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ROLES OF ENERGY STATUS, K_{ATP} CHANNELS AND CHANNEL ARREST IN FISH BRAIN K^+ GRADIENT DISSIPATION DURING ANOXIA

DAN JOHANSSON AND GÖRAN E. NILSSON

Vertebrate Physiology and Behaviour Unit, Department of Limnology, Uppsala University, Norbyvägen 20, S-752 36 Uppsala, Sweden

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Summary

The crucian carp (*Carassius carassius* L.) is one of the most anoxia-tolerant vertebrates known, being able to maintain ion homeostasis in its brain for many hours of anoxia. This study aims to clarify the importance of glycolysis during anoxia and also to investigate whether the extreme tolerance to anoxia could be due to down-regulation of K^+ permeability ('channel arrest') and/or activation of ATP-sensitive K^+ (K_{ATP}) channels. The latter was also tested in rainbow trout (*Oncorhynchus mykiss*).

The results suggest that, during anoxia, the crucian carp brain is completely dependent on glycolysis, since blocking glycolysis with iodoacetic acid (IAA) rapidly caused an increase in $[K^+]_o$ that coincided with a drastic drop in ATP level and energy charge. Testing the channel arrest hypothesis by measuring the K^+ efflux rate after Na^+/K^+-ATPase had been blocked by ouabain revealed no change in K^+ permeability in crucian carp brain in response to anoxia. Furthermore, superfusing the brain of anoxic crucian carp with the K_{ATP} channel blocker glibenclamide did not alter the efflux rate of K^+ after glycolysis had been inhibited with IAA. Glibenclamide had no effect on K^+ efflux rate in rainbow trout brain during anoxia.

Key words: anoxia, crucian carp, *Carassius carassius*, depolarisation, energy charge, glibenclamide, iodoacetic acid, $[K^+]_o$, ouabain, rainbow trout, *Oncorhynchus mykiss*.

Introduction

In fish (Nilsson *et al.* 1993) as well as mammals (Hansen, 1985), the brain is particularly sensitive to anoxia. The most critical problem for the anoxic brain is thought to be the matching of energy consumption with energy production. In the absence of oxygen, ATP can be produced only through anaerobic glycolysis, which yields about 8 % of the ATP produced from each mole of glucose during aerobic metabolism (Hochachka and Somero, 1984). In the mammalian brain, the glycolytic ATP production rate can apparently not be increased sufficiently to compensate for the loss of aerobic ATP production, since falling ATP levels are seen within one or a few minutes of anoxia/ischaemia.

The anoxic mammalian brain also shows a characteristic increase in extracellular K^+ activity ($[K^+]_o$), which starts during the first seconds of anoxia, when extracellular Ca^{2+} and Na^+ concentrations are still relatively unaffected (Hansen, 1985). When the anoxic brain depolarises, a cascade of degenerative events occurs, of which an increase in intracellular $[Ca^{2+}]$ appears to be particularly destructive, activating degrading processes and thus preventing recovery of the neurones (Siesjö, 1990).

Nevertheless, the crucian carp (*Carassius carassius* L.) and the congeneric goldfish (*Carassius auratus* L.) have the unique ability to survive anoxia for weeks at 10 °C and for months at temperatures close to 0 °C (Piironen and Holopainen, 1986). A similar degree of anoxia tolerance has also evolved, obviously separately, in some freshwater turtles (Ultsch, 1989). These anoxia-tolerant animals maintain their brain ATP levels when exposed to anoxia (Lutz *et al.* 1984; Van der Boon *et al.* 1992). This remarkable accomplishment shows that they have the ability to increase glycolytic ATP production rate and/or can lower their rate of ATP consumption in response to anoxia. The latter could be achieved by reducing ion permeability, as suggested by the channel arrest hypothesis proposed by Lutz *et al.* (1985) and Hochachka (1986).

In freshwater turtles, treatment with the glycolytic inhibitor iodoacetic acid (IAA) has been found to cause a rapid loss of brain K^+ gradients during anoxia (Sick *et al.* 1982). More recently, Chih *et al.* (1989*b*) found that blocking the Na^+/K^+ pump with ouabain resulted in an increase in $[K^+]_o$ that was slower to occur in anoxic than in normoxic turtles, which was taken as evidence for an anoxia-induced lowering of neuronal permeability (channel arrest) – neurones in general being more permeable to K^+ than to other cations. However, with regard to *Carassius*, there is no experimental information on the coupling between glycolytic ATP production and the maintenance of ion gradients. Indeed, there have been few experimental studies on the effects of anoxia on brain ion

gradients in fish. It was only recently shown that the anoxic crucian carp maintains a low $[K^+]_o$ for several hours in anoxia, while an anoxia-intolerant fish, such as the rainbow trout (*Oncorhynchus mykiss*), loses its brain ion gradients after only 30 min of anoxia (Nilsson *et al.* 1993).

By blocking ATP-sensitive K^+ channels (K_{ATP} channels) with glibenclamide in rats, Jiang *et al.* (1992) showed that as much as 40–45 % of the early outflow of K^+ during anoxia occurred through these channels. In mammals, the K_{ATP} channel opens in response to falling intracellular ATP levels and is thought to function as an energy-saving mechanism in energetically compromised neurones, allowing a large repolarizing current and, thus, inhibiting action potentials and neurotransmitter release (Krnjevic, 1990). Unfortunately, this strategy is only efficient as long as the cell is able to restore the energy charge. Hence, during more severe energy deficiency, resulting in longer periods (minutes) of low intracellular ATP levels, it becomes a dangerous scheme since rising $[K^+]_o$ will ultimately cause a depolarisation of the neurones.

Consequently, we formulated the following three aims for this study. (1) To determine whether there is a connection between K^+ balance and [ATP] maintenance through anaerobic glycolysis in the anoxic crucian carp brain. This was accomplished by blocking glycolysis during anoxia using the glycolytic inhibitor IAA. (2) To examine whether anoxia causes a change in K^+ permeability (i.e. channel arrest), as measured by the rate of K^+ efflux after Na^+/K^+ pump activity has been blocked by ouabain. (3) To investigate to what extent glibenclamide-sensitive K_{ATP} channels contribute to the K^+ permeability of the neuronal membranes in anoxic crucian carp brain. Here, comparative experiments were carried out on rainbow trout, an anoxia-intolerant species.

Materials and methods

Animals

Crucian carp, weighing 46±17 g (mean ± S.D.; *N*=60), were caught in osier baskets in a pond near Uppsala, Sweden, in August. The animals were kept indoors in 1000 l tanks with a continuous flow (3 l min^{-1}) of aerated Uppsala tapwater (9–12 °C). They were fed daily with Hikari Staple floating mini pellet (Kyorin Food Ind. Ltd). Rainbow trout, weighing 450±150 g (mean ± S.D.; *N*=25), were obtained from a local fish farm. They were kept under the same conditions as the crucian carp and were fed with commercial trout food (ET 90 no. 3, EWOS, Sweden).

The artificial light/dark cycle automatically followed Hamburg's latitude and longitude. The experiments were carried out in January and February (crucian carp) and June (rainbow trout).

Measurements of $[K^+]_o$

Crucian carp and rainbow trout were anaesthetised with pentobarbital (45 mg kg^{-1} intraperitoneally) and immobilised using Alloferin (alcuronium, 6 mg kg^{-1} intramuscularly). A hole (6 mm×5 mm) was opened in the skull above the telencephalon and the opening was kept moist with saline. The fish was placed in a holder and ventilated with aerated tapwater (500 ml min^{-1} for crucian carp, 1000 ml min^{-1} for rainbow trout) flowing over the gills through a tube inserted in the mouth. The temperature of the ventilating water was 10±1 °C (crucian carp) or 12±1 °C (rainbow trout). Anoxia was initiated and maintained by bubbling N_2 into the respiratory water. This caused the O_2 level in the water to fall below 0.1 mg l^{-1} within 5 min (as measured with an oxygen electrode). The holder was a rectangular polyvinylchloride box sealed during the experiment except for a small hole above the brain. The holding box was continuously flushed with air during normoxia or N_2 during anoxia. The position of the head was fixed with the mouth tube and two steel pins pressed against the sides of the skull. Using a micromanipulator, a double-barrelled K^+-selective glass microelectrode (4–6 μm tip diameter) was placed in the centre of one of the telencephalic hemispheres, with the tip 1 mm below the dorsal surface. The tip of one barrel had been sialinized and filled with ionophore (Fluka 60398 valinomycin-based potassium ionophore 1 – cocktail B). The remainder of this barrel was filled with 100 mmol l^{-1} KCl. The other barrel was filled with 3.0 mol l^{-1} NaCl and was used as a reference electrode to monitor local d.c. potentials. By the use of a differential amplifier, these d.c. potentials were electronically subtracted from the signal obtained from the K^+-sensitive barrel to yield a potential that varied with $[K^+]_o$. Further details of these procedures are given by Jiang *et al.* (1992) and Pérez-Pinzón *et al.* (1992).

Measurements of AMP, ADP and ATP levels in the telencephalon

A K^+ electrode was inserted into the telencephalon of a crucian carp that had been anaesthetized and subjected to surgery exactly as described above. Four different experiments were carried out. (1) After 60 min of normoxia, the telencephalon was rapidly lifted out and frozen in liquid N_2, the whole process taking less then 1 min. (2) After 60 min of anoxia, the telencephalon was lifted out and treated as described above. (3) After 60 min of normoxia, the brain surface was superfused with 10 mmol l^{-1} IAA. After another 60 min period of normoxia, the telencephalon was lifted out and treated as described above. (4) After 60 min of anoxia, the brain surface was superfused with 10 mmol l^{-1} IAA. When $[K^+]_o$ increased at more than 0.5 mmol l^{-1} min^{-1}, i.e. the start of phase 2 (see Results), the telencephalon was lifted out and treated as described above. While still frozen, the telencephalon was then weighed (14.3±4.5 mg; mean ± S.D., *N*=25) and sonicated in 300 μl of 6 % perchloric acid (PCA). After centrifugation at 15 000 ***g*** for 10 min, the supernatant was neutralised with 0.85 mol l^{-1} K_2CO_3 in the ratio 1:0.75 and centrifuged again as described above. The contents of ATP, ADP and AMP in 50 μl of each supernatant were analysed by HPLC as described by Van der Boon *et al.* (1992). The HPLC apparatus consisted of a reversed-phase column (4 mm×120 mm, Nucleosil 120, C_{18}, 3 μm; Macherey-Nagel, Düren, Germany), a SpectroMonitor 4100 detector and a ConstaMetric III pump (both from LDC

Analytical, Riviera Beach, FL, USA). The mobile phase contained 0.1 mmol l^{-1} NaH_2PO_4, 5 mmol l^{-1} tetrabutylammonium bromide (TBAB) (pH 6.0) and 10 % (v/v) acetonitrile. The energy charge was calculated as ([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP]).

Measurement of blood pressure

Rainbow trout were catheterised as described by Soivio *et al.* (1975). The blood pressure was measured with a Gould Statham P23 ID pressure transducer (Gould Inc., CA, USA) and registered on a chart recorder simultaneously with the measurement of $[K^+]_o$. Blood pressure in crucian carp could not be measured because of the animals' small size, which made catheterisation impossible.

Drugs

The telencephalon of the fish was situated approximately 3 mm below the dorsal surface of the skull. The opening made above the telencephalon therefore created an empty space of approximately 120 μl. The drugs were applied by filling this space with a saline solution containing the drug (or saline in controls). The concentrations used were 10 mmol l^{-1} for IAA, 10 mmol l^{-1} for ouabain and 80 μmol l^{-1} for glibenclamide. All drugs were obtained from Sigma Chemical Co.

Statistics

Statistical significance was tested with Mann–Whitney *U*-test, except for blood pressure in rainbow trout which was analysed with two-way analysis of variance (ANOVA). Values are presented as means ± S.E.M.

Results

General observations of $[K^+]_o$

Crucian carp displayed virtually no increase in brain $[K^+]_o$ during anoxia (Fig. 1A) unless the brain was superfused with inhibitors of glycolysis (IAA) or Na^+/K^+-ATPase (ouabain). Crucian carp treated with IAA did not display any significant elevation of $[K^+]_o$ until exposed to anoxia (Fig. 1B). The increases in $[K^+]_o$ in the telencephalon of anoxic rainbow trout and anoxic crucian carp treated with IAA were similar and they could be divided into the same three phases described for mammals by Hansen (1985). In both fish species, phase 1 was a slow increase from 2–3 mmol l^{-1} up to 4–7 mmol l^{-1} taking approximately 30 min. This was followed by the sudden onset of a steep increase (phase 2) when the increase in $[K^+]_o$ was greater than 0.5 mmol l^{-1} min^{-1}. Phase 2 was shortlasting and phase 3 was rapidly reached, being characterised by a decline in the velocity of K^+ efflux followed by a stabilisation of $[K^+]_o$, in most cases at a level between 30 and 50 mmol l^{-1}. However, during phase 3, a decrease in $[K^+]_o$ often occurred, possibly reflecting a removal of $[K^+]_o$ by blood circulation in the tissue surrounding the electrode. In most cases, the level of $[K^+]_o$ during phase 3 was above 30 mmol l^{-1} for at least 30 min. The mean K^+ efflux rate during phase 1, the length of phase 1 and the $[K^+]_o$ reached at the start of phase 2 during the different experiments described above are presented in Table 1.

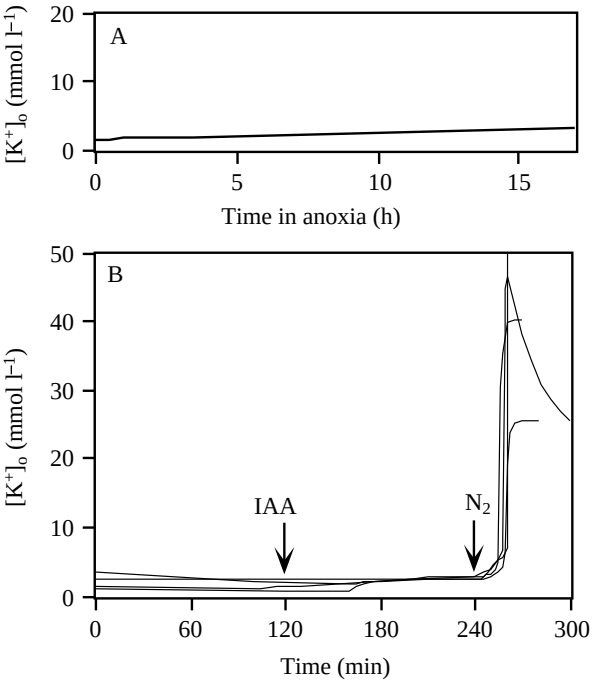

Fig. 1. $[K^+]_o$ in the telencephalon of crucian carp (A) during long-term anoxia and (B) during superfusion with iodoacetic acid (IAA) followed by exposure to anoxia. Results from four different preparations are shown in B.

Correlation between $[K^+]_o$ and energy status in crucian carp telencephalon

A comparison of the brain tissue content of AMP, ADP and ATP in normoxic crucian carp and in crucian carp kept anoxic for 120 min (Fig. 2A,B) showed no significant differences between the groups. Neither were there any significant differences between normoxic controls and normoxic crucian carp treated with IAA (Fig. 2C). In contrast, in anoxic crucian carp telencephalon undergoing depolarisation after IAA treatment (telencephalon sampled at the start of phase 2, Fig. 2D), the ATP level was 6.9 % and the AMP level 529.1 % of the anoxic control levels (Fig. 2B). As a result, energy charge had fallen to 18 % of the anoxic control value.

Effects of ouabain on $[K^+]_o$ in telencephalon of anoxic and normoxic crucian carp

The Na^+/K^+-ATPase blocker ouabain caused a rapid rise in $[K^+]_o$ when administered on the telencephalic surface of crucian carp. With regard to the time between ouabain exposure and the start of phase 2, there was no significant difference between anoxic and normoxic individuals in their response to ouabain (Table 1). Phase 2 occurred after 5.65±1.08 min at a $[K^+]_o$ of 3.50±0.40 mmol l^{-1} (Fig. 3A) and after 4.71±1.55 min at a $[K^+]_o$ of 3.12±0.62 mmol l^{-1} (Fig. 3B) in the anoxic and normoxic groups, respectively. Thus, the rate of rise in $[K^+]_o$ during phase 1 was not significantly affected by anoxia.

Table 1. *Characteristics of $[K^+]_o$ changes in crucian carp and rainbow trout telencephalon*

	Time between treatment and phase 2 (min)	$[K^+]_o$ at start of phase 2 (mmol l^{-1})	Rate of rise in $[K^+]_o$ during phase 1 (mmol l^{-1} min^{-1})
Anoxic crucian carp given ouabain (*N*=10)	5.65±1.08	3.50±0.40	0.13±0.02
Normoxic crucian carp given ouabain (*N*=7)	4.71±1.55	3.12±0.62	0.12±0.03
Anoxic crucian carp given glibenclamide followed by IAA (*N*=5)	36.20±4.69	4.24±0.46	0.07±0.02
Anoxic crucian carp given IAA (*N*=9)	32.97±4.16	5.06±0.34	0.09±0.01
Rainbow trout given glibenclamide followed by exposure to anoxia (*N*=11)	35.50±4.88	6.80±0.65	0.14±0.02
Rainbow trout exposed to anoxia (*N*=6)	32.58±5.57	6.30±0.92	0.13±0.01

Values are means ± S.E.M.
IAA, iodoacetic acid.

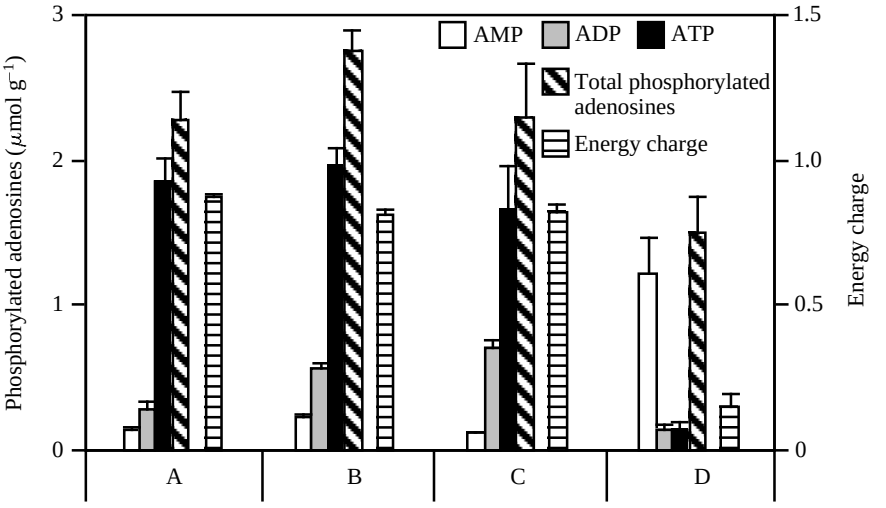

Fig. 2. Levels of phosphorylated adenosines and energy charge in the telencephalon of crucian carp during (A) normoxia (120 min), (B) anoxia (120 min), (C) normoxia combined with superfusion with IAA (120 min) and (D) anoxia (120 min) combined with IAA superfusion (telencephalon sampled at the start of phase 2). Values are means + S.E.M., *N*=10(A) or 5(B–D).

Effects of glibenclamide on $[K^+]_o$ in anoxic crucian carp brain

Superfusing the telencephalon of anoxic crucian carp with glibenclamide did not cause any prolongation of the time it took for IAA superfusion to cause the start of phase 2 (Table 1). The group treated with glibenclamide reached phase 2 after 36.20±4.69 min at a $[K^+]_o$ of 4.24±0.46 mmol l^{-1} (Fig. 4A), while phase 2 was reached in the control group after 32.97±4.16 min at a $[K^+]_o$ of 5.06±0.34 mmol l^{-1} (Fig. 4B).

Effects of glibenclamide on $[K^+]_o$ and blood pressure in rainbow trout

No significant difference was detected in the time course of $[K^+]_o$ increase during anoxia between the groups, the glibenclamide group reaching phase 2 after 35.50±4.88 min at a $[K^+]_o$ of 6.80±0.65 mmol l^{-1} and the control group reaching phase 2 after 32.58±5.57 min at a $[K^+]_o$ of 6.30±0.92 mmol l^{-1} (Table 1). Moreover, when superfused over the telencephalon, glibenclamide had no significant effect on systemic blood pressure in rainbow trout during normoxia and anoxia (results not shown), the blood pressure during anoxia following the same pattern of change as described previously (Nilsson *et al.* 1993).

Discussion

The present study shows that the crucian carp has an exceptional ability to maintain both ATP levels and ion homeostasis in its brain during anoxia and that this ability is dependent on glycolytic ATP production. The steep increase in telencephalic $[K^+]_o$ displayed by anoxic crucian carp treated with the glycolytic inhibitor IAA probably corresponds to the rapid increase in phase 2 $[K^+]_o$ occurring in the anoxic brain of rainbow trout (Nilsson *et al.* 1993) and mammals (Hansen, 1985), thus reflecting a general depolarisation of the telencephalon. A comparison of the telencephalic energy charge in IAA-treated anoxic crucian carp with that of anoxic controls showed that the phase 2 increase in $[K^+]_o$ in telencephalon coincided with a greater than 80 % decrease in energy charge in the telencephalon.

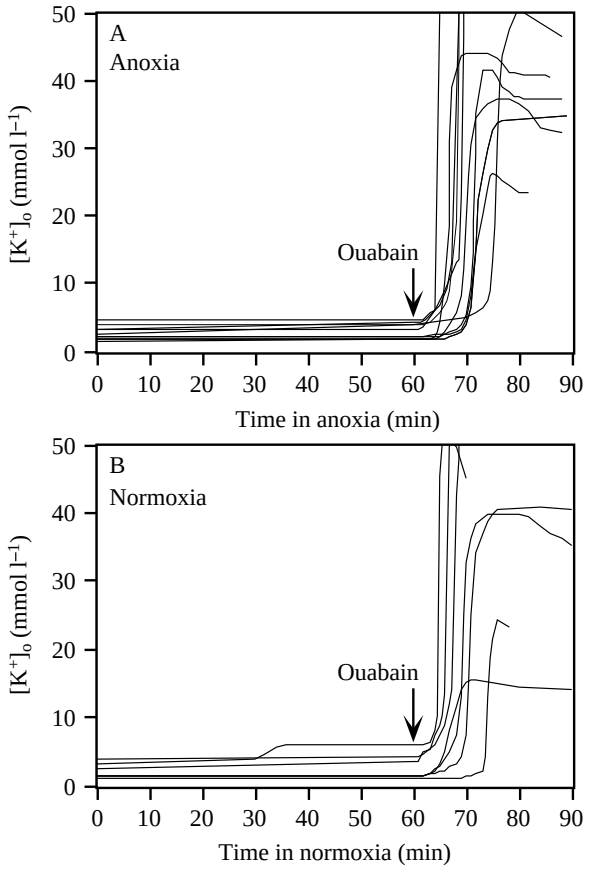

Fig. 3. $[K^+]_o$ in the telencephalon of crucian carp before and after superfusion with ouabain during (A) anoxia and (B) normoxia.

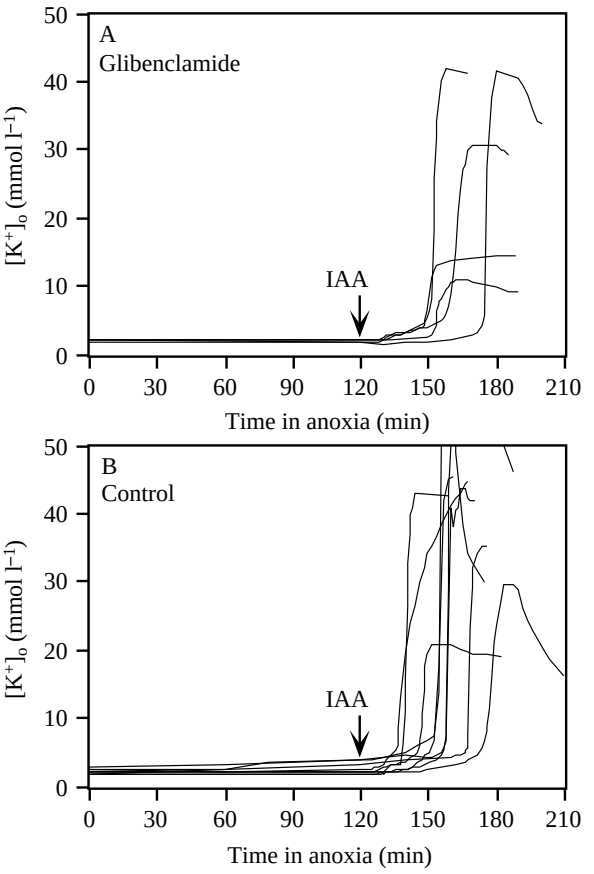

Fig. 4. $[K^+]_o$ in the telencephalon of anoxic crucian carp before and after superfusion with IAA. (A) Treated with glibenclamide. (B) Control.

This clearly indicates a causal connection between energy status and the ability of the brain to maintain ion balance, a connection that cannot be shown directly in the mammalian brain because it always loses its energy charge during anoxia. Thus, this study lends support to the hypothesis that loss of ion homeostasis is caused primarily by energy failure.

Like the brain of normoxic turtles (Sick *et al.* 1982; Chih *et al.* 1989*a*), the normoxic crucian carp brain was able to maintain a low $[K^+]_o$ when superfused with IAA, suggesting that substrates other than glucose can be utilized as energy sources. It is, however, also possible that some glycolytic activity remained after IAA treatment, and that this was sufficient to sustain a normal rate of oxidative ATP production, which yields about 13 times more ATP per mole of glucose than anaerobic glycolysis alone (Hochachka and Somero, 1984).

As expected, anoxic and normoxic crucian carp treated with the Na^+/K^+-ATPase blocker ouabain rapidly lost ion homeostasis in the telencephalon. As shown in earlier studies (Johansson *et al.* 1995), the crucian carp brain lowers its energy consumption in response to anoxia. Neurones are more permeable to K^+ than to any other cation, and reducing ion permeability ('channel arrest') has been hypothesised to be a major strategy for saving energy during anoxia (Lutz *et al.* 1985; Hochachka, 1986; Chih *et al.* 1989*b*; Doll *et al.* 1991). Thus, blocking the Na^+/K^+ pump and following the ensuing rise in $[K^+]_o$ should reveal whether there is a decreased K^+ permeability during anoxia. In fact, Chih *et al.* (1989*b*) used this approach to show an anoxia-induced reduction in K^+ permeability in the brain of the freshwater turtle *Trachemys scripta*. They found an almost 50 % reduction in initial K^+ leakage after 2 h of anoxia. However, our results show that, in crucian carp, the time between ouabain exposure and the rapid increase in $[K^+]_o$ does not differ between anoxic and normoxic individuals. Thus, in this respect, there appears to be a clear difference between crucian carp and turtles. A rational conclusion from this result is that there is no detectable down-regulation of K^+ permeability in crucian carp brain during anoxia, and there must therefore be other mechanisms responsible for the metabolic depression displayed by anoxic crucian carp brain tissue. One reason why the crucian carp does not down-regulate K^+ permeability could be that such a mechanism might be counterproductive in the context of metabolic depression. K^+ channels have a clamping effect on the membrane potential, and reducing K^+ permeability may move the membrane potential away from the K^+ equilibrium potential in the direction of depolarisation, thereby increasing the probability of action potential formation which, in turn, would cause an increased energy consumption in terms

of ion pumping. Nevertheless, it is possible that the crucian carp reduce the neural membrane 'leakiness' for ions such as Na^+ and Ca^{2+}, a strategy that should lead to reduced neuronal excitability.

It has been proposed that the K_{ATP} channel contributes to the maintenance of ion homeostasis in the mammalian brain during anoxia (Krnjevic, 1990). When brain slices from adult rats were exposed to anoxia in the presence of the K_{ATP} channel blocker glibenclamide (Jiang *et al.* 1992), the efflux rate of K^+ fell to 40–45 % of that in anoxic controls, indicating that this channel makes a major contribution to the efflux of K^+ during ATP depletion. However, anoxic crucian carp given the K_{ATP} blocker glibenclamide, followed by the glycolytic blocker IAA in order to inhibit ATP generation, displayed an increase in $[K^+]_o$ that followed the same pattern and time course as those of the corresponding control group. A likely explanation for these results would be that the K_{ATP} channel is of minor importance to the efflux of K^+ during ATP deficiency in crucian carp brain. Interestingly, Jiang *et al.* (1992) also found that anoxic brain tissue from neonatal rat and adult turtle, which both display an increased tolerance to anoxia, showed very little or no response to glibenclamide. Thus, these results may indicate that K_{ATP} channels are not in operation in anoxia-tolerant vertebrates.

However, the present study showed that the pattern of telencephalic $[K^+]_o$ rise in anoxic rainbow trout, an anoxia-sensitive ectotherm, fails to display any response to glibenclamide. This suggests that the lack of glibenclamide effects in crucian carp could have phylogenetic grounds rather than being a reflection of anoxia tolerance. It is, of course, also possible that teleost fish possess K_{ATP} channels that are insensitive to glibenclamide. Interestingly, recent studies on [^{3}H]glibenclamide binding in vertebrate cardiac membranes have indicated that neither chinook salmon nor carp possess K_{ATP} channels that bind glibenclamide (McKean *et al.* 1993).

In conclusion, the results show that the anoxia-tolerant crucian carp relies on maintained ATP levels and Na^+/K^+ pump activity to avoid a loss of ion homeostasis in brain during anoxia. Although the crucian carp brain, like the turtle brain, lowers its energy consumption in response to anoxia, it differs from the turtle brain in that a down-regulation of K^+ permeability does not seem to form part of this strategy. Furthermore, glibenclamide-sensitive K_{ATP} channels, which contribute to the maintenance of energy status in the mammalian brain, could not be detected in crucian carp or rainbow trout brain. However, whether fish brains have similar, but glibenclamide-insensitive, K_{ATP} channels remains to be determined. Furthermore, the results show that IAA can be used as a tool for inducing energy failure in the crucian carp brain. For future research, this opens up the opportunity of using specific channel blockers in order to study how different ion conductances contribute to ion fluxes in crucian carp brain.

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