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Substrate Cycling Between 5-Amino-4-Imidazolecarboxamide Riboside and its Monophosphate in Isolated Rat Hepatocytes

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ABSTRACT. AICA (5-amino-4-imidazolecarboxamide)-riboside is taken up by isolated rat hepatocytes and converted by adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) into AICAR (ZMP), an intermediate of the de novo synthesis of purine nucleotides. We investigated if, in these cells, a cycle analogous to the adenosine-AMP substrate cycle operates between AICAriboside and ZMP. When 50 μM ITu, an inhibitor of adenosine kinase, was added to hepatocytes that had metabolized AICAriboside for 30 min, the concentration of ZMP decreased immediately. This was mirrored by a reincrease of AICAriboside. Rates of the ITu-induced decrease of ZMP and the increase of AICAriboside, calculated at different concentrations of ZMP, were first order, up to the highest concentration of ZMP (approx. 5 μmol/g of cells). Dephosphorylation of ZMP added to crude cytosolic extracts of rat liver displayed hyperbolic kinetics, with a V_{max} of 0.65 μmol/min per g protein and an apparent K_m of 5 mM, and was markedly inhibited by Pi, an inhibitor of IMP-GMP 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5). We conclude that hepatocyte ZMP is continuously dephosphorylated, most likely by IMP-GMP 5′-nucleotidase, into AICAriboside, which is rephosphorylated into ZMP by adenosine kinase. Substrate cycling was also shown to occur between other nucleoside analogs and their phosphorylated counterparts. BIOCHEM PHARMACOL 52;7:999–1006, 1996.

KEY WORDS. adenosine kinase; cytosolic IMP-GMP 5′-nucleotidase; 5-iodotubericidin; 6-methyl-mercaptopurine riboside; 2-chloro-adenosine; Z-nucleotides

AICAriboside† is the dephosphorylated form of AICAribotide (AICAR or ZMP), an intermediate of the de novo synthesis of purine nucleotides. AICAriboside is taken up and metabolized by a variety of cell types, including human erythrocytes [1], Chinese hamster ovary fibroblasts [2, 3], dog, rat, and mouse cardiac and skeletal muscle [4, 5], isolated rat kidney tubules [6], human platelets and lymphoblasts [7], and isolated rat hepatocytes [8]. The first step in the metabolism of AICAriboside (Fig. 1) is phosphorylation into ZMP by adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20). Further metabolism of ZMP can occur in three directions: 1. conversion into IMP and, hence, into adenine and guanine nucleotides and their catabolites, initiated by the action of AICAR formyltransferase (10-formyltetrahydrofolate: 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase, EC 2.1.2.3), which requires the folate derivative 10-formyltetrahydrofolate; 2. conversion into SAICAR by reversal of the adenylosuccinate lyase (EC 4.3.2.2) reaction; and 3. phosphorylation into ZDP and ZTP by nucleoside monophosphate kinase (ATP: nucleosidemonophosphate phosphotransferase, EC 2.7.4.4) and nucleosidediphosphate kinase (ATP: nucleosidediphosphate phosphotransferase, EC 2.7.4.6), and/or reversal of the PRPP synthetase (ATP: D-ribose-5-phosphate pyrophosphotransferase, EC 2.7.6.1) reaction [9]. In most cell types that have been investigated, ZMP is predominantly channelled into the de novo synthesis of purine nucleotides, resulting in their catabolism. This is evidenced by limited accumulations of ZMP, minimal elevations of the concentrations of purine nucleotides, and substantial increases in the production of terminal purine catabolites. Isolated rat hepatocytes are an exception. Incubation of these cells with AICAriboside leads to accumulation of up to millimolar concentrations of ZMP, accompanied by formation of ZDP and ZTP, without significant modifications in the concentrations of purine nucleotides and the rate of production of purine catabolites [8].

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† *Abbreviations:* AICAriboside, 5-amino-4-imidazolecarboxamide riboside; AOPCP, α,β-methyleneadenosine 5′-diphosphate; DTT, dithiothreitol; ITu, 5-iodotubercidin; KRB, Krebs-Ringer bicarbonate buffer; LDH, lactate dehydrogenase; PEG, polyethyleneglycol; PRPP, 5-phosphoribosyl 1-pyrophosphate; SAICAR, succinyl AICAribotide; ZMP (AICAribotide), AICAriboside 5′-monophosphate; ZDP, AICAriboside 5′-diphosphate; ZTP, AICAriboside 5′-triphosphate.

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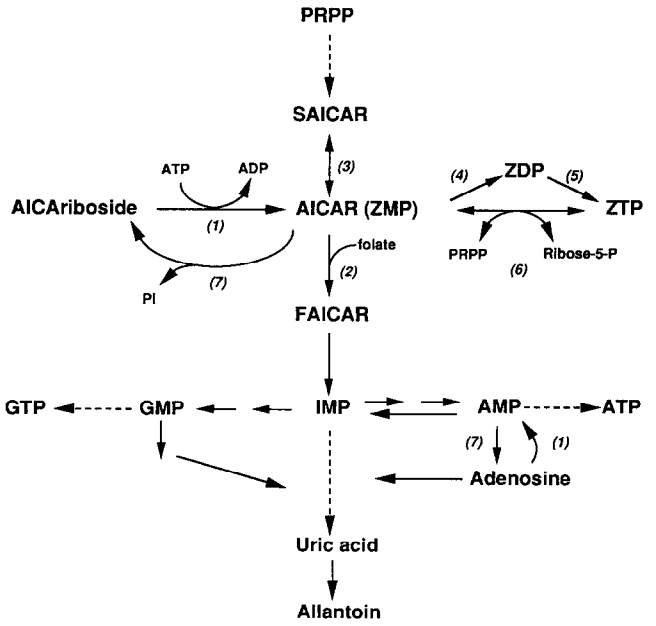

FIG. 1. Metabolism of AICAriboside. AICAriboside, 5-amino-4-imidazolecarboxamide riboside; PRPP, 5-phosphoribosyl 1-pyrophosphate; FAICAR, formyl AICAribotide; SAICAR, succinyl AICAribotide; AICAR (ZMP), AICAriboside 5′-monophosphate; ZDP, AICAriboside 5′-diphosphate; ZTP, AICAriboside 5′-triphosphate. (1), adenosine kinase; (2), AICAR formyltransferase; (3), adenylosuccinate lyase; (4), nucleosidemonophosphate kinase; (5), nucleosidediphosphate kinase; (6), PRPP synthetase; (7), cytosolic 5′-nucleotidase(s). The *de novo* synthesis of purine nucleotides involves conversion of PRPP into IMP.

This can be explained by the association in the liver of a highly active adenosine kinase [10] with a poorly active AICAR formyltransferase [8].

Up to now, a fourth direction in the metabolism of ZMP, namely its dephosphorylation into AICAriboside, had not been considered. Previous studies in this laboratory have shown that in isolated normoxic rat hepatocytes, AMP is continuously dephosphorylated by cytosolic 5′-nucleotidase(s) (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) into adenosine, which is immediately reconverted into AMP by adenosine kinase [11]. More recently, interruption of this substrate cycle has been shown to play a major role in the anoxia-induced elevation of adenosine, both in isolated rat hepatocytes [12] and in rabbit cardiomyocytes [13]. The aim of the present work was to investigate if a substrate cycle operates between AICAriboside and ZMP when the latter accumulates to substantial concentrations in isolated rat hepatocytes that have been incubated with the nucleoside. The possibility that substrate cycling might occur between pharmacologic nucleoside analogs and their nucleosidemonophosphate counterparts was also investigated.

MATERIALS AND METHODS

Materials

ITu was from Warner Lambert (Detroit, MI, USA). AICAriboside, ZMP, AOPCP, 6-methyl-mercaptopurine riboside, 2-chloro-adenosine and bovine serum albumin (fraction V, essentially fatty acid free) were purchased from Sigma (St Louis, MO, USA). Albumin was dialyzed overnight against 25 volumes of KRB, with two changes. PEG 6000 was from UCB (Brussels, Belgium). All chemicals were of analytical grade.

Isolated Hepatocytes

Hepatocytes were prepared from fed male Wistar rats as previously described [14]. The cells were incubated at 37°C in KRB supplemented with 10 mM glucose and 1% bovine serum albumin, and gassed with O_2/CO_2 (95:5). Their concentration (50–90 mg cells/mL, equivalent to 7–12.5 × 10^6 cells/mL) was determined at the beginning of the incubations by weighing the pellet obtained by centrifuging 1 mL of the cell suspension. Cell viability was assessed by measuring the proportion of LDH in the extracellular medium. It reached 5–15% of total LDH at the end of the incubations. The cell suspensions were preincubated for 15 to 20 min before addition of AICAriboside. ITu, when used, was added either immediately before or 30 min after the addition of AICAriboside, as indicated. ATP depletion was induced by addition of 5 mM KCN.

Analytical Methods

AICAriboside and purine nucleotides were measured as described previously [8] on neutralized perchloric acid extracts prepared from the whole suspension or, when indicated, from the extracellular medium after centrifugation of the cells, as described previously [11]. Intracellular inorganic phosphate was determined by the method of Itaya and Ui [15] in extracts obtained by centrifuging 0.5 mL of the hepatocyte suspension through a 0.5-mL layer of silicone oil (DC 550, Serva Heidelberg, Germany) into 0.5 mL ice-cold 10% perchloric acid. LDH was measured by the method of Vassault [16].

Dephosphorylation of ZMP in Rat Liver Cytosolic Extracts

For preparation of cytosolic fractions, rat liver was homogenized in 4 vol of 0.25 M sucrose containing 25 mM Hepes, pH 7.4, 100 mM KCl, 1mM DTT, and 1 mM EDTA. After centrifugation at 12,000 rpm for 10 min, the supernatant was fractionated with 5 to 30% PEG. The final pellet was resuspended in 20 mL of the buffer described above (except that sucrose was omitted) and dialyzed. Dephosphorylation of ZMP was determined by measuring the production of AICAriboside. Incubations were performed at 37°C in a medium containing 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, various concentrations of ZMP, other compounds as noted in Results, and 40 μL of PEG extract in a total volume of 0.1 mL. At various time intervals, 30-μL aliquots were transferred into 10 μL of 10% $HClO_4$. After centrifugation and neutralization of the supernatant with 3 M K_2CO_3,

AICAriboside was measured by HPLC [8]. It was verified that, under our conditions, the formation of AICAriboside was linear as a function of time and the amount of protein present in the assay.

RESULTS

Effect of ITu on the Metabolism of AICAriboside in Hepatocytes

In accordance with previous results [8], AICAriboside, added at 500 μM concentration to isolated hepatocyte suspensions incubated in the absence of ITu, was taken up by the cells (Fig. 2A, open circles), and metabolized into ZMP (Fig. 2B), ZDP (not shown), and ZTP (Fig. 2C). The concentration of ZMP reached 5 μmol/g of cells after 30 min and decreased slightly thereafter, at the rate of 24 ± 7 nmol/min/g of cells (mean ± SEM for $N = 7$). The concentration of ZTP, which increased almost linearly for 30 min, tended to plateau thereafter, at a level of approx. 1 μmol/g of cells. The concentration of ZDP reached approx. 0.1 μmol/g of cells after 30 min (not illustrated). Characteristically, AICAriboside did not disappear completely from the cell suspension: its concentration tended to plateau after 30 min, reaching approx. 10% of its initial value. At 60 min, the residual concentration of AICAriboside, expressed as μmol/mL of cell suspension, corresponded to 0.9 ± 0.09% ($N = 4$) of that of ZMP, expressed as μmol/g of hepatocytes. The steady state observed between ZMP and AICAriboside provided a first indication that a substrate cycle might operate between the nucleotide and its corresponding nucleoside.

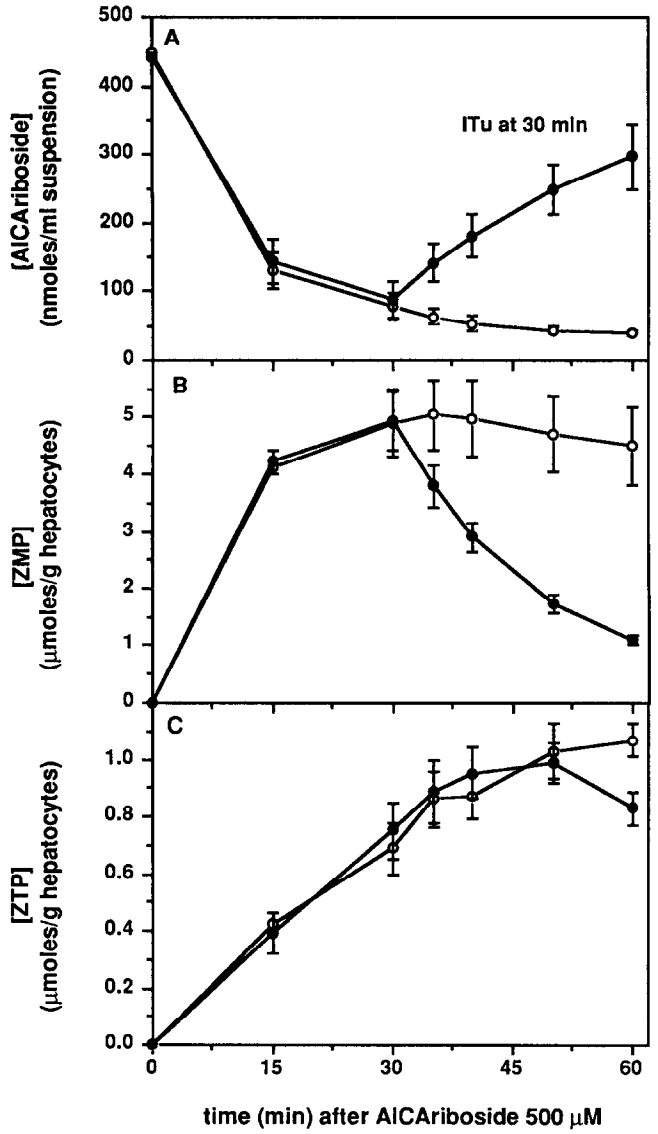

FIG. 2. Time-course of the utilisation of AICAriboside and formation of ZMP and ZTP, and the effect of ITu. Hepatocytes were incubated in the presence of 500 μM AICAriboside added at zero time, without (○) or with (●) 50 μM ITu added at 30 min. Results are means ± SEM of 4 separate experiments.

To investigate this hypothesis, experiments were performed with ITu, a powerful inhibitor of adenosine kinase [17], the enzyme responsible for the phosphorylation of AICAriboside [3]. In accordance with previous results [8], addition of ITu to the cell suspension, immediately before AICAriboside, completely blocked the metabolism of the nucleoside: AICAriboside remained at its initial concentration and neither ZMP nor ZTP could be detected (not illustrated). When ITu was added 30 min after AICAriboside (Fig. 2A, closed circles), the concentration of AICAriboside in the cell suspension reincreased immediately. This increase was mirrored by a concomitant immediate decrease in the concentration of ZMP (Fig. 2B). These effects of ITu clearly indicate that, in the absence of the inhibitor, hepatocyte ZMP is continuously recycled (i.e. dephosphorylated into AICAriboside which, in turn, is rephosphorylated into ZMP). Addition of ITu at 30 min did not influence the accumulation of ZTP (Fig. 2C) before an additional 20 min. After this time interval, the concentration of ZTP started to decline.

To obtain an estimate of the influence of the concentration of ZMP on the velocity of its dephosphorylation, rates of ITu-induced decreases of ZMP and increases of AICAriboside were calculated over 10 min at different concentrations of ZMP, obtained by incubating hepatocyte suspensions with various concentrations of AICAriboside ranging from 50 to 500 μM. As shown in Fig. 3, both rates were first order, up to the highest concentration of intracellular ZMP obtained (approx. 5 μmol/g of cells). The higher rates of decrease of ZMP, compared with the rates of increase of AICAriboside, are most likely explained by the continuing conversion of ZMP into ZTP (Fig. 2C) during the initial stage of the dephosphorylation of ZMP.

Extracellular Dephosphorylation of ZMP by Hepatocytes

Similarly to that of AMP, dephosphorylation of intracellularly accumulated ZMP could be accomplished either inside the cells by cytosolic 5′-nucleotidase(s) or, after release of ZMP from broken cells in the extracellular medium, by

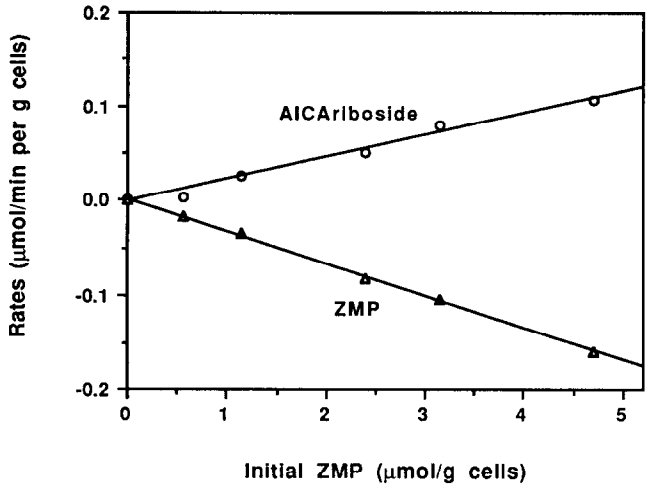

FIG. 3. Correlation between the concentration of ZMP and the rate of ITu-induced reincrease of AICAriboside (○) and decrease of ZMP (△). ZMP concentrations were measured 20 min after the addition to hepatocyte suspensions of various concentrations of AICAriboside (50, 100, 200, 300, and 500 µM). At this time interval, 50 µM ITu was added and the rates of reincrease of AICAriboside and decrease of ZMP were measured over 15 min.

ecto-5′-nucleotidase. We, first, measured the concentration of ZMP in the extracellular medium of isolated hepatocytes incubated in the presence of 500 µM AICAriboside. Extracellular ZMP was undetectable at the beginning of the experiments. During incubation, it accumulated almost linearly for 30 min, reaching 5–10 nmol/mL or 2–5% of total ZMP at this time point, and plateaued thereafter (results not shown). To determine if ZMP could be degraded extracellularly by hepatocytes, we investigated its fate upon addition to the cell suspension. At a concentration of 50 µM, extracellular ZMP disappeared at the rate of 27 nmol/min per g of cells (results not shown). This disappearance was inhibited by 95% by a mixture of 2.5 mM AOPCP, a potent inhibitor of membranous 5′-nucleotidase [18], and 10 mM β-glycerophosphate, a competitive inhibitor of aspecific phosphatases. Taken together, these results demonstrated that a small amount of ZMP was released by the cells and slowly dephosphorylated extracellularly.

To assess the role of this extracellular dephosphorylation in the AICAriboside–ZMP substrate cycle, we tested the effect of the inhibitory mixture on the ITu-induced increase of AICAriboside and decrease of ZMP in an experiment similar to that depicted in Fig. 2. Addition of AOPCP and β-glycerophosphate modified neither the uptake of AICAriboside nor the accumulation of ZMP. It also failed to influence the ITu-induced reappearance of AICAriboside and degradation of ZMP (results not shown). This demonstrated that the dephosphorylation of ZMP was not due to the action of ecto-5′-nucleotidase and/or aspecific phosphatases, but to an intracellular process.

Dephosphorylation of ZMP in Cytosolic Liver Extracts

To assess the overall dephosphorylation of ZMP by the various cytosolic 5′-nucleotidases known to be present in rat liver, the production of AICAriboside from ZMP added to crude cytosolic extracts of this tissue was measured. The influence on this production of known effectors of the various cytosolic 5′-nucleotidases was also investigated.

As depicted in Fig. 4A, dephosphorylation of ZMP displayed hyperbolic kinetics. From a double reciprocal plot (Fig. 4B), a V_{max} of 0.65 µmol/min per g protein and an apparent K_m for ZMP of 5 mM were determined. Physiological concentrations of Pi, a potent inhibitor of cytosolic IMP-GMP 5′-nucleotidase [19], had a marked inhibitory effect. The double reciprocal plots revealed mixed-type inhibition, with a K_i of 1.7 mM; at 5 mM Pi, the V_{max} decreased to 0.26 µmol/min per g protein, and the K_m increased to 8.2 mM. 2,3-Bisphosphoglycerate, like ATP a potent stimulator of cytosolic IMP-GMP 5′-nucleotidase from various sources [20, 21], increased dephosphorylation of 5 mM ZMP by 51 ± 18% ($N = 4$) at 3 mM concentration. Dephosphorylation was not affected by 2.5 mM AOPCP, corroborating that the cytosol preparation was free of con-

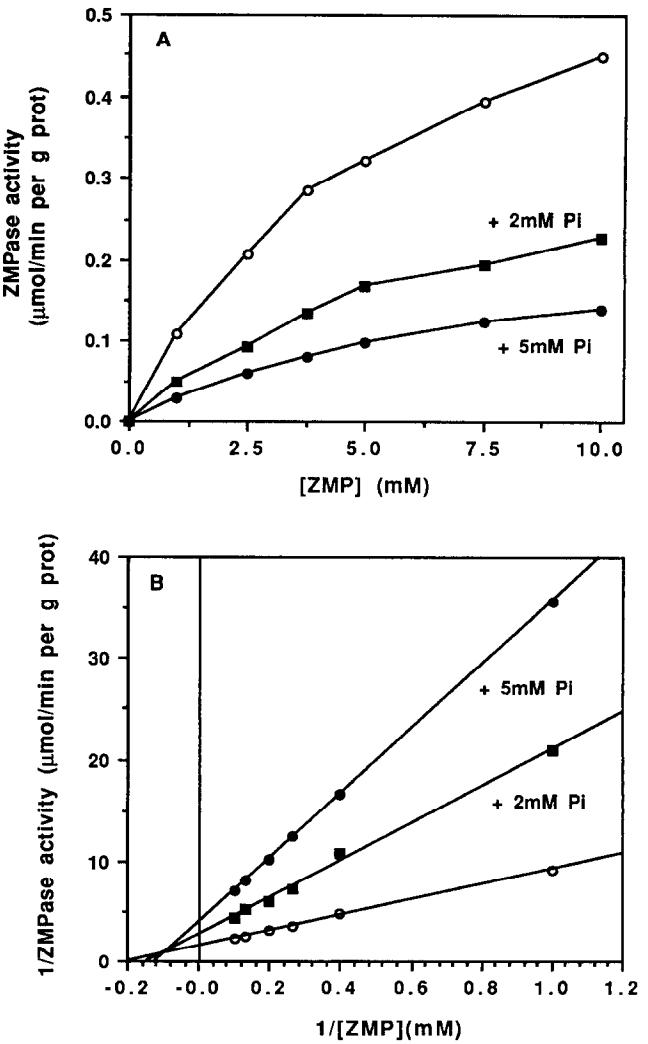

FIG. 4. Dephosphorylation of ZMP in rat liver cytosolic extract. Saturation curves for ZMP were measured in the absence (○) or presence of 2 mM (■) or 5 mM (●) Pi. Direct (A) and double reciprocal plots (B) are shown.

tamination by the membranous ecto-5′-nucleotidase. Taken together, these results suggested that intracellular ZMP was dephosphorylated by IMP-GMP 5′-nucleotidase.

Effect of ATP Depletion and Pi Elevation on Dephosphorylation of ZMP in Hepatocytes

To further substantiate the role of IMP-GMP 5′-nucleotidase in the dephosphorylation of ZMP into AICAriboside, experiments were performed in isolated hepatocytes in which, after preincubation for 30 min with 500 μM AICAriboside, ATP depletion and Pi elevation were induced by addition of KCN. In hepatocytes preincubated with 500 μM AICAriboside, the concentration of ATP was 2.3 ± 0.3 μmol/g of cells (N = 4), a value not different from that recorded in the absence of AICAriboside. Preincubation with 500 μM AICAriboside decreased the intracellular concentration of Pi from 4.6 ± 0.9 to 3.0 ± 0.8 μmol/g of cells (N = 4; $P \leq 0.01$). As shown in Fig. 5, addition of 10 mM KCN provoked a 70% decrease in the concentration of ATP within 10 min (Fig. 5A); intracellular Pi was elevated 2-fold over the same time interval, and continued to increase progressively thereafter (Fig. 5B). On addition of ITu alone, the concentration of ATP tended to decrease slowly, and that of intracellular Pi was not significantly modified. ITu barely modified the decrease of ATP and the elevation of intracellular Pi induced by KCN.

Figure 6 shows the effects of KCN and/or ITu, added 30 min after AICAriboside, on the concentrations of AICAriboside and Z-nucleotides. Addition of KCN induced a reaccumulation of AICAriboside (Fig. 6A) which was, conspicuously, 4-fold slower than that provoked by ITu in the absence of KCN, and not influenced by addition of ITu.

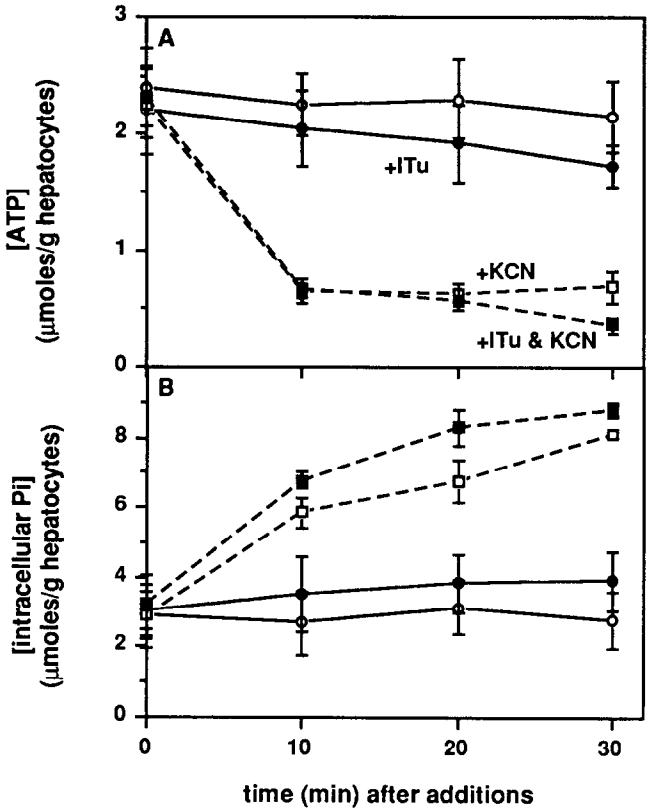

FIG. 5. Effect of ITu and/or KCN on the concentrations of ATP (A) and intracellular Pi (B) in hepatocytes preincubated in the presence of AICAriboside. AICAriboside was added 30 min before zero time at the concentration of 500 μM. Cells were then incubated without further additions (○), in the presence of 50 μM ITu (●), 10 mM KCN (□), or both (■). Results are means ± SEM of 4 separate experiments.

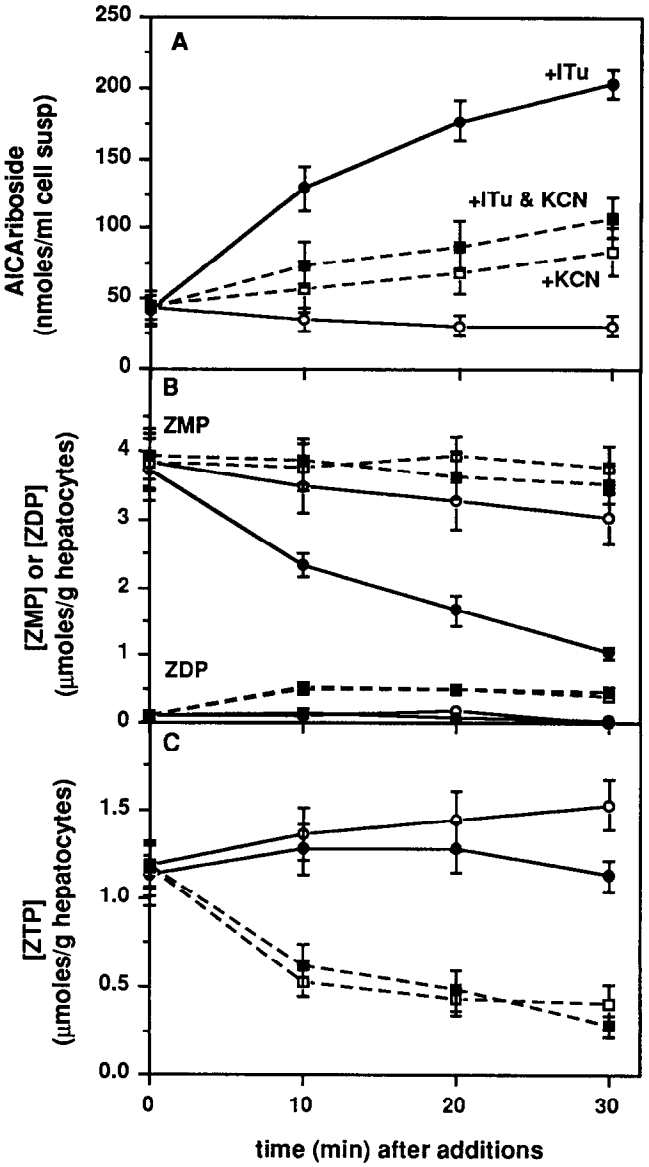

FIG. 6. Effect of ITu and/or KCN on the concentrations of AICAriboside (A), ZMP and ZDP (B), and ZTP (C) in hepatocytes preincubated in the presence of AICAriboside. AICAriboside was added 30 min before zero time at the concentration of 500 μM. Cells were then incubated without further additions (○), in the presence of 50 μM ITu (●), 10 mM KCN (□), or both (■). Results are means ± SEM of 4 separate experiments.

Moreover, the accumulation of AICAriboside induced by KCN, also irrespective of the presence of ITu, was not accompanied by a decrease in the concentration of ZMP (Fig. 6B) as was observed with ITu in the absence of KCN. Consequently, the concentration of ZMP at 30 min was approx. 20% higher in KCN-treated cells than in the absence of KCN and ITu. Measurements of the other Z-nucleotides revealed that KCN induced a 4-fold increase in the concentration of ZDP (Fig. 6B) and a 50% decrease in that of ZTP over 30 min (Fig. 6C), modifications which were also not influenced by addition of ITu.

Over 30 min, the sum of cellular ZMP, ZDP, and ZTP decreased by 8% in the absence of KCN and ITu, whereas addition of ITu provoked a 57% decrease of this sum (results not shown). In cells treated with KCN, the sum of the Z-nucleotides decreased by 11% in the absence of ITu, but only by 17% in its presence (not illustrated). During the same time interval, the sum of AICAriboside, ZMP, ZDP, and ZTP slowly diminished by 10% in the absence of KCN, and this decrease was not modified by the addition of ITu. In the presence of KCN, no decrease of the sum of the Z-compounds was recorded, irrespective of the presence of ITu (not illustrated). Taken together, these results indicated that the degradation of ZMP in isolated hepatocytes preincubated with AICAriboside was markedly diminished when the intracellular concentration of ATP, a stimulator of IMP-GMP 5′-nucleotidase, decreased, and that of Pi, an inhibitor of the enzyme, increased. They also showed that the degradation of ZTP via ZDP played a role in the maintenance of the concentration of ZMP.

Effect of ITu on the Metabolism of Other Nucleosides

To verify that recycling can also proceed between other nucleoside–nucleoside 5′-monophosphate pairs, in addition to adenosine–AMP and AICAriboside–ZMP, additional experiments were performed with two other nucleoside analogs. After verification that their phosphorylation was inhibited by ITu, 6-methyl-mercaptopurine riboside and 2-chloro-adenosine were used. When added at 500 μM concentration to isolated rat hepatocyte suspensions, 6-methyl-mercaptopurine riboside was 85% metabolized after 30 min. This resulted in the accumulation of 4.6 ± 0.7 μmol/g of cells of 6-methylmercaptopurine riboside 5′-monophosphate at this time point; no 6-methylmercaptopurine riboside 5′-triphosphate could be detected ($N = 3$, not illustrated). After addition of ITu at 30 min, the concentration of 6-methylmercaptopurine riboside 5′-monophosphate decreased to nearly zero over 30 min, whereas that of its nucleoside counterpart reincreased to 200 μM (results not shown).

Owing to its markedly slower utilization, 2-chloro-adenosine was used at a 100 μM concentration. Under this condition, more than 90% was utilized after 30 min, 2-chloro-AMP was below detection, and the corresponding 5′-triphosphate accumulated to 0.8 μmoles/g hepatocytes. Addition of ITu at 30 min provoked a slow elevation of 2-chloro-adenosine at the rate of 3 nmoles/min per g hepatocytes, accompanied by a decrease of the 5′-triphosphate derivative from 0.80 to 0.65 μmoles/g hepatocytes (results not shown).

DISCUSSION

The present work provides new insights into the metabolism of ZMP, an intermediate of the *de novo* synthesis of purine nucleotides that we have reported previously to markedly accumulate in isolated rat hepatocytes on addition of AICAriboside, its dephosphorylated form [8]. We now show that, in addition to its limited phosphorylation into ZDP and ZTP and its minimal conversion into intermediates of the *de novo* pathway, hepatic ZMP is dephosphorylated at a high rate into AICAriboside, most likely by cytosolic IMP-GMP 5′-nucleotidase. This catabolism is, however, counteracted by continuous rephosphorylation of AICAriboside into ZMP by adenosine kinase. Consequently, a substrate cycle operates between AICAriboside and ZMP, one similar to the cycle we have previously shown to function between adenosine and AMP in control hepatocytes [11] and cardiomyocytes [13], and to be abolished by ATP depletion [12, 13]. We will discuss successively: 1. the evidence in favor of the existence of a substrate cycle between AICAriboside and ZMP; 2. the enzyme responsible for the dephosphorylation of ZMP; and 3. the implications of this cycle for the pharmacokinetics of nucleoside analogs.

Substrate Cycle Between AICAriboside and ZMP

The observation that AICAriboside was never completely removed from the cell suspension on addition to isolated hepatocytes, and plateaued as a constant percentage of intracellularly accumulated ZMP, led to the suspicion that the residual concentration of AICAriboside resulted from an equilibrium between its phosphorylation into ZMP and the dephosphorylation of the latter (i.e. from the operation of a substrate cycle between AICAriboside and ZMP). Phosphorylation of AICAriboside is catalyzed by adenosine kinase, as evidenced by the observation that adenosine kinase-deficient Chinese hamster ovary fibroblasts [3] and human B lymphoblasts [7] are unable to metabolize AICAriboside. Furthermore, in accordance with previous work [8], we found that the accumulation of ZMP induced by the addition of AICAriboside to isolated hepatocytes was completely blocked by ITu, a powerful inhibitor of adenosine kinase [17]. ITu could therefore be used as a tool to assess the existence of a substrate cycle between AICAriboside and ZMP. Indeed, when added to hepatocytes that had been preincubated with AICAriboside and consequently accumulated ZMP, ITu provoked an immediate decrease of ZMP, mirrored by an elevation of AICAriboside (Fig. 2). This clearly indicates that, in the absence of the adenosine kinase inhibitor, ZMP is continuously dephos-

phorylated into AICAriboside, and that the latter is reconverted into ZMP by adenosine kinase.

The operation of a substrate cycle between AICAriboside and ZMP implies that the latter will accumulate up to the moment when a steady-state is reached between phosphorylation of AICAriboside and dephosphorylation of ZMP. At steady-state, the rate of substrate cycling should be equal to the velocity of the ITu-induced decrease of ZMP and increase of AICAriboside (Fig. 3). As mentioned above, the higher rates of decrease of ZMP, compared with the rates of increase of AICAriboside depicted in this figure, are most likely explained by further conversion of ZMP into ZTP. Nevertheless, both rates are, as expected from the substrate saturation curves of the dephosphorylation of ZMP in crude liver extracts (Fig. 4), linearly dependent on the concentration of ZMP, which is, itself, determined by the concentration of AICAriboside in the cell suspension. From the data depicted in Fig. 3, it can be concluded that the rate of substrate cycling reaches approx. 25 nmol/min per g of cells at 1 μmol of ZMP per g of cells, and approx. 115 nmol/min at 5 μmol/g of ZMP. The former rate is comparable to that of the substrate cycle between adenosine and AMP, which reaches 20 nmol/min per g of hepatocytes [11]. The AICAriboside–ZMP cycle consumes ATP for phosphorylation of AICAriboside by adenosine kinase. This consumption of ATP is, nevertheless, very low when compared to the rate of synthesis of ATP by mitochondrial phosphorylation, which reaches approx. 15 μmol/min per g cells [22]. Accordingly, incubation of hepatocytes with AICAriboside did not provoke a decrease in their ATP concentration.

The Enzyme Responsible for the Dephosphorylation of ZMP

The observation that the ITu-induced dephosphorylation of ZMP was not influenced by inhibition of the membranous ecto-5′-nucleotidase by AOPCP indicated that it is an intracellular process. Liver cytosol contains at least two enzymes, termed according to their preferred substrates, that could theoretically dephosphorylate ZMP. IMP-GMP 5′-nucleotidase has an approx. 10-fold higher affinity for the latter nucleoside monophosphates compared with AMP, is stimulated by ATP, ADP, and 2,3-bisphosphoglycerate, and markedly inhibited by Pi [19, 21, 23]. AMP 5′-nucleotidase has only been investigated in detail in rat [24], pigeon [25], and rabbit [26] heart, but its presence in rat liver has been observed by Bontemps *et al.* [27]. It is stimulated by ADP, but not by ATP and 2,3-bisphosphoglycerate, and only slightly inhibited by Pi. The marked inhibitory effect of physiological concentrations of Pi on the dephosphorylation of ZMP in crude rat liver cytosolic extracts (Fig. 4) suggests that it is catalyzed by IMP-GMP 5′-nucleotidase, rather than by AMP 5′-nucleotidase. From the substrate saturation curve for ZMP obtained in the absence of Pi (Fig. 4), it can be tentatively concluded that the K_m of rat liver IMP-GMP 5′-nucleotidase for ZMP (5 mM) is higher than the K_m for IMP (1 mM), but lower than that for AMP (10 mM) [19].

The experiments performed in hepatocytes that had been preincubated with AICAriboside to build up ZMP, and thereafter incubated with KCN to provoke ATP depletion and accumulation of intracellular Pi, are in agreement with the results obtained in liver extracts. The strikingly less pronounced rate of accumulation of AICAriboside induced by KCN, as compared to that provoked by ITu in cells not treated with KCN (Fig. 6A), can be explained by the decrease in ATP (Fig. 5A), a stimulator of IMP-GMP 5′-nucleotidase, associated with the accumulation of Pi (Fig. 5B), a potent inhibitor of this enzyme. That ZMP was nearly not modified in KCN-treated cells, whereas it decreased slightly in control cells in the absence of ITu, can be explained by degradation of accumulated ZTP (Fig. 6C) into ZDP (Fig. 6B) and, hence, into ZMP. The absence of effect of ITu on the production of AICAriboside (Fig. 6A) in KCN-treated cells, and on the concentrations of ZMP, ZDP (Fig. 6B), and ZTP (Fig. 6C), indicates that rephosphorylation of AICAriboside by adenosine kinase had already been suppressed by ATP depletion. This finding is in accord with our previous observations that ATP depletion abolishes recycling of adenosine into AMP in isolated rat hepatocytes [12] and rabbit cardiomyocytes [13].

Implications for the Pharmacokinetics of Nucleoside Analogs

Our experiments with 6-methyl-mercaptopurine riboside and 2-chloro-adenosine indicate that pharmacological nucleoside analogs that are phosphorylated into the corresponding 5′-monophosphate, as well as those which are further phosphorylated into 5′-triphosphates, may also be subject to substrate recycling. This was evidenced by the ITu-induced reincrease of the nucleoside analogs and by the ensuing decrease in the concentration of their phosphorylated derivatives. The markedly slower reincrease of 2-chloro-adenosine as compared with AICAriboside and 6-methyl-mercaptopurine riboside after addition of ITu can be explained by the very low concentration of its immediate precursor, 2-chloro 5′-monophosphate. Also, in accordance with the general occurrence of nucleoside-nucleoside 5′-monophosphate substrate cycling, continuous phosphorylation and dephosphorylation has been reported to proceed between pyrimidine deoxyribonucleosides and their corresponding 5′-monophosphates in mouse fibroblasts [28]. Substrate cycles probably also operate between nucleoside 5′-monophosphates and -diphosphates, and between the latter and nucleoside 5′-triphosphates. Accumulation of di- and triphosphates might result from either a higher rate of phosphorylation of the mono- and diphosphates, or a slower rate of dephosphorylation of the tri- and diphosphates. Ascertainment of these cycles will require the availability of potent inibitors of nucleoside mono- and diphosphate kinases. From the observation that ZTP continued to

build up for nearly 20 min after addition of ITu (Fig. 2C) while ZMP decreased (Fig. 2B), it can be concluded that ITu has no effect on the nucleosidephosphate kinases. Development of inhibitors of the various 5′-nucleotidases responsible for dephosphorylation of the mono-, di- and triphosphates could also be useful in potentiating the action of pharmacological nucleoside analogs.

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References

1. Zimmerman TP and Deeprose RD, Metabolism of 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide and related five-membered heterocycles to 5′-triphosphates in human blood and L5178Y cells. *Biochem Pharmacol* **27:** 709–716, 1978.
2. Thomas CB, Meade JA and Holmes EW, Aminoimidazole carboxamide ribonucleoside toxicity: A model for study of pyrimidine starvation. *J Cell Physiol* **107:** 335–344, 1981.
3. Sabina RL, Patterson D and Holmes EW, 5-Amino-4-imidazolecarboxamide riboside (Z-riboside) metabolism in eukaryotic cells. *J Biol Chem* **260:** 6107–6114, 1985.
4. Swain JL, Hines JJ, Sabina RL and Holmes EW, Accelerated repletion of ATP and GTP pools in postischemic canine myocardium using a presursor of purine de novo synthesis. *Circ Res* **51:** 102–105, 1982.
5. Sabina RL, Kernstine KH, Boyd RL, Holmes EW and Swain JL, Metabolism of 5-amino-4-imidazolecarboxamide riboside in cardiac and skeletal muscle. Effects on purine nucleotide synthesis. *J Biol Chem* **257:** 10178–10183, 1982.
6. Nissim I, Yudkoff M and Segal S, Effect of 5-amino-4-imidazolecarboxamide riboside on renal ammoniagenesis. Study with [^{15}N]aspartate. *J Biol Chem* **261:** 6509–6514, 1986.
7. Jimenez R, Gruber HE and Baranckiewicz J, AICA-riboside (5-amino-4-imidazole carboxamide riboside) metabolism in human lymphoblasts, red blood cells and platelets. *Int J Purine Pyrimidine Res* **1:** 51–60, 1990.
8. Vincent MF, Marangos PJ, Gruber HE and Van den Berghe G, Inhibition by AICA riboside of gluconeogenesis in isolated rat hepatocytes. *Diabetes* **40:** 1259–1266, 1991.
9. Sabina RL, Holmes EW and Becker MA, The enzymatic synthesis of 5-amino-4-imidazolecarboxamide riboside triphosphate (ZTP). *Science* **223:** 1193–1195, 1984.
10. Arch JRS and Newsholme ER, Activities and some properties of 5′-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. *Biochem J* **174:** 965–977, 1978.
11. Bontemps F, Van den Berghe G and Hers HG, Evidence for a substrate cycle between AMP and adenosine in isolated hepatocytes. *Proc Natl Acad Sci USA* **80:** 2829–2833, 1983.
12. Bontemps F, Vincent F and Van den Berghe G, Mechanisms of elevation of adenosine levels in anoxic hepatocytes. *Biochem J* **290:** 671–677, 1993.
13. Wagner DR, Bontemps F and Van den Berghe G, Existence and role of substrate cyling between AMP and adenosine in isolated rabbit cardiomyocytes under control conditions and in ATP depletion. *Circulation* **90:** 1343–1349, 1994.
14. Van den Berghe G, Bontemps F and Hers HG, Purine catabolism in isolated rat hepatocytes. Influence of coformycin. *Biochem J* **188:** 913–920, 1980.
15. Itaya K and Ui M, A new micromethod for the colorimetric determination of inorganic phosphate. *Clin Chim Acta* **14:** 361–366, 1966.
16. Vassault A, Lactate dehydrogenase: UV Method with pyruvate and NADH. In: *Methods of Enzymatic Analysis*, 3rd ed. (Ed. Bergmeyer HU) Vol. 3, pp. 118–126. Verlag Chemie, Weinheim, 1987.
17. Henderson JF, Paterson ARP, Caldwell IC, Paul B, Chan MC and Lau KF, Inhibitors of nucleoside and nucleotide metabolism. *Cancer Chemother Rep Part 2* **3:** 71–85, 1972.
18. Burger RM and Lowenstein JM, Preparation and properties of 5′-nucleotidase from smooth muscle of small intestine. *J Biol Chem* **245:** 6274–6280, 1970.
19. Van den Berghe G, van Pottelsberghe C and Hers HG, A kinetic study of the soluble 5′-nucleotidase of rat liver. *Biochem J* **162:** 611–616, 1977.
20. Bontemps F, Van den Berghe G and Hers HG, 5′-Nucleotidase activities in human erythrocytes: Identification of a purine 5′-nucleotidase stimulated by ATP and glycerate 2,3-bisphosphate. *Biochem J* **250:** 687–696, 1988.
21. Bontemps F, Vincent MF, Van den Bergh F, van Waeg G and Van den Berghe G, Stimulation by glycerate 2,3-bisphosphate: A common property of cytosolic IMP-GMP 5′-nucleotidase in rat and human tissues. *Biochim Biophys Acta* **997:** 131–134, 1989.
22. Krebs HA, Hems R, Lund P, Halliday D and Read WWC, Sources of ammonia for mammalian urea synthesis. *Biochem J* **176:** 733–737, 1978.
23. Itoh R, Purification and some properties of cytosol 5′-nucleotidase from rat liver. *Biochim Biophys Acta* **657:** 402–410, 1981.
24. Truong VL, Collinson AR and Lowenstein JM, 5′-Nucleotidases in rat heart. Evidence for the occurrence of two soluble enzymes with different specificities. *Biochem J* **253:** 117–121, 1988.
25. Newby AC, The pigeon heart 5′-nucleotidase responsible for ischaemia-induced adenosine formation. *Biochem J* **253:** 123–130, 1988.
26. Yamazaki Y, Truong VL and Lowenstein JM, 5′-Nucleotidase I from rabbit heart. *Biochemistry* **30:** 1503–1509, 1991.
27. Bontemps F, Mimouni M and Van den Berghe G, Phosphorylation of adenosine in anoxic hepatocytes by an exchange reaction catalysed by adenosine kinase. *Biochem J* **290:** 679–684, 1993.
28. Nicander B and Reichard P, Evidence for the involvement of substrate cycles in the regulation of deoxyribonucleoside triphosphate pools in 3T6 cells. *J Biol Chem* **260:** 9216–9222, 1985.