ACTION OF ADENOSINE ON ENERGETICS, PROTEIN SYNTHESIS AND K+ HOMEOSTASIS IN TELEOST HEPATOCYTES

GERHARD KRUMSCHNABEL*, CHRISTINA BIASI AND WOLFGANG WIESER

Institut für Zoologie, Abteilung für Ökophysiologie, Universität Innsbruck, Technikerstraße 25, A-6020 Innsbruck, Austria

*e-mail: Gerhard.Krumschnabel@uibk.ac.at

Summary

In a comparative study, we analysed the effects of adenosine on the energetics, protein synthesis and K+ homeostasis of hepatocytes from the anoxia-tolerant goldfish *Carassius auratus* and the anoxia-intolerant trout *Oncorhynchus mykiss*. The rate of oxygen consumption did not respond immediately to the addition of adenosine to the cells from either species, but showed a significant decrease in trout hepatocytes after 30 min. The anaerobic rate of lactate formation was not significantly affected by adenosine in goldfish hepatocytes, but was increased in trout cells. We also studied the effects of adenosine on the two most prominent ATP consumers in these cells, protein synthesis and Na+/K+-ATPase activity. Under aerobic conditions, adenosine inhibited protein synthesis of hepatocytes from goldfish by 51 % and of hepatocytes from trout by 32 %. During anoxia, the rate of protein synthesis decreased by approximately 50 % in goldfish hepatocytes and by 90 % in trout hepatocytes, and this decrease was not altered by the presence of adenosine. Adenosine inhibited normoxic Na+/K+-ATPase activity and K+ efflux by 20–35 % in the cells of both species. An investigation into the mechanism underlying the inhibition of protein synthesis by adenosine indicated that, in the goldfish cells, adenosine acts *via* a membrane receptor-mediated pathway, i.e. the effect of adenosine was abolished by applying the A1 receptor antagonist 8-phenyltheophylline. In the trout, however, the uptake of adenosine into hepatocytes seems to be required for an effect on protein synthesis. [Ca^{2+}]$_i$ does not seem to be involved in the inhibition of protein synthesis by adenosine.

Key words: oxygen consumption, lactate production, K+ flux, intracellular free Ca^{2+}, metabolic depression, *Carassius auratus*, *Oncorhynchus mykiss*.

Introduction

Adenosine exerts an array of regulatory effects on cellular metabolism, many of which have been found to be beneficial under energy-limited conditions. The previously described effects of adenosine include (i) a decrease in anaerobic ATP production (Bernier et al., 1996; Nilsson, 1991; Fralix et al., 1993), (ii) an inhibition of ATP consumption by protein synthesis (Tinton et al., 1995) and (iii) a reduction in cellular ion leakage (Fralix et al., 1993; Buck and Bickler, 1998; Pék and Lutz, 1997). The common denominator among these effects is the fact that they aid in prolonging anoxic survival by decreasing cellular energy turnover, while at the same time maintaining a constant intracellular milieu. It is this very capability of coordinately shutting down ATP-producing and ATP-consuming functions that is characteristic of anoxia-tolerant cells and organisms. Thus, as suggested by Nilsson (1991), adenosine appears to be a likely candidate for a role in metabolic depression.

On the basis of this hypothesis, in the present study, we have compared the effects of adenosine on the energetics of hepatocytes from anoxia-tolerant goldfish *Carassius auratus* and hepatocytes from the anoxia-intolerant rainbow trout *Oncorhynchus mykiss*. The variables studied include oxygen consumption and lactate production, as an estimate of aerobic and anaerobic ATP production, the size of the cellular ATP pool, as a measure of the energetic state of the hepatocytes, protein synthesis and K+(Rb+) uptake by the Na+ pump, as representatives of ATP consumers, and the rate of K+ efflux.

In addition, the effects of blockage of adenosine receptors on the inhibition of protein synthesis by adenosine and the effects of adenosine on intracellular free Ca^{2+} levels were investigated. These experiments should provide some insight into the mechanistic basis of the effects of adenosine and into potential species-related differences in the transduction pathway for the action of adenosine.

Materials and methods

Chemicals

l-[4,5-^{3}H]leucine was purchased from Amersham. Collagenase (Type VIII), bovine serum albumin (BSA), Leibovitz L-15 medium, adenosine, 8-phenyltheophylline

(8-PT), nitrobenzyl thioinosine (NBTI), Fura 2-AM, thapsigargin and luciferase–luciferin were obtained from Sigma. All other chemicals were of analytical grade and were purchased from local suppliers.

Animals and preparation of hepatocytes

Experiments were conducted on hepatocytes isolated from goldfish *Carassius auratus* acclimated to 20 °C and from trout *Oncorhynchus mykiss* (Walbaum) acclimated to 15 °C, applying preparation procedures described previously (Krumschnabel et al., 1994b, 1996). Prior to experimentation, hepatocytes were incubated for 1 h in a shaking water bath thermostatted to the respective acclimation temperature, which was also the temperature used during the experiments. Cell media used for most studies consisted of (in mmol l^{-1}, for goldfish) 10 Hepes, 135 NaCl, 3.8 KCl, 1.3 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 10 NaHCO$_3$ and 1 % BSA, pH 7.6 at 20 °C, and (in mmol l^{-1}, for trout) 10 Hepes, 136.9 NaCl, 5.4 KCl, 1 MgSO$_4$, 0.33 NaH$_2$PO$_4$, 0.44 KH$_2$PO$_4$, 5 NaHCO$_3$, 1.5 CaCl$_2$, 5 glucose, and 1 % BSA, pH 7.6 at 20 °C. For K$^+$(Rb$^+$) flux measurements, cells were transferred to a medium containing Rb$^+$ instead of K$^+$ immediately before the experiments. Hepatocytes used for measurements of protein synthesis were maintained in modified Leibovitz L-15 medium (L-15 medium with 10 mmol l^{-1} Hepes, 5 mmol l^{-1} NaHCO$_3$, 1 % BSA, pH 7.6) for 1 h before experimentation. Cell viability was determined from Trypan Blue exclusion and was maintained at levels greater than 95 % throughout the experiments described.

Analytical procedures

The rate of oxygen consumption ($\dot{V}_{O_2}$), lactate, ATP and intracellular free Ca^{2+} ([Ca^{2+}]i) concentrations and rates of K$^+$(Rb$^+$) uptake and K$^+$ efflux were determined applying methods described previously (Krumschnabel et al., 1994b, 1996). Cycloheximide, used for the indirect estimation of protein synthesis, was applied at 15 mmol l^{-1}, the dose required to inhibit oxygen consumption maximally in teleost hepatocytes (Pannevis and Houlihan, 1992; Krumschnabel et al., 1994a).

For measurements of protein synthesis, hepatocytes were washed after 1 h of pre-incubation and resuspended at 20×10^6 cells ml^{-1} in modified L-15 medium devoid of BSA. Protein synthesis was determined from the incorporation of [^{3}H]leucine into cellular protein over 1 h. To this end, [^{3}H]leucine (18.5×10^3 Bq ml^{-1}) was added to cell suspensions, and duplicate 20 µl samples were removed onto Whatman GF/A filters after 5, 15, 30, 45 and 60 min. After three samplings, adenosine and/or NaCN were added, and sampling was continued for another 30 min. In this way, each [^{3}H]leucine incorporation curve could serve as its own control by comparing the rates before and after addition of the respective agent. Further treatment of the sample filters and measurement of precipitated radioactivity were as described in detail by Kwast and Hand (1993).

Experimental protocols

In all experiments, adenosine was applied at a concentration of 100 µmol l^{-1}, taken from a 30 mmol l^{-1} stock dissolved in dimethyl sulphoxide (DMSO). Wherever appropriate, control cells received an equal amount of DMSO alone so that the effect of the solvent on the variable under investigation could be assessed. The adenosine receptor antagonist 8-PT (100 µmol l^{-1} in DMSO) and the adenosine transport inhibitor NBTI (100 µmol l^{-1} in DMSO) were given either alone or 5 min prior to addition of adenosine. Anoxic conditions were created by gassing experimental flasks with a gas mixture of 99 % N$_2$/1 % CO$_2$ prepared by a Wösthoff gas-mixing pump (Wösthoff, Germany). The incubation flasks were closed with a rubber cap equipped with openings for the inlet and outlet of gas, the latter also serving for removal of samples as required. Time zero given in the figures corresponded to the initiation of anoxic gassing and was also the time when the first samples were removed. The time before fully anoxic conditions were established was approximately 5 min. Chemical anoxia, used in some experiments for methodological reasons, was established by addition of 2 mmol l^{-1} sodium cyanide (NaCN) from a neutralised stock solution to cells maintained in aerobic incubation medium.

Statistical analyses

Data are presented as means ± S.E.M. of N independent preparations. Statistical differences were evaluated applying Student's t-test or analysis of variance (ANOVA) as appropriate. In the latter case, if there was a significant difference among the treatments, Tukey's procedure was used to test the differences between individual means. $P<0.05$ was considered to be significant.

Results
Effects of adenosine on aerobic energetics

Fig. 1 summarises the effects of incubation with adenosine on total $\dot{V}_{O_2}$ ($\dot{V}_{O_2tot}$) and on $\dot{V}_{O_2}$ sensitive to the protein synthesis inhibitor cycloheximide ($\dot{V}_{O_2cs}$; an indirect estimate of the activity of protein synthesis) of hepatocytes from goldfish and trout. Exposure times of 10 and 30 min were chosen because, as will be shown below, most effects of adenosine determined in this study became apparent within this period.

In hepatocytes from goldfish, neither adenosine alone nor adenosine in combination with the adenosine receptor antagonist 8-PT had a significant impact on $\dot{V}_{O_2tot}$ within 10 min of incubation (Fig. 1A). When adenosine was given in the presence of the adenosine uptake inhibitor NBTI, a non-significant decrease in $\dot{V}_{O_2tot}$ was noted. Similarly, $\dot{V}_{O_2cs}$ showed no significant response to any of these treatments, although a slight (but not significant) decrease was again seen with adenosine plus NBTI.

After a 30 min incubation, control cells and cells in the presence of both 8-PT plus adenosine showed no changes in $\dot{V}_{O_2tot}$ and $\dot{V}_{O_2cs}$, but there was a tendency for both variables to be reduced by adenosine alone. The effect of NBTI was not

measured at this time point since, as will be shown below, NBTI alone was found to produce a drastic reduction in the rate of protein synthesis.

$\dot{V}_{O_2tot}$ of trout hepatocytes was unaltered by a 10 min incubation with adenosine, irrespective of the additional presence of 8-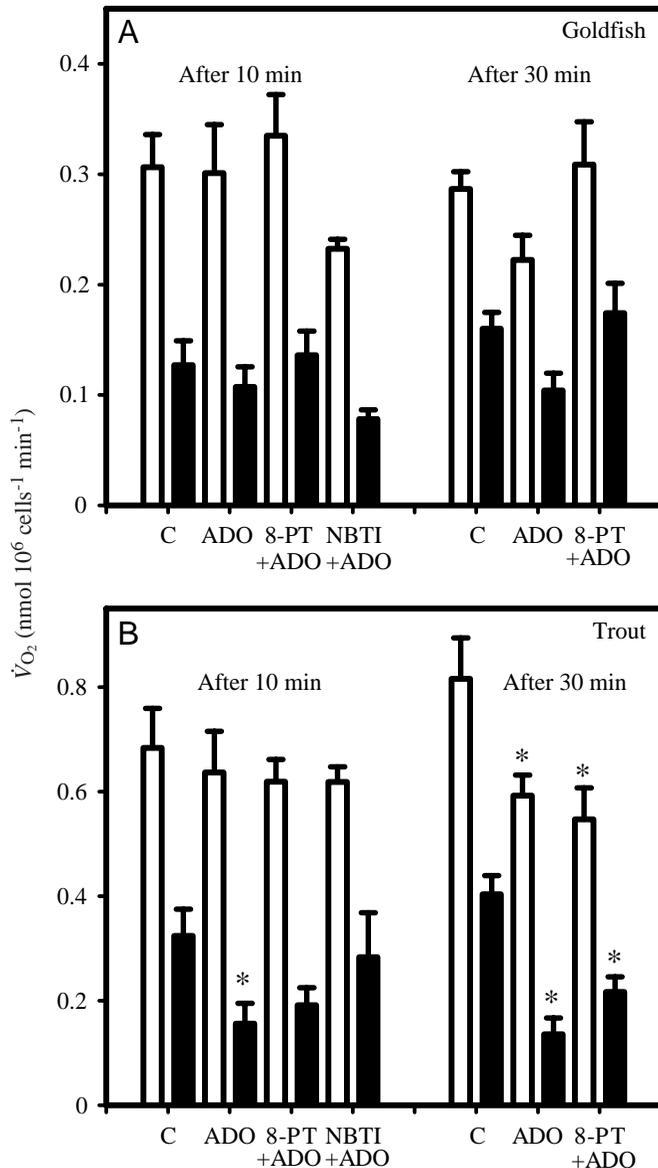PT or NBTI. In contrast, $\dot{V}_{O_2cs}$ was significantly decreased by exposure of the cells to adenosine alone, whereas no significant change was induced by adenosine in the presence of either 8-PT or NBTI. After 30 min of incubation, $\dot{V}_{O_2tot}$ of controls had increased slightly, whereas that of cells treated with adenosine or with 8-PT plus adenosine had decreased slightly, these alterations resulting in significant differences between controls and both groups of treated cells (Fig. 1B). $\dot{V}_{O_2cs}$, like $\dot{V}_{O_2tot}$, increased slightly in controls and remained significantly depressed in the presence of adenosine and 8-PT plus adenosine.

Effects of adenosine on anaerobic energetics

Cellular ATP content and anaerobic rates of ATP production, as estimated from lactate accumulation, were determined in anoxic cells (99 % N_2/1 % CO_2) in the absence and presence of adenosine. In goldfish hepatocytes in the absence of adenosine, ATP content showed a slight but non-significant decrease after 60 min but was, overall, largely unaffected over the 2 h of anoxic exposure studied (Fig. 2A). Adenosine-treated cells, while showing significantly elevated
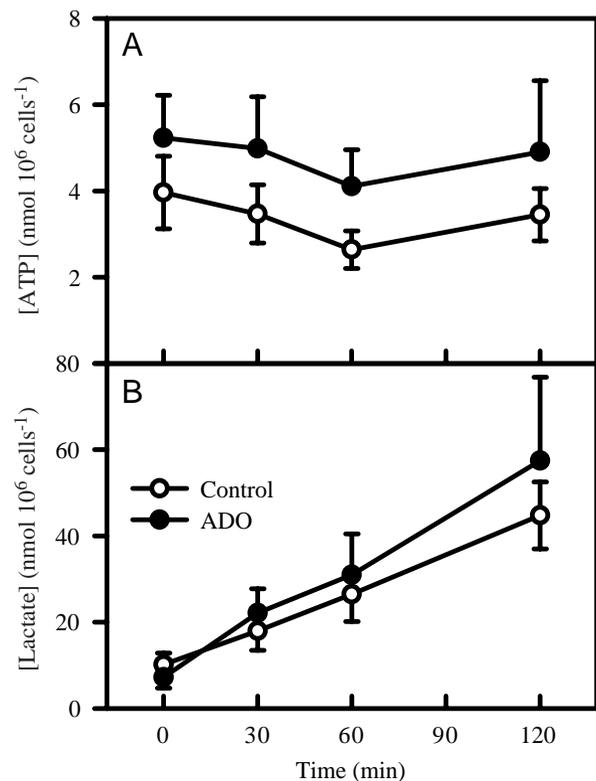

Fig. 1. Rates of total oxygen consumption (open columns) and cycloheximide-sensitive oxygen consumption (filled columns) in hepatocytes from (A) goldfish and (B) trout. Cells were incubated without further additions (C), with $100\,\mu mol\,l^{-1}$ adenosine (ADO), with $100\,\mu mol\,l^{-1}$ 8-phenyltheophyline + adenosine (8-PT+ADO) and with $100\,\mu mol\,l^{-1}$ nitrobenzyl thioinosine + adenosine (NBTI+ADO), and rates of oxygen consumption were determined after 10 and 30 min (10 min only for NBTI). Values are means + S.E.M. of 5–7 (four for NBTI) independent preparations for hepatocytes from goldfish and 7–10 (three for NBTI) independent preparations for hepatocytes from trout. An asterisk indicates a significant difference (*P*<0.05) from the respective control value.

Fig. 2. Cellular ATP content (A) and lactate production (B) of goldfish hepatocytes incubated under anoxia (99 % N_2/1 % CO_2) either in the absence (Control) or in the presence of $100\,\mu mol\,l^{-1}$ adenosine (ADO). Values are means ± S.E.M. of six independent preparations. Duplicate samples of each preparation were taken at the times indicated and analysed for ATP using the luciferase–luciferin method and for lactate using a standard enzymatic technique. The ATP concentration of adenosine-treated cells was significantly elevated compared with that of controls (*P*<0.05, repeated-measures ANOVA).
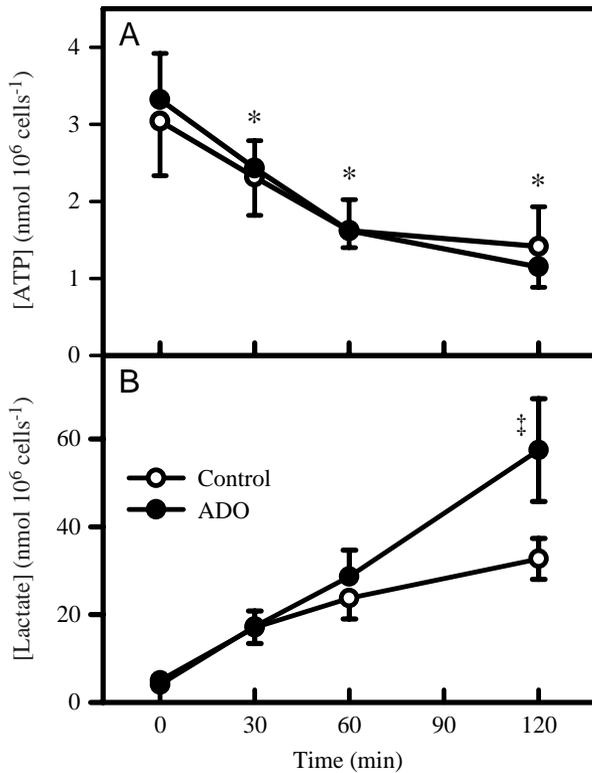
Fig. 3. Cellular ATP content (A) and lactate production (B) of trout hepatocytes incubated as described for Fig. 2. Values are means ± S.E.M. of six independent preparations. *$P<0.05$ compared with the value at time zero. ‡$P<0.05$ compared with the control value.

levels of ATP compared with controls ($P<0.05$), followed an essentially identical response pattern and at no point showed a significant decrease in ATP content.

The same was true for rates of lactate accumulation, which were almost linear in both groups of cells with a slight tendency to be higher in the presence of adenosine (Fig. 2B).

In trout hepatocytes, anoxia caused ATP levels to decline, following an almost identical pattern in the absence and presence of adenosine, from a starting value of approximately $3\,\mathrm{nmol}\,10^6\,\mathrm{cells}^{-1}$ to approximately $1.5\,\mathrm{nmol}\,10^6\,\mathrm{cells}^{-1}$ after 2 h of anoxia (Fig. 3A). In contrast, while rates of lactate production decreased with an increase in the duration of anoxia in the absence of adenosine, lactate accumulated at near-linear rates over 2 h of anoxia in the presence of adenosine, resulting in significantly elevated lactate concentrations in suspensions of adenosine-treated compared with untreated cells at 2 h (Fig. 3B).

Effects of adenosine on protein synthesis

The effect of adenosine, under normoxic conditions, on the incorporation of [^{3}H]leucine into cellular protein of goldfish hepatocytes is shown in Fig. 4: protein synthesis was impaired within 15 min after the addition of the nucleoside. A more detailed analysis of the effects of adenosine on the rate of protein synthesis is presented in Fig. 5A,B.

Expressed in relative terms, the inhibition of [^{3}H]leucine incorporation by adenosine amounted to approximately 51 %
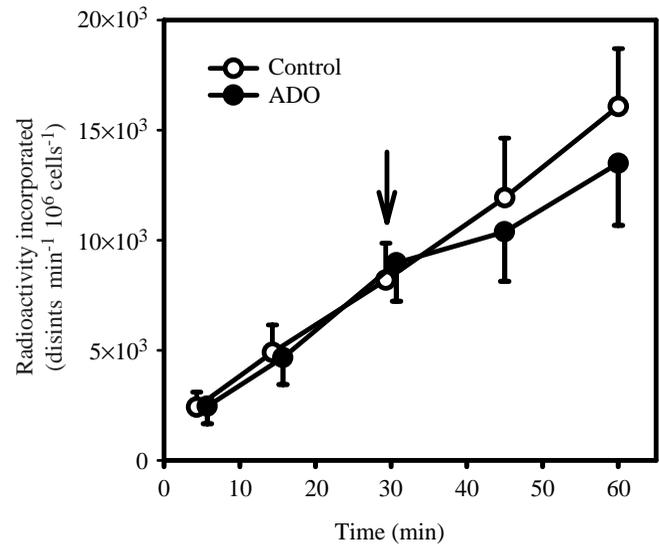

Fig. 4. Incorporation of [^{3}H]leucine into trichloroacetic-acid-precipitable protein in normoxic goldfish hepatocytes. After 30 min of incubation, cells were treated with dimethyl sulphoxide (DMSO) (Control) or $100\,\mu\mathrm{mol}\,\mathrm{l}^{-1}$ adenosine dissolved in DMSO (ADO), as indicated by the arrow. Values are means ± S.E.M. of eight independent preparations.

in hepatocytes from goldfish and 32 % in hepatocytes from trout. When adenosine was added to cells after a 5 min pre-incubation period with the specific A1 receptor antagonist 8-PT, a remarkable difference in the responses of the cells from the two species was observed. While in the goldfish cells, 8-PT completely prevented the inhibition of protein synthesis by adenosine, no such effect was seen in trout hepatocytes. On its own, 8-PT did not inhibit, but slightly stimulated, protein synthesis (in goldfish hepatocytes), an effect similar to that of the vehicle DMSO observed in the controls. In contrast, both alone and in combination with adenosine, NBTI strongly inhibited [^{3}H]leucine incorporation into hepatocytes from both goldfish and trout.

The effect of chemical anoxia, produced by the inhibition of oxidative phosphorylation with cyanide, was also tested. In goldfish hepatocytes, cyanide alone caused an inhibition of protein synthesis amounting to approximately 50 %; in combination with adenosine, protein synthesis was inhibited by 60 %, a value similar in magnitude to that observed in the presence of adenosine alone. Addition of 8-PT prior to cyanide did not prevent the reduction in the rate of protein synthesis caused by chemical anoxia. In trout hepatocytes, the inhibitory effects of chemical anoxia on [^{3}H]leucine incorporation were significantly more pronounced both with and without adenosine (approximately 90 % inhibition) compared with the effect of adenosine alone. As in goldfish hepatocytes, 8-PT was without effect on this reduction in the rate of protein synthesis.

Effects of adenosine on K$^+$ fluxes

The maintenance of K$^+$ homeostasis in goldfish hepatocytes and the decoupling of unidirectional K$^+$ fluxes in trout
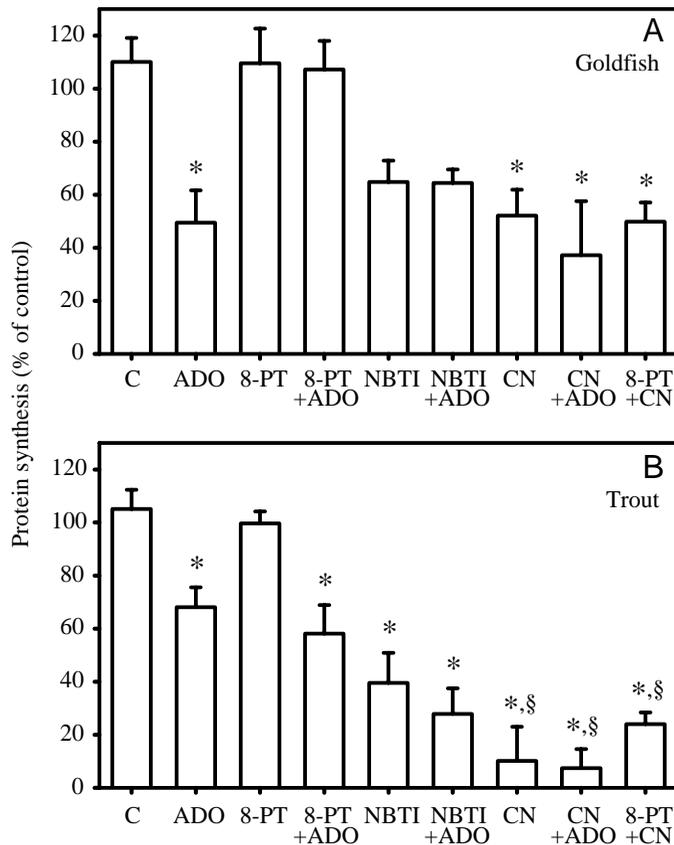
Fig. 5. Protein synthesis, as estimated from the rate of [3H]leucine incorporation, in hepatocytes from (A) goldfish and (B) trout. Measurements were performed as shown in Fig. 4, and the data are expressed as a percentage of the rates determined before the addition of the respective agents. Treatments were: dimethyl sulphoxide (DMSO) (C), $100\,\mu mol\,l^{-1}$ adenosine (ADO), $100\,\mu mol\,l^{-1}$ 8-phenyltheophylline (8-PT), 8-phenyltheophylline + adenosine (8-PT+ADO), $100\,\mu mol\,l^{-1}$ nitrobenzyl thioinosine (NBTI), nitrobenzyl thioinosine + adenosine (NBTI+ADO), $2\,mmol\,l^{-1}$ cyanide (CN), cyanide + adenosine (CN+ADO) and 8-phenyltheophylline + cyanide (8-PT+CN). Values are means + s.e.m. of 4–8 independent preparations. *$P<0.05$ compared with controls. §$P<0.05$ compared with adenosine alone.

hepatocytes have been found to be critical events under anoxic conditions (Krumschnabel et al., 1996). In consequence, in a further series of experiments, the effects of adenosine on Rb^+ uptake and K^+ efflux were tested. Rb^+ uptake served both as a measure of K^+ uptake and, since it is inhibited by more than 95 % in the presence of ouabain (Krumschnabel et al., 1996, 1998), of Na^+ pump activity.

As shown in Fig. 6 for normoxic cells, adenosine elicited significant reductions in unidirectional K^+ fluxes in hepatocytes from goldfish and trout, with two exceptions (K^+ efflux from goldfish cells at 30 min, and K^+ efflux from trout cells at 10 min) caused by large scattering of the data. The decrease in ion fluxes was noted both 10 min and 30 min after addition of adenosine, was similar in magnitude (20–35 %) in cells from both species and showed no increase with time of exposure.
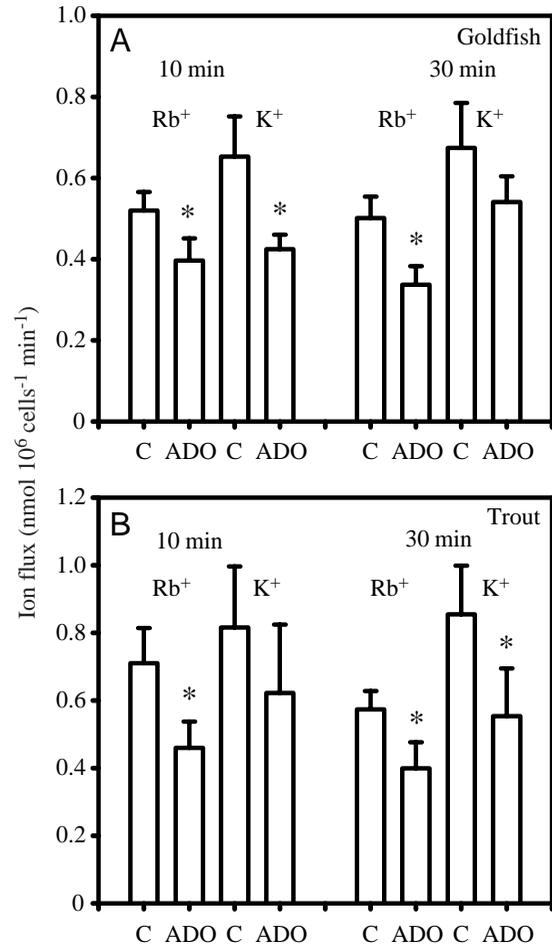

Fig. 6. Unidirectional $K^+(Rb^+)$ fluxes in hepatocytes from (A) goldfish and (B) trout. Cells were incubated with dimethyl sulphoxide (DMSO) (C) or with $100\,\mu mol\,l^{-1}$ adenosine dissolved in DMSO (ADO), and Rb^+ uptake (Rb^+) and K^+ efflux (K^+) were determined after 10 and 30 min. Values are means + s.e.m. of eight (goldfish) and six (trout) independent preparations. *$P<0.05$ compared with controls.

Effects of adenosine on $[Ca^{2+}]_i$

In an attempt to elucidate the transduction pathway for the action of adenosine, its effects on hepatocyte $[Ca^{2+}]_i$ were investigated. Examples of $[Ca^{2+}]_i$ measurements in Fura-2-loaded hepatocytes from goldfish and trout are depicted in Fig. 7. In the goldfish cells (Fig. 7A), adenosine caused a slight increase in $[Ca^{2+}]_i$ to a new elevated steady-state level that was maintained for at least another 10 min. Subsequent addition of thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, resulted in a further and much more pronounced elevation of $[Ca^{2+}]_i$. In trout hepatocytes (Fig. 7B), the increase in $[Ca^{2+}]_i$ evoked by adenosine occurred rapidly and, in contrast to goldfish hepatocytes, was of a transient nature. As in the goldfish cells, thapsigargin caused a further increase in $[Ca^{2+}]_i$ with no subsequent recovery within the time tested. A summary of these and related data is given in Table 1.
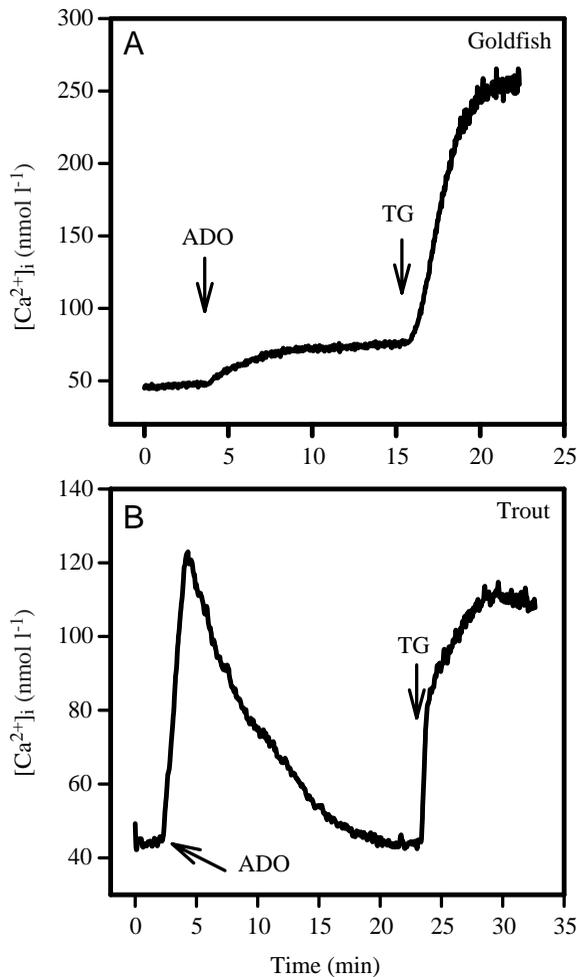
Fig. 7. Representative tracings of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in hepatocytes from (A) goldfish and (B) trout. Where indicated by arrows, $100\,\mu mol\,l^{-1}$ adenosine (ADO) and $100\,nmol\,l^{-1}$ thapsigargin (TG) were added.

Table 1. *Resting levels of $[Ca^{2+}]_i$ and increases in $[Ca^{2+}]_i$ in hepatocytes from goldfish and trout treated with adenosine, with adenosine in the presence of 8-phenyltheophylline, with 2-chloro-adenosine, with thapsigargin or with adenosine in the absence of extracellular Ca^{2+}*

| | $[Ca^{2+}]_i$ ($nmol\,l^{-1}$) | | | |
	Control	Treatment	Increase	N
Goldfish				
Adenosine	49±4	72±6	23±3*	13
8-PT+adenosine	53±7	69±8	16±4*	7
2-Cl-adenosine	66±15	74±15	8±3*·‡	10
Thapsigargin	64±7	151±17	87±14*·‡	9
$[Ca^{2+}]_e=0$	46±3	49±4	3±0	3
Trout				
Adenosine	102±11	165±15	63±13*	15
8-PT+adenosine	123±12	205±30	82±23*	6
2-Cl-adenosine	78±11	80±11	1±2	6
Thapsigargin	125±28	242±58	117±31*·‡	6
$[Ca^{2+}]_e=0$	59±6	61±6	2±3	6

Values are means ± S.E.M. of *N* independent preparations.

* denotes a significant increase in $[Ca^{2+}]_i$, $P<0.05$; ‡ denotes an increase in $[Ca^{2+}]_i$ significantly different from that for adenosine-treated cells.

8-PT, 8-phenyltheophylline; 2-Cl-adenosine, 2-chloro-adenosine.

In hepatocytes from both goldfish and trout, adenosine caused a significant increase in $[Ca^{2+}]_i$ that was neither prevented nor significantly reduced by the presence of 8-PT. The adenosine receptor agonist 2-chloro-adenosine evoked a smaller increase in $[Ca^{2+}]_i$ in goldfish hepatocytes, but no increase in trout hepatocytes. In cells incubated in the absence of extracellular Ca^{2+}, adenosine had no effect on $[Ca^{2+}]_i$. Thapsigargin caused $[Ca^{2+}]_i$ to increase in cells from both species, and this increase was significantly larger than that induced by adenosine.

Discussion

In the present study, adenosine was used at a concentration of $100\,\mu mol\,l^{-1}$ to assess the impact of this compound on cellular energetics. Although the *in vivo* concentration of adenosine in the anoxic liver is not known, we believe the level used to be justified for the following reasons. First, in agreement with numerous other studies (Buck and Bickler,

1995; Land et al., 1997; Tinton et al., 1995), we wanted to provide a qualitative picture of the effects that adenosine exerts on the metabolic variables studied, neglecting lower concentrations that might produce submaximal effects. Second, in brain tissue from both the anoxia-sensitive rat (Hagberg et al., 1987) and the anoxia-tolerant turtle (Nilsson et al., 1994), extracellular adenosine was found to reach levels of up to $40\,\mu mol\,l^{-1}$, which is similar in magnitude to the concentration applied in our study. Given that blood flow through the liver may become significantly reduced during anoxia, a large build-up of extracellular adenosine levels can be easily envisaged.

Adenosine and ATP production

The various effects of adenosine on cellular energetics described in this study are in line with its hypothetical role as a protective agent during anoxia and/or ischaemia, with some species-specific differences being evident. First, in hepatocytes from both goldfish and trout, adenosine was found to lower the rate of important ATP-requiring processes such as protein synthesis and active ion transport, reducing the total cellular ATP demand. Second, in trout hepatocytes, adenosine exerted an effect on both aerobic and anaerobic ATP production, whereas no such effects occurred in goldfish cells. A significant depression of the rate of oxygen consumption by adenosine has recently been described for the marine invertebrate *Sipunculus nudus* (Reipschlager et al., 1997). However, in this case, adenosine most probably acted by exerting its established inhibitory effects on neuronal

excitability and neurotransmitter release, since no decrease in the rate of oxygen consumption was observed in isolated body wall musculature treated with adenosine. In trout, however, adenosine affected the aerobic metabolism of parenchymal cells directly, without the intermediary of nervous tissue.

Assuming, as is generally accepted, that oxygen consumption reflects mitochondrial ATP generation, the question arises as to the benefit of the effects of adenosine in decreasing the rate of oxygen consumption. A possible answer may be derived from the fact that both Rb^+ uptake (Fig. 6) and $\dot{V}_{O_2cs}$ (Fig. 1) were more rapidly affected by adenosine than was $\dot{V}_{O_2tot}$, indicating that the decreased rate of aerobic ATP production was a consequence of a diminished cellular ATP requirement and, thus, merely an indirect effect of adenosine. It should be noted in this context that the estimation of the rate of protein synthesis from the inhibition of oxygen consumption with cycloheximide, as applied in this study, provides only a qualitative, and not a quantitative, measure (Wieser and Krumschnabel, 1999), a problem that is under current investigation.

In contrast to aerobic ATP production, anaerobic ATP production, measured by following lactate accumulation, was increased, rather than decreased in the presence of adenosine (Figs 2B, 3B). In relation to this finding, conflicting results have been reported in the literature. In perfused heart preparations, for example, adenosine was found to increase lactate accumulation in one study (Wyatt et al., 1989) but to decrease it in another (Fralix et al., 1993). In contrast, in both crucian carp (*Carassius carassius*) and trout, adenosine decreased the rate of anaerobic metabolism, a conclusion derived from the observation that the accumulation of anaerobic end product (ethanol for crucian carp, lactate for trout) was greater in the presence of adenosine-receptor antagonists than in their absence (Nilsson, 1991; Bernier et al., 1996). The decreased anaerobic rate, however, was deduced from measurements made on the whole fish or from blood samples, so that an increased rate in a single organ, such as the liver, cannot be excluded. In principle, both an increase and a decrease in the rate of anaerobic ATP production may aid in prolonging anoxic survival, the first by sustaining critical ATP-consuming functions under short-term anoxia, the second by preserving carbohydrate reserves for prolonged anoxia. Thus, in trout, which are not resistant to prolonged anoxia, an increased rate of lactate production may be beneficial, whereas in goldfish, in which the anoxia-tolerance is based on the ability of the fish to down-regulate its metabolic rate, the requirement to supply glucose from the liver to other organs may be the determinant of the glycolytic rate (Shoubridge and Hochachka, 1983; Rahman and Storey, 1988).

In trout hepatocytes, despite the increase in the rate of anaerobic ATP production, the cellular ATP content decreased to the same extent and followed the same dynamics in the absence of adenosine and in its presence (Fig. 3A), showing that ATP supply and ATP demand were not matched in these anoxia-sensitive cells. In comparison, hepatocyte ATP levels in the anoxia-tolerant goldfish were preserved both with and without adenosine (Fig. 2A). In the presence of adenosine, ATP levels were significantly elevated compared with untreated cells. This may have been due both to the increased rate of glycolytic ATP production and to the uptake and subsequent conversion of adenosine into ATP. Uptake of adenosine into goldfish hepatocytes (Schwarzbaum et al., 1998) and rat hepatocytes (Bontemps et al., 1983), as well as an increase in ATP levels due to adenosine uptake (Bontemps et al., 1983; Tinton and Buc-Calderon, 1995), have been described before. For the present data, however, such an interpretation is somewhat marred by the fact that ATP level was elevated from the very beginning in adenosine-treated cells.

Adenosine and ATP consumption

As outlined above, a reduction in cellular ATP consumption is of pivotal importance for anoxic survival. Given that protein synthesis is the most important ATP-consuming process in a number of cells, it appears to be a reasonable target for down-regulation to achieve significant energy savings. Indeed, in the hepatocytes from goldfish and trout, both the indirect ($\dot{V}_{O_2cs}$, Fig. 1) and the direct ([³H]leucine incorporation, Fig. 5) measurement of protein synthesis indicate that this process was inhibited in the presence of adenosine. Interestingly, in goldfish hepatocytes, the extent of this inhibition was roughly the same under normoxia and chemical anoxia, with no apparent additive effects of adenosine and cyanide. Furthermore, given that 8-PT prevented the inhibition of protein synthesis by adenosine under normoxia but had no effect on the inhibition caused by chemical anoxia, it appears that adenosine and cyanide act independently.

In trout cells, inhibition of protein synthesis was much more pronounced under chemical anoxia than under normoxia, irrespective of the presence of adenosine (Fig. 5). Thus, in trout cells more than in goldfish hepatocytes, the energetic deficiency created by anoxia is a more critical determinant of the rate of protein synthesis than is the inhibitory effect of adenosine.

Another significant contributor to cellular ATP turnover, namely Na^+/K^+-ATPase activity, was also found to be partially inhibited in the presence of adenosine (Fig. 6). In goldfish cells, both true (G. Weiß and G. Krumschnabel, unpublished observation) and chemical anoxia (Krumschnabel et al., 1994b) have been found to result in an approximately 50 % reduction in Na^+ pump activity, so that the 25 % reduction observed under normoxia in the present study would account for approximately half of this decrease. The hypothetical beneficial effect of adenosine that may be inferred from this observation is further emphasized by the fact that K^+ efflux is also down-regulated, resulting in the maintenance of ion homeostasis at diminished costs in terms of ATP consumption. In principle, the same could also be concluded for trout hepatocytes. However, since Na^+/K^+-ATPase activity is inhibited by more than 80 % during anoxia in these cells (Krumschnabel et al., 1996), the benefit of the adenosine effect appears to be marginal. The effect may,

however, be more important during hypoxia, when Na^+/K^+-ATPase activity is reduced to a lesser extent (Krumschnabel et al., 2000).

A question that cannot be clarified on the basis of the present data is whether adenosine is capable of directly interacting with Na^+/K^+-ATPase or whether the decrease in Na^+ pump activity is secondary to an effect on K^+ efflux mechanisms. While, to our knowledge, the former has not yet been described, there is evidence that adenosine is involved in K^+ channel arrest in the anoxic turtle brain (Pérez-Pinzón et al., 1993; Pék and Lutz, 1997).

Mechanism of action of adenosine

An important goal pursued in the present study was to identify potential mechanisms through which adenosine could exert its effects on cellular processes. In the case of protein synthesis, we present clear evidence that adenosine inhibits this process *via* interaction with an A1 receptor in goldfish hepatocytes and by a different mechanism in trout hepatocytes. The former finding is remarkable since, to our knowledge, only the A2 receptor has been described to date in liver tissue (Olsson and Pearson, 1990), with the possible exception of a study in which 8-PT was found to affect Ca^{2+} efflux from the hepatocytes of an anoxia-tolerant turtle (Land et al., 1997). However, this finding was not systematically pursued, so that a non-specific side-effect cannot be excluded.

The absence of an effect of 8-PT on the adenosine-induced inhibition of protein synthesis in trout hepatocytes suggests that in this case (i) adenosine acts on some other receptor or (ii) adenosine is taken up into the cells and acts intracellularly. Unfortunately NBTI, a well-known adenosine transport inhibitor, was itself a significant inhibitor of protein synthesis, thereby preventing a clear conclusion. The same observation has been described for rat hepatocytes (Tinton and Buc-Calderon, 1995) but, in this study, a number of other adenosine receptor antagonists were applied without effect, leaving the intracellular action of adenosine as the more likely option.

One effect in hepatocytes that has repeatedly been associated with the presence of adenosine is an increase in $[Ca^{2+}]_i$ (Tinton and Buc-Calderon, 1995; Diaz et al., 1991). This was also found in the present study, with two species-specific responses to adenosine being measured. In goldfish hepatocytes, adenosine induced an increase in $[Ca^{2+}]_i$ that was comparatively small but persistent (Fig. 7A), consisting of a presumably receptor-mediated component (an increase in $[Ca^{2+}]_i$ in the presence of 2-Cl-adenosine) and a receptor-independent component (an increase in $[Ca^{2+}]_i$ in the presence of 8-PT plus adenosine; Table 1). Since 8-PT abolished the inhibition of protein synthesis caused by adenosine (Fig. 5) but not the increase in $[Ca^{2+}]_i$, the increase in $[Ca^{2+}]_i$ does not appear to be involved in the inhibition of protein synthesis, corroborating findings previously reported for rat hepatocytes (Tinton and Buc-Calderon, 1995). In trout hepatocytes, in comparison, the increase in $[Ca^{2+}]_i$ evoked by adenosine was larger but transient (Fig. 7B) and was presumably not receptor-

mediated (no increase in the presence of 2-chloro-adenosine and no reduction in the increase in the presence of 8-PT plus adenosine; Table 1). In the cells from both species, the increase in $[Ca^{2+}]_i$ must have been due to an increased rate of Ca^{2+} entry (there was no increase in the absence of extracellular Ca^{2+}) and not to the release of Ca^{2+} from intracellular stores (because Ca^{2+} was still being released by thapsigargin after the adenosine-induced increase in $[Ca^{2+}]_i$ and because the increase induced by thapsigargin was significantly greater than that caused by adenosine). Again, these observations agree with those reported for rat hepatocytes (Tinton and Buc-Calderon, 1995; Tinton et al., 1996), but the physiological significance of the increase in $[Ca^{2+}]_i$ induced by adenosine in relation to the phenomenon of metabolic depression clearly requires further study.

In summary, the results of the present study provide further evidence for the hypothetical role of adenosine as a metabolite promoting prolonged anoxic survival and suggest that it evokes distinct response patterns in anoxia-sensitive and anoxia-tolerant cells. In goldfish hepatocytes, ATP-consuming functions were significantly depressed, the inhibitory effect on protein synthesis being mediated *via* interaction between adenosine and a membrane receptor, probably of the A1 subtype. The production of ATP appears to be unresponsive to adenosine in these cells. In contrast, in trout hepatocytes, inhibition of ATP consumers by adenosine was accompanied by a simultaneous increase in the rate of anaerobic ATP production, although this was not reflected in better maintenance of cellular ATP levels. The effects on trout cells appear to require uptake of adenosine into the cells. The role of $[Ca^{2+}]_i$ elevation induced by adenosine remains unresolved at this stage.

References

Bernier, N. J., Harris, J., Lessard, J. and Randall, D. J. (1996). Adenosine receptor blockage and hypoxia-tolerance in rainbow trout and Pacific hagfish. *J. Exp. Biol.* **199**, 485–495.

Bontemps, F., van-den Berghe, G. and Hers, H. G. (1983). Evidence for a substrate cycle between AMP and adenosine in isolated hepatocytes. *Proc. Natl. Acad. Sci. USA* **80**, 2829–2833.

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

Buck, L. T. and Bickler, P. E. (1998). Adenosine and anoxia reduce N-methyl-D-aspartate receptor open probability in turtle cerebrocortex. *J. Exp. Biol.* **201**, 289–297.

Diaz, A., Guinzberg, R., Uribe, S. and Pina, E. (1991). Metabolic responses of isolated hepatocytes to adenosine; dependence on external calcium. *Life Sci.* **49**, 505–510.

Fralix, T. A., Murphy, E., London, R. E. and Steenbergen, C. (1993). Protective effects of adenosine in the perfused rat heart: changes in metabolism and intracellular ion homeostasis. *Am. J. Physiol.* **264**, C986–C994.

Hagberg, H., Andersson, P., Lacarewicz, J., Jacobson, I., Butcher, S. and Sandberg, M. (1987). Extracellular adenosine, inosine, hypoxanthine and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. *J. Neurochem.* **49**, 227–231.

Krumschnabel, G., Biasi, C., Schwarzbaum, P. J. and Wieser, W. (1996). Membrane–metabolic coupling and ion homeostasis in anoxia-tolerant and anoxia-intolerant hepatocytes. *Am. J. Physiol.* **270**, R614–R620.

Krumschnabel, G., Frischmann, M. E., Schwarzbaum, P. J. and Wieser, W. (1998). Loss of K^+ homeostasis in trout hepatocytes during chemical anoxia: a screening study for potential causes and mechanisms. *Arch. Biochem. Biophys.* **353**, 199–206.

Krumschnabel, G., Malle, S., Schwarzbaum, P. J. and Wieser, W. (1994a). Glycolytic function in goldfish hepatocytes at different temperatures: relevance for Na^+ pump activity and protein synthesis. *J. Exp. Biol.* **192**, 285–290.

Krumschnabel, G., Schwarzbaum, P. J., Lisch, J., Biasi, C. and Wieser, W. (2000). Oxygen-dependent energetics of anoxia-tolerant and anoxia-intolerant hepatocytes. *J. Exp. Biol.* **203**, 951–959.

Krumschnabel, G., Schwarzbaum, P. J. and Wieser, W. (1994b). Coupling of energy supply and energy demand in isolated goldfish hepatocytes. *Physiol. Zool.* **67**, 438–448.

Kwast, K. E. and Hand, S. C. (1993). Regulatory features of protein synthesis in isolated mitochondria from *Artemia* embryos. *Am. J. Physiol.* **265**, R1238–R1246.

Land, S. C., Sanger, R. H. and Smith, P. J. (1997). O_2 availability modulates transmembrane Ca^{2+} flux *via* second-messenger pathways in anoxia-tolerant hepatocytes. *J. Appl. Physiol.* **82**, 776–783.

Nilsson, G. E. (1991). The adenosine receptor blocker aminophylline increases anoxic ethanol excretion in crucian carp. *Am. J. Physiol.* **261**, R1057–R1060.

Nilsson, G. E., Hylland, P. and Lofman, C. O. (1994). Anoxia and adenosine induce increased cerebral blood flow rate in crucian carp. *Am. J. Physiol.* **267**, R590–R595.

Olsson, R. A. and Pearson, J. D. (1990). Cardiovascular purinoceptors. *Physiol. Rev.* **70**, 761–845.

Pannevis, M. C. and Houlihan, D. F. (1992). The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Physiol.* B **162**, 393–400.

Pék, M. and Lutz, P. L. (1997). Role for adenosine in channel arrest in the anoxic turtle brain. *J. Exp. Biol.* **200**, 1913–1917.

Pérez-Pinzón, M. A., Lutz, P. L., Sick, T. J. and Rosenthal, M. (1993). Adenosine, a 'retaliatory' metabolite, promotes anoxia tolerance in turtle brain. *J. Cereb. Blood Flow Metab.* **13**, 728–732.

Rahman, M. S. and Storey, K. B. (1988). Role of covalent modification in the control of glycolytic enzymes in response to environmental anoxia in goldfish. *J. Comp. Physiol.* B **157**, 813–820.

Reipschlager, A., Nilsson, G. E. and Pörtner, H. O. (1997). A role for adenosine in metabolic depression in the marine invertebrate *Sipunculus nudus*. *Am. J. Physiol.* **272**, R350–R356.

Schwarzbaum, P. J., Frischmann, M. E., Krumschnabel, G., Rossi, R. C. and Wieser, W. (1998). Functional role of ecto-ATPase activity in goldfish hepatocytes. *Am. J. Physiol.* **274**, R1031–R1038.

Shoubridge, E. A. and Hochachka, P. W. (1983). The integration and control of metabolism in the anoxic goldfish. *Mol. Physiol.* **4**, 165–195.

Tinton, S. and Buc-Calderon, P. M. (1995). Inhibition of protein synthesis induced by adenine nucleotides requires their metabolism into adenosine. *Biochem. Pharmac.* **50**, 481–488.

Tinton, S. A., Chow, S. C., Buc-Calderon, P. M. and Kass, G. E. (1996). Adenosine stimulates calcium influx in isolated rat hepatocytes. *Eur. J. Biochem.* **238**, 576–581.

Tinton, S. A., Chow, S. C., Buc-Calderon, P. M., Kass, G. E. and Orrenius, S. (1995). Adenosine inhibits protein synthesis in isolated rat hepatocytes. Evidence for a lack of involvement of intracellular calcium in the mechanism of inhibition. *Eur. J. Biochem.* **229**, 419–425.

Wieser, W. and Krumschnabel, G. (1999). Interrelationship and hierarchy of ATP consuming processes in teleost hepatocytes. *Zoology* **102** (Suppl. II), 75.

Wyatt, D. A., Edmunds, M. C., Rubio, R., Berne, R. M., Lasley, R. D. and Mentzer, R. M. J. (1989). Adenosine stimulates glycolytic flux in isolated perfused rat hearts by A1-adenosine receptors. *Am. J. Physiol.* **257**, H1952–H1957.