Cell-type specificity of inhibition of glycolysis by 5-amino-4-imidazolecarboxamide riboside

Lack of effect in rabbit cardiomyocytes and human erythrocytes, and inhibition in FTO-2B rat hepatoma cells

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The nucleoside AICARiboside (5-amino-4-imidazolecarboxamide riboside) has been shown to inhibit glycolysis in isolated rat hepatocytes [Vincent, Bontemps and Van den Berghe (1992) *Biochem. J.* **281**, 267–272]. The effect is mediated by AICARibotide (ZMP), the product of the phosphorylation of AICARiboside by adenosine kinase. To assess the cell-type specificity of the effect, studies were conducted in rabbit cardiomyocytes, human erythrocytes and rat hepatoma FTO-2B cells. AICARiboside had no effect on glycolysis in cardiomyocytes, and a slight stimulatory effect in erythrocytes, but inhibited glycolysis by 65 % at 250 μ M concentration in FTO-2B cells, although only when tissue-culture medium was replaced by Krebs–Ringer bicarbonate buffer. At 500 μ M AICARiboside, ZMP remained undetectable in cardiomyocytes, but reached 0.65 mM in erythro-

cytes and 5 mM in FTO-2B cells. In the latter, AICARiboside provoked up to 2-fold elevations of glucose 6-phosphate and fructose 6-phosphate, accompanied by a decrease in fructose 1,6-bisphosphate. This indicated inhibition of 6-phosphofructo-1-kinase (PFK-1). Accordingly, in FTO-2B cell-free extracts, the activity of PFK-1, measured under physiological conditions, was inhibited by approx. 70 % by 5 mM ZMP. ZMP had a less pronounced effect on the activity of PFK-1 in normal rat liver; it did not influence the activity of PFK-1 in rat muscle, rabbit heart and human erythrocytes. It is concluded that the inhibitory effect of AICARiboside on glycolysis is dependent on both (1) the capacity of the cells to accumulate ZMP and (2) the presence of target enzymes which are sensitive to ZMP.

INTRODUCTION

AICARiboside (5-amino-4-imidazolecarboxamide ribofuranoside) is the nucleoside corresponding to AICARiboside 5'-monophosphate (AICAR or ZMP), an intermediate of the 'de novo' pathway of purine biosynthesis. AICARiboside is taken up and metabolized by various cell types, including erythrocytes and lymphoma cells [1], fibroblasts [2], isolated kidney tubules [3] and isolated hepatocytes [4]. The first step of the metabolism of AICARiboside is its phosphorylation to ZMP by adenosine kinase [1]. Thereafter, ZMP can be metabolized in two directions: (i) conversion into IMP by the sequential action of AICAR formyltransferase, which requires 10-formyl-tetrahydrofolate, and IMP cyclohydrolase; (ii) phosphorylation to the corresponding di- and tri-phosphates, ZDP and ZTP.

We have reported previously that addition of AICARiboside to suspensions of isolated rat hepatocytes causes a ZMP-mediated inhibition of glycolysis [5]. The inhibition was shown to result from a diminution of the phosphorylation of glucose to glucose 6-phosphate, accompanied by an inactivation of 6-phosphofructo-2-kinase (PFK-2). This inactivation provokes a decrease in fructose 2,6-bisphosphate (Fru-2,6- P_2), the main physiological stimulator of liver 6-phosphofructo-1-kinase (PFK-1).

Hepatic phosphorylation of glucose is mainly accomplished by the high- K_m isoenzyme of hexokinase, glucokinase. Liver also contains a PFK-2 isoenzyme specific for this tissue [6]. We therefore decided to investigate the effect of AICARiboside on glycolysis in other cell types, which possess different isoforms of

these two enzymes. Isolated rabbit cardiomyocytes, human erythrocytes and FTO-2B cells, a rat hepatoma cell line, were chosen for this purpose.

MATERIALS AND METHODS

Materials

Collagenase was from Wako (Osaka, Japan), BSA from Serva (Heidelberg, Germany), and fatty-acid-free BSA from Sigma (St. Louis, MO, U.S.A.). Cell-culture components were purchased from Gibco (Paisley, Scotland, U.K.). AICARiboside and 6-phosphofructokinase (pyrophosphate-dependent) were from Sigma. [3 H]Glucose (17.5 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.) and was re-purified by paper chromatography before use, to free it from a metabolizable contaminant [7]. 5-Iodotubercidin was from RBI (Natick, MA, U.S.A.). Auxiliary enzymes used in the spectrophotometric assays were from Boehringer (Mannheim, Germany). Sephadex G-25 (fine grade) was from Pharmacia (Uppsala, Sweden).

Isolation of cardiomyocytes

Cardiac ventricular myocytes were isolated from male New Zealand White rabbits weighing 1.7–2.3 kg, by a modification of the procedure of Altschuld et al. [8]. Hearts were perfused by the Langendorff method and myocytes isolated by addition of collagenase. After isolation, the preparations were enriched in

Abbreviations used: AICARiboside, 5-amino-4-imidazolecarboxamide riboside; ZMP (AICARibotide), AICARiboside 5'-monophosphate; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Fru-2,6- P_2 , fructose 2,6-bisphosphate; KRB, Krebs–Ringer bicarbonate buffer; FBPase-1, fructose-1,6-bisphosphatase; FBPase-2, fructose-2,6-bisphosphatase.

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viable myocytes by three sequential sedimentations in 4% BSA. Before incubations, the myocytes were counted in a Nageotte cell chamber and their viability was assessed by Trypan Blue exclusion. Myocytes were adjusted to approx. 400 000 cells/ml. The protein content of cell suspensions was determined as described by Lowry et al. [9]. Myocytes were incubated in Hepes-buffered Krebs–Henseleit solution (pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1 mM CaCl_2 and 25 mM Hepes, supplemented with 11 mM glucose and 1% BSA (fatty-acid-free), and gassed with 100% O_2 . Incubations were performed at 37 °C in a gently shaking water bath. Cells were preincubated for 20 min before starting the experiments.

Incubation of human erythrocytes

Fresh blood taken from a cubital vein of healthy human volunteers was collected on heparin. Isolation and washing of erythrocytes were performed in Krebs–Ringer bicarbonate buffer (KRB), pH 7.4, containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 and 25 mM NaHCO_3 , supplemented with 5 mM glucose and gassed with O_2/CO_2 (19:1) as described in [10]. The cells were resuspended in the same medium at a 20% haematocrit. Cells were incubated in a shaking water bath. To avoid modifications of pH of the incubation medium by loss of CO_2 , vials were carefully regassed after removal of each sample.

Cell cultures

Monolayer cultures of rat hepatoma FTO-2B cells were grown in Dulbecco's Modified Eagle's Medium/Ham's F12 (v/v) medium supplemented with 10% fetal-calf serum, 1% penicillin (5000 i.u./ml)/streptomycin (5000 µg/ml) and 2 mM glutamine, at 37 °C, in an atmosphere of air containing 5% CO_2 .

Incubation of FTO-2B cells

The day before the experiment, plastic Petri dishes (60 mm diameter) were seeded with 1×10^6 cells. The day of the experiment, the medium was removed and the cells were washed with 2 ml of KRB. Experiments were started by the addition of 5 or 10 mM glucose, immediately followed by AICariboside. When used, 10 µM 5-iodotubercidin was added immediately before AICariboside. For measurement of the release of $^3\text{H}_2\text{O}$ from [^3H]glucose, 0.02 µCi of labelled glucose/ml of KRB was added simultaneously with unlabelled glucose. When Fru-2,6- P_2 was measured, the cells were preincubated with glucose for 1 h before the addition of AICariboside.

Measurements of metabolites

Lactate and $^3\text{H}_2\text{O}$ were measured in samples of the incubation medium deproteinized in 2% HClO_4 and neutralized with 3 M K_2CO_3 . For nucleotide measurements, the incubation medium was removed and the cells were treated with 1 ml of 2% HClO_4 , scraped with a rubber spatula, and the extracts neutralized with 3 M K_2CO_3 . For measurement of glycolytic intermediates, the cells were treated with 0.5 ml of 2% trichloroacetic acid, scraped, and centrifuged. The supernatants were neutralized by extractions with 3 × 5 vol. of ether. For the determination of Fru-2,6- P_2 , the cells were extracted with 1 ml of 50 mM NaOH containing 1% (v/v) Triton X-100, scraped, heated for 15 min at 80 °C and frozen in acetone/solid CO_2 .

Lactate [11], triose phosphates and Fru-1,6- P_2 [12] and hexose 6-phosphates [13] were determined spectrophotometrically, and nucleotides and nucleosides were quantified by h.p.l.c. as de-

scribed in [4]. $^3\text{H}_2\text{O}$ was separated from [^3H]glucose on Dowex AG-1 (borate form) columns [7]. Fru-2,6- P_2 was measured by its stimulatory effect on potato 6-phosphofructokinase (pyrophosphate-dependent), as described by Van Schaftingen [14], except that commercial enzyme was used.

Enzyme assays

For measurement of enzyme activities in FTO-2B cells, approx. 400×10^6 cells were trypsin-treated, washed twice in PBS (150 mM NaCl/2.5 mM KCl/8 mM Na_2HPO_4 /1.5 mM KH_2PO_4 , pH 7.4) and resuspended in 6 ml of buffer A (50 mM Hepes/100 mM KF/15 mM EGTA, pH 7.1). The suspension was sonicated and centrifuged for 15 min at 60 000 g. The supernatant was filtered on a Sephadex G-25 (fine grade) column (20 cm × 1 cm), using buffer A for equilibration and elution. For the measurement of PFK-1 activity, 1 µM ATP and 1 µM Fru-2,6- P_2 were added in buffer A in order to stabilize the enzyme (buffer B). Hexokinase, PFK-1 and PFK-2 activities were measured as described in [7], [15] and [16] respectively. Fructose-1,6-bisphosphatase (FBPase-1) was assayed as given in [17]. Protein concentration was measured with the Bio-Rad assay [18].

For the assay of PFK-1 in rat liver and muscle, and in rabbit heart, tissues were homogenized in 4 vol. of buffer B. The homogenate was centrifuged for 15 min at 11 000 g, and the supernatant filtered on glass-wool, followed by centrifugation for 30 min at 60 000 g. The supernatant was filtered on Sephadex G-25 (fine), using buffer A for equilibration and buffer B for elution. PFK-1 activity was measured as given in [15].

For the assay of PFK-1 in human erythrocytes, fresh blood was collected on heparin. After three washes in 0.9% NaCl, the cell pellet was frozen in acetone/solid CO_2 , followed by thawing at room temperature, and addition of 4 vol. of 10 mM Hepes (pH 7.0)/10 mM KCl/1 µM Fru-2,6- P_2 /1 µM ATP. The activity of PFK-1 was measured as in [15], but with 50 mM Tris buffer (pH 7.4)/200 µM Mg-ATP/1 mM magnesium acetate/50 µM fructose 6-phosphate/150 µM glucose 6-phosphate/10 µM AMP.

Statistics

Unless stated otherwise, experiments shown are representative of at least three studies that gave similar results. Results of averaged experiments are given as means ± S.E.M. Significance was tested by Student's *t* test.

RESULTS

Effects of AICariboside in rabbit cardiomyocytes

In isolated rabbit cardiomyocytes incubated in Hepes-buffered Krebs–Henseleit solution, the production of lactate from 11 mM glucose was 90 ± 10 µmol/h per g of cells ($n = 3$). Addition of 100–500 µM AICariboside to the cell suspensions did not modify this rate. The rate of disappearance of 250 µM AICariboside was 2.4 ± 0.3 µmol/h per g of cells ($n = 3$). Both in the absence and in the presence of 100–500 µM AICariboside, Z-nucleotides remained undetectable over the whole duration (60 min) of the incubations.

Effects of AICariboside in human erythrocytes

In human erythrocytes incubated in KRB with 5 mM glucose, the production of lactate was 1.56 ± 0.11 µmol/h per ml of packed cells ($n = 3$). Addition of 500 µM AICariboside slightly stimulated this production, to 2.09 ± 0.15 µmol/h per ml of packed cells ($P = 0.046$). In the absence of AICariboside Z-

Figure 1 Time course of the effect of AICArriboside on the production of lactate in FTO-2B cells

Cells were incubated in KRB medium. Glucose (5 mM) was added simultaneously with various concentrations of AICArriboside. The insert shows the effect of AICArriboside on the rate of production of lactate. Production of lactate was calculated over the 2–8 h time interval after addition of the nucleoside.

Figure 2 Dose-dependent accumulation of Z-nucleotides in FTO-2B cells

Cells were preincubated in KRB medium with 10 mM glucose for 1 h before addition of various concentrations of AICArriboside. ZMP (a), ZDP and ZTP (b) were measured after 6 h of incubation.

nucleotides were undetectable. AICArriboside, added at 500 μM concentration, disappeared at the rate of approx. 0.6 μmol/h per ml of packed cells; ZMP accumulated to 0.6, ZDP to 0.06, and ZTP to 0.25 μmol/g of packed cells after 3 h.

Effects of AICArriboside in FTO-2B cells

Incubated in KRB medium with 5 mM glucose, FTO-2B cells produced lactate at a rate of 27.2 ± 2.4 μmol/h per g of cells ($n = 3$). Similar rates were recorded when the cells were incubated with 1, 2, 10 or 15 mM glucose, indicating that glucose was phosphorylated by low- K_m hexokinase, rather than by high- K_m glucokinase. When AICArriboside was added, the production of lactate from glucose was inhibited after a latency of approx. 2 h

Figure 3 Influence of 5-iodotubercidin (ITu) on the production of lactate

FTO-2B cells were incubated in KRB medium with 10 mM glucose, without (○) or with additions of AICArriboside (250 μM) and/or ITu (10 μM), as indicated.

(Figure 1). The dose-dependency of the effect (Figure 1, insert) shows that half-maximal inhibition was obtained with a AICArriboside concentration between 50 and 100 μM, and maximal (65%) inhibition with a concentration of 250 μM. In FTO-2B cells incubated in tissue-culture medium, AICArriboside had no effect on the production of lactate.

In FTO-2B cells incubated in tissue-culture medium or in KRB supplemented with 10 mM glucose, Z-nucleotides were undetectable in the absence of AICArriboside. In tissue-culture medium, incubation of the cells with 250 μM AICArriboside provoked accumulation of ZMP, up to 1.5 μmol/g of cells after 4 h; ZDP remained undetectable and ZTP accumulated to 0.09 μmol/g of cells. In cells incubated in KRB, AICArriboside induced much higher increases in Z-nucleotides. This is most likely due to the absence in KRB of folate compounds, which allow conversion of ZMP into IMP in tissue medium. Figure 2 depicts the effect of the addition of 25–500 μM AICArriboside on the level of Z-nucleotides after 6 h of incubation. At 500 μM AICArriboside, ZMP (Figure 2a), ZDP and ZTP (Figure 2b) reached approx. 5.5, 0.035 and 0.6 μmol/g of cells respectively. The accumulation of Z-nucleotides reached a plateau after 2 h of incubation and persisted for as long as 24 h without much decline, provided that AICArriboside was still present (results not shown). The rate of disappearance of 50 μM AICArriboside was 1.04 ± 0.05 μmol/h per g of cells ($n = 3$). Since inhibition of glycolysis by AICArriboside was only observed in FTO-2B cells incubated in KRB, subsequent experiments were performed in this cell type and incubation medium.

Effect of inhibition of adenosine kinase in FTO-2B cells

To study if AICArriboside by itself or, as in isolated hepatocytes [5], its metabolite ZMP, was responsible for the inhibition of the glycolytic pathway, FTO-2B cells were incubated with 250 μM AICArriboside, without or with 10 μM 5-iodotubercidin, a powerful inhibitor of adenosine kinase [19]. As expected, incubation with 5-iodotubercidin completely prevented the formation of ZMP from AICArriboside (results not shown). 5-Iodotubercidin by itself had a slight inhibitory effect on the production of lactate (Figure 3). When the cells were incubated with 250 μM AICArriboside and 10 μM 5-iodotubercidin, the inhibition of glycolysis from glucose was suppressed. This result indicates that the inhibitory effect of AICArriboside on glycolysis requires its phosphorylation.

Figure 4 Effect of AICArriboside on the concentration of Fru-2,6-P₂

FTO-2B cells were preincubated for 1 h in KRB medium with 10 mM glucose, before the addition of various concentrations of AICArriboside. Fru-2,6-P₂ was measured 6 h after addition of the nucleoside (*** $P \leq 0.001$). The concentration of Fru-2,6-P₂ in the absence of AICArriboside was 2.1 ± 0.4 nmol/g of cells.

Influence of AICArriboside on the concentration of Fru-2,6-P₂

In isolated rat hepatocytes [5], AICArriboside provokes a rapid decrease of Fru-2,6-P₂, the main physiological stimulator of hepatic glycolysis (reviewed in 20,21). The effect of AICArriboside on the concentration of Fru-2,6-P₂ was therefore investigated in FTO-2B cells. The usual preparation procedure of the cells, i. e. removal of tissue-culture medium and washing in KRB, resulted in a 75% decrease of the concentration of Fru-2,6-P₂, independently of the presence of glucose (results not shown). To restore Fru-2,6-P₂ to physiological levels, cells were preincubated for 1 h in KRB containing 10 mM glucose. It was verified that this preincubation did not modify the inhibitory effect of AICArriboside on the production of lactate (results not shown). As shown in Figure 4, addition of 25–50 μM AICArriboside had no significant effect on Fru-2,6-P₂. Incubation with higher concentrations of AICArriboside diminished Fru-2,6-P₂ in a dose-dependent manner, up to a 40% decrease with 250–500 μM of the nucleoside. Noteworthy, this effect was only observed after a latency period of 4–6 h (results not shown).

In isolated rat hepatocytes, addition of AICArriboside provokes conversion into its inactive form of PFK-2 [5], the enzyme that synthesizes Fru-2,6-P₂ from fructose 6-phosphate. In FTO-2B cells, AICArriboside did not influence the activation state of PFK-2. Also, in FTO-2B cell extracts, the activity of PFK-2 was not influenced by ZMP (results not shown). Taken together, these results indicate that in FTO-2B cells, in contrast with isolated hepatocytes, decreased formation of Fru-2,6-P₂ plays little or no role in the inhibition of glycolysis.

Influence of AICArriboside on lactate production from dihydroxyacetone

As a further attempt to determine the site of action of AICArriboside along the glycolytic pathway in FTO-2B cells, the production of lactate from dihydroxyacetone was measured. This compound is phosphorylated by triokinase to dihydroxyacetone phosphate, which is converted into the other triose phosphate, glyceraldehyde 3-phosphate, by triose phosphate isomerase. FTO-2B cells incubated with 10 mM dihydroxyacetone produced lactate at the rate of approx. 33 μmol/h per g of cells; there was no production of glucose. The production of lactate was not modified by addition of 250 μM AICArriboside (results not shown). This indicates that AICArriboside does not exert its inhibitory effect on the enzymes that convert triose

Figure 5 Time course of the effect of AICArriboside on the release of ³H₂O from [2-³H]glucose

FTO-2B cells were incubated in KRB medium with glucose (5 mM) and [2-³H]glucose (0.02 μCi/ml of medium) added simultaneously with AICArriboside. The insert shows the effect of various concentrations of AICArriboside on the rate of release of ³H₂O calculated over the first 8 h of incubation with the nucleoside.

phosphates into lactate, but on one or more of the first steps of glycolysis.

Effect of AICArriboside on ³H₂O release from [2-³H]glucose

The release of ³H₂O from [2-³H]glucose allows assessment of glucose transport and the first two steps of glucose metabolism, namely phosphorylation to glucose 6-phosphate and subsequent isomerization to fructose 6-phosphate [22]. As shown in Figure 5, after a latency of approx. 2 h, AICArriboside dose-dependently inhibited the release of ³H₂O from [2-³H]glucose in FTO-2B cells. Half-maximal inhibition was reached with 75 μM, and near-maximal (65%) inhibition with 250 μM AICArriboside (Figure 5, insert).

Influence of AICArriboside on glycolytic intermediates

In order to define more precisely the site of action of AICArriboside on the first steps of glycolysis, glycolytic intermediates were measured in FTO-2B cells incubated with 10 mM glucose and 500 μM AICArriboside. Cross-over plots revealed that after 2 h of incubation (Figure 6a) glucose 6-phosphate and fructose 6-phosphate increased by 246% and 174% respectively, whereas Fru-1,6-P₂ decreased by 30%. After 6 h of incubation (Figure 6b), the decreases in Fru-1,6-P₂ (–70%) and triose phosphates (–30%) were even more pronounced. Taken together, these results indicate inhibition of glycolysis at the level of PFK-1.

Effect of ZMP on the activity of PFK-1

The activity of PFK-1, measured in cell-free extracts of FTO-2B cells under physiological conditions, namely at pH 7.4 in the presence of 50 μM fructose 6-phosphate, 2.5 mM ATP, 100 μM AMP and 2.5 μM Fru-2,6-P₂, was 32.5 ± 1.2 μmol/min per g of protein ($n = 5$). Figure 7 shows that ZMP inhibited the activity of PFK-1 in a dose-dependent manner, reaching 70% with 5 mM ZMP.

The finding that ZMP had an inhibitory effect on PFK-1 in FTO-2B cells prompted a study of its influence on the activity of PFK-1 in other cell types, namely normal rat liver and muscle, rabbit heart, and human erythrocytes. In cell-free extracts of rat liver, ZMP inhibited the activity of PFK-1 to a lesser extent than in FTO-2B cells: 5 mM ZMP provoked only 30% inhibition.

Figure 6 Effect of AICariboside on the concentrations of glycolytic intermediates

FTO-2B cells were incubated in KRB medium with 10 mM glucose. Metabolite concentrations in cells incubated for (a) 2 h or (b) 6 h with 500 μ M AICariboside are plotted as percentages of concentrations measured in the absence of the nucleoside. Control values (expressed as nmol/ml of medium for lactate, and as nmol/g of cells for other metabolites) were: at 2 h, glucose 6-phosphate (G6P) 34, fructose 6-phosphate (F6P) 15, Fru-1,6- P_2 (F1,6P2) 65, triose phosphates (TP) 108, and lactate (LAC) 239; at 6 h, G6P 30, F6P 11, F1,6P2 172, TP 242, and LAC 485. Values are means $\pm$ S.E.M. ($n = 3$) (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Figure 7 Effect of ZMP on the activity of PFK-1

The enzyme activity was measured at pH 7.4 in a Sephadex-G-25-filtered high-speed supernatant of FTO-2B cells in the presence of 50 μ M fructose 6-phosphate, 2.5 mM ATP, 100 μ M AMP, 2.5 μ M Fru-2,6- P_2 and various concentrations of ZMP (* $P \leq 0.05$).

The activities of PFK-1 in extracts of rat muscle, rabbit heart and human erythrocytes were not influenced by up to 5 mM ZMP.

Influence of ZMP on other glycolytic and gluconeogenic enzymes

To explain the inhibitory effect of AICariboside on the release of $^3\text{H}_2\text{O}$ from $[2\text{-}^3\text{H}]\text{glucose}$, the influence of ZMP on the phosphorylation of glucose was measured in FTO-2B cell extracts. That the K_m for glucose was 40–50 μ M, and phosphorylation was feedback-inhibited by glucose 6-phosphate, confirmed that it was accomplished not by glucokinase but by hexokinase. Its activity, measured in the presence of a physiological concentration of glucose 6-phosphate, was approx. 1 μ mol/min per g of protein. No effect of AICariboside, ZMP or ZTP on

hexokinase activity could be detected under various conditions (results not shown).

The activity of phosphoglucose isomerase, measured with 20 μ M glucose 6-phosphate as substrate, was 82.4 ± 1.7 μ mol/min per g of protein ($n = 3$) in FTO-2B cell extracts. It was inhibited by 70% by 5 mM ZMP (results not shown).

As evidenced by the absence of production of glucose from dihydroxyacetone, FTO-2B cells lack glucose-6-phosphatase and/or FBPase-1. The activity of FBPase-1, measured at 50 μ M Fru-1,6- P_2 , was 0.6 μ mol/min per g of protein in FTO-2B cell extracts, which is approx. 100-fold lower than in normal hepatocytes. This confirms the known predominance of the glycolytic over the gluconeogenic pathway in hepatoma cells. As in isolated rat hepatocytes [4], 5 mM ZMP inhibited FBPase-1 by 95% (results not shown).

DISCUSSION

This study shows that the inhibitory effect of AICariboside on glycolysis, initially observed in normal rat hepatocytes [5], is not seen in isolated rabbit cardiomyocytes and human erythrocytes, but can be evidenced in FTO-2B cells, a rat hepatoma cell line, although only under certain conditions. From the data presented it appears that the inhibitory effect of AICariboside depends on: (1) the capacity of the cells to accumulate ZMP; (2) the presence of target enzymes that are sensitive to inhibition and/or inactivation by ZMP. These points will be discussed separately.

Accumulation of ZMP

We have shown previously that in normal rat hepatocytes AICariboside induces a dose- and time-dependent build-up of ZMP, reaching approx. 3 μ mol/g of cells after 20 min in the presence of 500 μ M nucleoside [4]. In the present study, incubation with 500 μ M AICariboside resulted in undetectable ZMP in isolated rabbit cardiomyocytes after 60 min, and in 0.6 μ mol of ZMP/g of cells after 3 h in human erythrocytes. In FTO-2B cells, 250 μ M AICariboside induced accumulation of ZMP to 1.5 μ mol/g of cells after 4 h of incubation in tissue-culture medium, and to 5 μ mol/g of cells after 1 h in KRB (Figure 2a). The variability of the capacity of the different cell types to accumulate ZMP is most likely explained by differences in the activity of adenosine kinase, the enzyme that phosphorylates AICariboside to ZMP [1]. Indeed, its activity is several-fold lower in heart and erythrocytes than in liver [23]. Accordingly, the rate of utilization of 500 μ M AICariboside which we measured in rabbit cardiomyocytes and human erythrocytes was approx. 20- and 60-fold lower than in isolated hepatocytes [4]. The absence of detectable accumulation of ZMP in rabbit cardiomyocytes accords with the observation that, in dog hearts *in vivo*, ZMP concentration reached only approx. 0.07 μ mol/g of cells after a 24 h perfusion with AICariboside [24].

The rate of conversion of ZMP into IMP also plays a role in the accumulation of ZMP: that in FTO-2B cells ZMP build-up was several-fold higher in KRB than in tissue-culture medium can be explained by the presence of folate compounds in the latter, which allow ZMP to be formylated and subsequently converted into IMP and other purine compounds. The lower build-up of ZMP in the presence of folates in FTO-2B cells raised the possibility that the accumulation of ZMP in isolated rat hepatocytes, recorded upon incubation in KRB [4,5], might also be decreased in the presence of folates. However, the observation that, on administration of AICariboside to rats *in vivo*, hepatic ZMP accumulates rapidly to concentrations which are comparable with those observed in isolated hepatocytes (M. F. Vincent and G. van den Berghe, unpublished work) indicates

that even in the presence of physiological levels of folates there is little conversion of ZMP into IMP in liver. This may be due to the 2–4-fold lower activity of AICAR transformylase in liver compared with other tissues [4]. In cancerous liver cells, with higher rates of synthesis *de novo*, the accumulation of ZMP could be enhanced by inhibition of AICAR transformylase by antifolates such as methotrexate.

The inhibitory effect of AICARiboside on glycolysis is dependent on the capacity of the cells to accumulate ZMP, as demonstrated by suppression of the effect when adenosine kinase is inhibited by 5-iodotubercidin (Figure 3). The absence of effect of AICARiboside on glycolysis in rabbit cardiomyocytes can thus be explained by their inability to accumulate measurable amounts of ZMP. However, even in cells that are able to accumulate ZMP, there is no clear correlation between ZMP concentration and inhibition of glycolysis: in isolated rat hepatocytes, half-maximal inhibition occurred at a ZMP concentration of 0.25 $\mu\text{mol/g}$ of cells [4,5], whereas in FTO-2B cells incubated in KRB it required 2 $\mu\text{mol/g}$ of cells (compare Figures 1 and 2a); in erythrocytes and in FTO-2B cells incubated in tissue-culture medium, which accumulated ZMP to 0.6 and 1.5 $\mu\text{mol/g}$ of cells respectively, glycolysis was not inhibited. This indicates that the target enzymes for ZMP, and/or their sensitivity, vary from one cell type to another.

Target enzymes for ZMP

In normal rat hepatocytes, AICARiboside inhibits glycolysis [5] by both decreasing the phosphorylation of glucose, which is predominantly effected by glucokinase in this cell type, and inactivating PFK-2, the enzyme that catalyses the synthesis of Fru-2,6- P_2 , the principal stimulator of hepatic glycolysis. That in FTO-2B cells the rate of production of lactate remained constant when the concentration of glucose was decreased from 15 to 1 mM, and that in FTO-2B cell extracts glucose was phosphorylated with a K_m of 40–50 μM , shows that these cells contain only hexokinase. Similarly to other hepatoma cells, FTO-2B cells, although displaying a highly differentiated hepatic phenotype [25], thus lack glucokinase. That AICARiboside did not decrease glucose 6-phosphate and fructose 6-phosphate in FTO-2B cells, as in normal hepatocytes [5], indicates that it does not act primarily on hexokinase. The observation that ZMP accumulation did not inhibit glycolysis in erythrocytes, which lack glucokinase, also accords with the proposal that the latter is a target for ZMP, whereas hexokinase is not.

The decrease in the release of $^3\text{H}_2\text{O}$ from [2- ^3H]glucose after addition of AICARiboside to FTO-2B cells could, in theory, be explained by inhibition of hexokinase and/or phosphoglucose isomerase. Implication of the 70% inhibition of phosphoglucose isomerase by 5 mM ZMP in the inhibition of detritiation and glycolysis can be ruled out, because residual isomerase activity remained 25-fold higher than that of hexokinase. The inhibition of the release of $^3\text{H}_2\text{O}$ is thus probably a consequence of feedback inhibition of hexokinase by the increased concentration of glucose 6-phosphate resulting from the inhibition of PFK-1.

FTO-2B cells also differ from hepatocytes with respect to the bifunctional PFK-2/fructose-2,6-bisphosphatase (FBPase-2), the enzyme that synthesizes and degrades Fru-2,6- P_2 . Compared with the normal liver enzyme, the FTO-2B enzyme has a 4-fold higher ratio of kinase to phosphatase activity [26], no inactivating cyclic-AMP-dependent phosphorylation site [27], and a different cloned cDNA, resulting from alternative splicing of the gene encoding the liver enzyme [27]. The small and delayed decrease in Fru-2,6- P_2 which AICARiboside induced in FTO-2B cells

(Figure 4) indicates that, in contrast with normal hepatocytes, their PFK-2/FBPase-2 is not a target for ZMP. The mechanism of the retarded decrease in Fru-2,6- P_2 is not immediately apparent, since the concentration of its precursor, fructose 6-phosphate, was still twice as high as in control conditions after 6 h of incubation with AICARiboside (Figure 6).

The metabolite cross-over at the level of PFK-1, combined with the absence of effect of AICARiboside on the production of lactate from dihydroxyacetone, indicated that AICARiboside inhibits glycolysis at the level of PFK-1 in FTO-2B cells. Accordingly, the activity of PFK-1 in FTO-2B cell extracts was inhibited by 70% by concentrations of ZMP reached in cells incubated with 250 μM AICARiboside. The observation that ZMP inhibited the activity of PFK-1 in FTO-2B cells and in normal rat hepatocytes, but not in rat muscle, rabbit heart and human erythrocytes, suggests that only the liver isoenzyme of PFK-1 is ZMP-sensitive. That the inhibitory effect was more pronounced in FTO-2B cell extracts than in hepatocyte extracts accords with the finding of modified PFK-1 isoenzymes in hepatomas (reviewed in [28]).

Taken together, our previous study in isolated rat hepatocytes [5] and the present work indicate that glucokinase and PFK-2 of normal liver are most sensitive to ZMP, whereas hexokinase is insensitive. In FTO-2B cells, which lack glucokinase and have a different PFK-2 which is not sensitive to ZMP, glycolysis can nevertheless also be inhibited by AICARiboside via ZMP. This is due to the inhibitory effect of ZMP on PFK-1. Why liver PFK-1 is sensitive to ZMP, whereas the skeletal-muscle, heart and erythrocyte enzymes are not, is unknown.

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