l^{-1} ($P<0.01$) after 4 h anoxia (Table 1; Fig. 3). Measurement of $[Ca^{2+}]_i$ with BTC produced similar results, with a significant increase in $[Ca^{2+}]_i$ after 4 h anoxia ($P<0.01$) and the highest $[Ca^{2+}]_i$ measured of 31.8 ± 8.0 µmol l^{-1} (Table 1; Fig. 4). Using the combination of fura-2 for estimating resting calcium levels and fura 2-FF or BTC for estimating maximal calcium levels during anoxia, the overall change in $[Ca^{2+}]_i$ during a 4–6 h exposure to anoxia produced an approximate 200 to 600-fold increase in $[Ca^{2+}]_i$.

Discussion

There are two important findings from our study. First, large increases in $[Ca^{2+}]_i$ occur in anoxic tadpole forebrain cells, but this is not associated with a significant increase in acute cell death. Second, the use of several calcium indicators allowed us to obtain reasonable estimates of resting cell calcium levels and the large (600-fold) increase in $[Ca^{2+}]_i$ during anoxia. Measurements over such a large range would not be possible using a single calcium indicator. The results indicate that large increases in $[Ca^{2+}]_i$ are not necessarily associated with acute cell death during anoxia.

Calcium and cell survival during anoxia

The major results from this study demonstrate that tadpole forebrain cells survive and recover from prolonged anoxia while experiencing relatively large increases in $[Ca^{2+}]_i$. Compared to mammalian neurons, which show evidence of increased $[Ca^{2+}]_i$ and cell death rapidly (within minutes to hours) with brief (<10 min) hypoxia, tadpole forebrain cells endured prolonged increases in $[Ca^{2+}]_i$ associated with anoxia without evidence of membrane damage. This finding is remarkable because it has been widely assumed that increases in $[Ca^{2+}]_i$ during anoxia are a central cause of cellular injury and death during severe hypoxia (Kristian and Siesjö, 1998). Indeed, preventing increases in $[Ca^{2+}]_i$ has been a common goal of therapies to treat brain ischemia or hypoxia (Choi, 1995). Survivable anoxia associated with large increases in $[Ca^{2+}]_i$ is concordant with data showing that anoxia-tolerant neurons from freshwater turtles (*Chrysemys*) undergo a 2–3-fold increase of $[Ca^{2+}]_i$ during prolonged and survivable hypoxia (Bickler, 1998; Bickler and Buck, 1998). Note that $[Ca^{2+}]_i$ estimates made by Buck and Bickler were all performed with fura-2 and thus true $[Ca^{2+}]_i$ may have been considerably higher (see below). Therefore, relatively large increases in $[Ca^{2+}]_i$ are not necessarily associated with cellular injury.

A number of studies involving mammalian neurons demonstrate that increases in $[Ca^{2+}]_i$ are associated with neuroprotective consequences. For example, some forms of the phenomenon of ischemic preconditioning (wherein a mild stress induces tolerance of a later severe ischemic insult) depend on increases in $[Ca^{2+}]_i$ produced by activation of NMDA receptors, and can be blocked by calcium chelators, calcium channel blockers or NMDA receptor blockers (Kato et al., 1992). Furthermore, there is good evidence that survival mechanisms associated with ischemic preconditioning involve calcium-dependent processes such as activation of protein kinase C (Raval et al., 2003), increased expression of MAP kinase cascades, nitric oxide (Nandagopal et al., 2001) and the survival factors bax and bcl-2 activated by Akt (protein kinase B; Mattson, 1997). In mammalian hippocampal neurons, moderate increases in $[Ca^{2+}]_i$ produced by calcium-selective ionophores protect by activating calcium-dependent signal cascades (Bickler and Fahlman, 2004). Furthermore, it is clear that too little calcium at critical times induces apoptosis in neurons and other cells (Lee et al., 1999). Altogether, the available information suggests that moderate increases in $[Ca^{2+}]_i$ may actually be crucial to surviving hypoxic stress.

Intracellular calcium estimates during anoxia

We used several different calcium indicator compounds to show that survivable anoxia is associated with relatively large increases in $[Ca^{2+}]_i$ in tadpole forebrain cells. This approach greatly increases the probability that we are correct in the assertion that the calcium increases were substantial. Fura-2

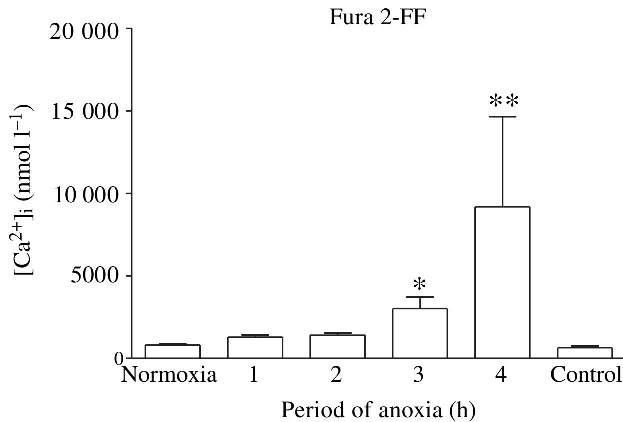

Fig. 3. Intracellular calcium $[Ca^{2+}]_i$ (nmol l^{-1}) measured with fura-2FF. Measurements were taken from forebrain cells after exposure to normoxia, and 1–4 h anoxia and 4 h normoxic control. Values are means ± S.E.M., N=21–172 cells; *P<0.05, **P<0.01 from normoxia or control.

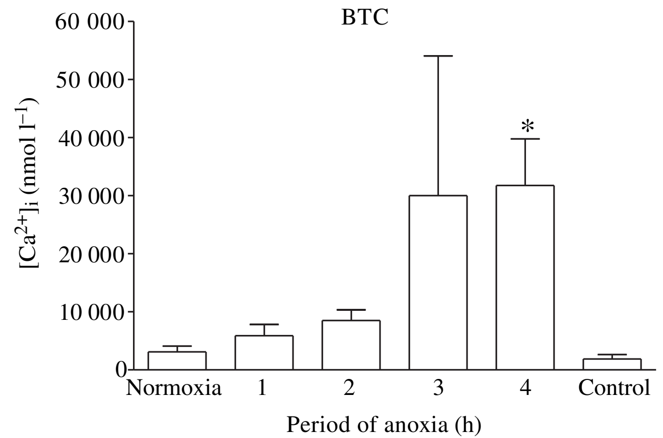

Fig. 4. Intracellular calcium $[Ca^{2+}]_i$ (nmol l^{-1}) measured with BTC. Measurements were taken from forebrain cells after exposure to normoxia, and 1–4 h anoxia and 4 h normoxic control. Values are means ± S.E.M., N=12–36 cells; *P<0.01 from normoxia or control.

has a K_D of approximately 300 nmol l^{-1} and is therefore an appropriate dye for estimating calcium levels in the low nanomolar range. The estimate of resting $[Ca^{2+}]_i$ of 50 nmol l^{-1} using fura-2 is in the same range as estimates of resting $[Ca^{2+}]_i$ in snail neurons made using Ca^{2+} microelectrodes (Kennedy and Thomas, 1996) and in bullfrog sympathetic neurons using the ratiometric indicators fura-2 (Nohmi et al., 1988) and fluo-3 (Yu et al., 1994). Estimates of resting calcium in turtle neurons, measured using fura-2, are higher (100–180 nmol l^{-1}) than we have measured in tadpole cells (Bickler, 1998; Buck and Bickler, 1995; Bickler et al., 2000). In addition, our estimate of $[Ca^{2+}]_i$ in anoxic forebrain cells of about 0.83 μmol l^{-1} (Table 1) measured with fura-2 is similar to maximal values (1 μmol l^{-1}) measured with fura-2 in bullfrog sympathetic neurons permeabilized with digitonin (Tokimasa et al., 1997).

During hypoxia maximal calcium concentrations estimated with fura-2 are limited because of at least two factors. First, significant labile background fluorescence occurs at the same wavelengths employed with fura-2, particularly that from NADH. Second, the K_D of fura-2 makes detection of $[Ca^{2+}]_i$ changes problematic when $[Ca^{2+}]_i$ exceeds 600 nmol l^{-1} (Hyrc et al., 1997). BTC avoids the first problem because the excitation and emission wavelengths are greater than those of fura dyes. Fura-2FF, with a K_D of approximately 8 μmol l^{-1}, avoids the second problem. However, both low-affinity calcium indicators are poor for estimating resting calcium levels, evidenced by the very high resting values obtained with these indicators (Table 1). Regardless of the type of calcium indicator used, all results agree with the conclusion that survivable anoxia in tadpole forebrain cells is associated with increases in $[Ca^{2+}]_i$ on the order of hundreds of nmol l^{-1}.

The finding that large increases in $[Ca^{2+}]_i$ are not associated with increased cell death is also consistent with recent studies in adult frogs showing that energy loss and neuronal death are not tightly linked. For example, exposure of *Rana pipiens* to anoxia causes a large (80%) fall in brain ATP in about 1 h, but the frog survives an additional 1–2 h before the release of excitotoxic neurotransmitters (Lutz and Reiners, 1997). Anoxia tolerance in the adult frog is also extended by maintaining ion homeostasis during anoxia (Knickerbocker and Lutz, 2001). An emerging view from recent studies with amphibians is that anoxia results in a ‘slow death’ of neurons with the same sequence of degenerative events as seen in mammals, but on a longer time scale (Milton et al., 2003; Lutz and Nilsson, 2004).

Natural history observations suggest that early-stage *Rana* tadpoles are more tolerant to extended periods of hypoxia or anoxia than late-stage tadpoles or adults (Bradford, 1983; Crowder et al., 1998), and perhaps part of the increased hypoxia tolerance relates to tolerance of large increases in $[Ca^{2+}]_i$ during cellular anoxia. By contrast, adult amphibians are relatively intolerant of anoxia or severe hypoxia for longer than a few days at 3°C (Hutchison and Dady, 1964; Christiansen and Penney, 1973; Lillo, 1980; Stewart et al., 2004) or a few hours at room temperature. These survival rates are consistent with studies at the cellular level (Lutz and Reiners, 1997; Knickerbocker and Lutz, 2001).

The increases in $[Ca^{2+}]_i$ observed in tadpole forebrain cells are similar in magnitude, but different in time scale, to those seen in more anoxia-tolerant neurons from Western painted turtles *Chrysemys picta*. Painted turtles survive several months of anoxia during winter dormancy (Ultsch and Jackson, 1982). In laboratory-induced dormancy, $[Ca^{2+}]_i$ in the cerebrocortex of this species increases to about twice normal over the course of several hours (Bickler et al., 2000) and remains elevated for weeks to months (Bickler, 1998). It should be noted, however, that previous estimates of $[Ca^{2+}]_i$ in turtle neurons were made exclusively with fura-2, perhaps underestimating the maximal increases in $[Ca^{2+}]_i$ that might occur during anoxia. In light of the results obtained using ratiometric indicators with low calcium affinities (fura-2FF and BTC), it might be useful to re-

examine the degree to which $[Ca^{2+}]_i$ increases in anoxia-tolerant species such as turtles. Thus, even though the increase in $[Ca^{2+}]_i$ during the first several hours of hypoxia is rather similar in tadpole and turtle neurons, the long-term survival is much more pronounced in turtle neurons. It is of interest to know more about how moderate increases in $[Ca^{2+}]_i$ relate to neuroprotective signaling.

In conclusion, we have demonstrated that anoxia tolerance in tadpole forebrain cells is associated with moderate to large increases in $[Ca^{2+}]_i$, but is not associated with increased cell death. The large increases in $[Ca^{2+}]_i$ were measurable only by the use of ratiometric indicators with varying affinities for calcium. The large increase in $[Ca^{2+}]_i$ may play a neuroprotective role for tadpole forebrain cells in anoxia.

These studies were supported by NIH-GM 52212 (P.E.B.) and NIH-SO6 GM 48135 (M.S.H.).

References

Aarts, M., Iihara, K., Wei, W. L., Xiong, Z. G., Arundine, M., Cerwinski, W., MacDonald, J. F. and Tymianski, M. (2003). A key role for TRPM7 channels in anoxic neuronal death. *Cell* **115**, 863-877.

Bickler, P. E. (1998). Reduction of NMDA receptor activity in cerebrocortex of turtles (*Chrysemys picta*) during 6 wk of anoxia. *Am. J. Physiol*. **275**, R86-R91.

Bickler, P. E. (2004). Clinical perspectives: neuroprotection lessons from hypoxia- tolerant organisms. *J. Exp. Biol*. **207**, 3243-3249.

Bickler, P. E. and Buck, L. T. (1998). Adaptations of vertebrate neurons to hypoxia and anoxia: maintaining critical calcium concentrations. *J. Exp. Biol*. **201**, 1141-1152.

Bickler, P. E. and Donohoe, P. H. (2002). Adaptive responses of vertebrate neurons to hypoxia. *J. Exp. Biol.* **205**, 3579-3586.

Bickler, P. and Fahlman, C. S. (2004). Moderate increases in intracellular calcium activate neuroprotective signals in hippocampal neurons. *Neurosci.* **127**, 673-683.

Bickler, P. E. and Hansen, B. M. (1998). Hypoxia-tolerant neonatal CA1 neurons: Relationship of survival to evoked glutamate release and glutamate-receptor mediated calcium changes in hippocampal slices. *Dev. Brain Res*. **106**, 57-69.

Bickler, P. E., Donohoe, P. H. and Buck, L. T. (2000). Hypoxia-induced silencing of NMDA receptors in turtle neurons. *J. Neurosci.* **20**, 3522-3528.

Bradford, D. F. (1983). Winterkill, oxygen relations, and energy metabolism of a submerged dormant amphibian, *Rana muscosa*. *Ecology* **64**, 1171-1183.

Buck, L. T. and Bickler, P. E. (1995). Role of adenosine in NMDA receptor modulation in the cerebral cortex of anoxia-tolerant turtle (*Chrysemys picta belli*). *J. Exp. Biol*. **198**, 1621-1628.

Choi, D. W. (1995) Calcium: still center stage in hypoxic-ischemic neuronal death. *Trends Neurosci*. **18**, 58-60.

Christiansen, J. and Penney, D. (1973). Anaerobic glycolysis and lactic acid accumulation in cold-submerged *Rana pipiens*. *J. Comp. Physiol*. **87**, 237-245.

Crowder, W. C., Nie, M. and Ultsch, G. R. (1998). Oxygen uptake in bullfrog tadpoles (*Rana catesbeiana*). *J. Exp. Zool*. **280**, 121-134.

Duffy, T. E., Kohle, S. J. and Vannucci, R. C. (1975). Carbohydrate and energy metabolism in perinatal rat brain: relation to survival in anoxia. *J. Neurochem.* **24**, 271-276.

Firestone, S., Firestone, L. L., Ferguson, C. and Blanck, D. (1993). Staurosporine, a protein kinase C inhibitor, decreases the general anesthetic requirement in *Rana pipiens* tadpoles. *Anesth. Analg*. **77**, 1026-1030.

Hochachka, P. W. and Lutz, P. L. (2001). Mechanism, origin and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol.* **130B**, 435-459.

Hutchison, V. H. and Dady, M. J. (1964). The viability of *Rana pipiens* and *Bufo terrestris* submerged at different temperatures. *Herpetologica* **20**, 149-162.

Hyrc, K., Handran, D. S., Rothman, S. M. and Goldberg, M. P. (1997). Ionized intracellular calcium concentration predicts excitotoxic neuronal death: observations with low affinity fluorescent calcium indicators. *J. Neurosci*. **17**, 6669-6677.

Hyrc, K. L., Bownik, J. M. and Goldberg, M. P. (2000). Ionic selectivity of low-affinity ratiometric calcium indicators: mag-Fura-2, Fura-2FF and BTC. *Cell Calcium* **27**, 75-86.

Kato, H., Liu, Y., Araki, T. and Kogure, K. (1992). MK-801, but not anisomycin, inhibits the induction of tolerance to ischemia in the gerbil hippocampus. *Neurosci. Lett*. **139**, 118-121.

Kennedy, H. J. and Thomas, R. C. (1996). Effects of injecting calcium-buffer solution on $[Ca^{2+}]_i$ in voltage-clamped snail neurons. *Biophys. J*. **70**, 2120-2130.

Knickerbocker, D. L. and Lutz, P. L. (2001). Slow ATP loss and the defense of ion homeostasis in the anoxic frog brain. *J. Exp. Biol*. **204**, 3547-3551.

Kristian, T. and Siesjö, B. K. (1998). Calcium in ischemic cell death. *Stroke* **29**, 705-718.

Lee, J. M., Zipfel, G. J. and Choi, D. W. (1999). The changing landscape of ischaemic brain injury mechanisms. *Nature* **399 suppl.**, A7-A14.

Lillo, R. S. (1980). Heart rate and blood pressure in bullfrogs during prolonged maintenance in water at low temperature. *Comp. Biochem. Physiol*. **65A**, 251-253.

Lipton, P. (1999). Ischemic cell death in neurons. *Physiol. Rev*. **79**, 1432-1568.

Lutz, P. L. and Nilsson, G. E. (2004). Vertebrate brains at the pilot light. *Respir. Physiol. Neurobiol*. **141**, 285-296.

Lutz, P. L. and Reiners, R. (1997). Survival of energy failure in the anoxic frog brain: delayed release of glutamate. *J. Exp. Biol*. **200**, 2913-2917.

Mattson, M. P. (1997). Neuroprotective signal transduction: Relevance to stroke. *Neurosci. Biobehav. Rev*. **21**, 193-206.

Milton, S. L., Manuel, L. and Lutz, P. L. (2003). Slow death in the leopard frog *Rana pipiens*: neurotransmitters and anoxia tolerance. *J. Exp. Biol*. **206**, 4021-4028.

Nandagopal, K., Dawson, T. M. and Dawson, V. L. (2001). Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. *J. Pharmacol. Exp. Ther*. **297**, 474-478.

Nohmi, M., Kuba, K., Ogura, A. and Kudo, Y. (1988). Measurement of intracellular Ca^{2+} in bullfrog sympathetic ganglion cells using fura 2 fluorescence. *Brain Res*. **438**, 175-187.

Raval, A. P., Dave, K. R., Mochly-Rosen, D., Sick, T. J. and Perez-Pinzon, M. A. (2003). Epsilon PKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. *J. Neurosci*. **23**, 384-391.

Selyanko, A. A., Smith, P. A. and Zidichouski, J. A. (1990). Effects of muscarine and adrenaline on neurones from *Rana pipiens* sympathetic ganglia. *J. Physiol.* **425**, 471-500.

Stewart, E. R., Reese, S. A. and Ultsch, G. R. (2004). The physiology of hibernation in Canadian leopard frogs (*Rana pipiens*) and bullfrogs (*Rana catesbeiana*). *Physiol. Biochem. Zool*. **77**, 65-73.

Taylor, A. C. and Kollros, J. J. (1946). Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec*. **94**, 7-24.

Tokimasa, T., Shirasaki, T. and Kuba, K. (1997). Evidence for the calcium-dependent potentiation of M-current obtained by the ratiometric measurement of the fura-2 fluorescence in bullfrog sympathetic neurons. *Neurosci. Lett*. **236**, 123-126.

Ultsch, G. R. and Jackson, D. C. (1982). Long-term submergence at 3°C of the turtle, *Chrysemys picta belli*, in normoxic and severely hypoxic water. I. Survival, gas exchange and acid–base status. *J. Exp. Biol*. **96**, 11-28.

Wegner, G. and Krause, U. (1993). Environmental and exercise anaerobiosis in frogs. In *Surviving Hypoxia* (ed. P. W. Hochachka, P. L. Lutz, T. J. Sick, M. Rosenthal and G. van den Thillart), pp. 217-236. Boca Raton, FL: CRC Press.

Winmill, R. E. and Hedrick, M. S. (2003). Gap junction blockade with carbenoxolone differentially affects fictive breathing in larval and adult bullfrogs. *Respir. Physiol. Neurobiol*. **138**, 239-251.

Yu, S. P., O'Malley, D. M. and Adams, P. R. (1994). Regulation of M current by intracellular calcium in bullfrog sympathetic ganglion neurons. *J. Neurosci*. **14**, 3487-3499.

Yu, S. P., Canzoniero, L. M. T. and Choi, D. W. (2001). Ion homeostasis and apoptosis. *Curr. Opin. Cell Biol.* **13**, 405-411.

Zar, J. H. (1974). *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice-Hall.