Existence and Role of Substrate Cycling Between AMP and Adenosine in Isolated Rabbit Cardiomyocytes Under Control Conditions and in ATP Depletion

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Background Adenosine, a physiological coronary vasodilator, has been proposed to regulate coronary circulation according to myocardial oxygen demand. In the present study, we investigated the mechanisms of adenosine formation and utilization in isolated rabbit cardiomyocytes and, in particular, the existence and the role of substrate cycling between AMP and adenosine in the regulation of its concentration.

Methods and Results Rabbit cardiomyocytes were isolated by collagenase perfusion and incubated in HEPES-buffered Krebs-Henseleit solution at 37°C, pH 7.4, in control conditions and in ATP depletion achieved by inhibiting glycolysis with 5 mM/L iodoacetate. Under control conditions, adenosine accumulated at a rate of 4 pmol·min⁻¹·10⁻⁶ cells. The 13-fold elevation of adenosine accumulation induced by iodoacetidin (ITu), an inhibitor of adenosine kinase, proves that adenosine is normally recycled into AMP. This recycling involves 95% of the adenosine formed. In ATP depletion, adenosine accumulated at the rate of 335 pmol·min⁻¹·10⁻⁶ cells and was no longer rephosphorylated after 20 minutes, as shown by the absence of effect of ITu after this time interval. Moreover, adenosine was deaminated, as indicated by the twofold increase of its accumulation induced by deoxycoformycin (dCF), an inhibitor of adenosine deaminase. Both in control conditions and in ATP depletion, adenosine-dialdehyde, an inhibitor of S-adenosylhomocysteine (SAH) hydrolase, had no significant effect on adenosine formation, indicating that the transmethylation pathway is not an important source of adenosine in rabbit cardiomyocytes.

Conclusions The results indicate that recycling of adenosine into AMP is essential for the maintenance of low, nonvasodilatory concentrations of the nucleoside under control conditions and that interruption of recycling plays an important role in elevating adenosine during ATP depletion. (Circulation. 1994;90:1343-1349.)

Key Words: adenosine · myocardium

In the heart, as well as in several other organs, elevation of extracellular adenosine provoked by hypoxia has been proposed to induce vasodilation, aimed at restoration of the oxygen supply.¹ The physiological role of adenosine and its properties as a cardiovascular agent have been characterized in great detail over the past decade.² However, the exact mechanisms of adenosine formation and utilization in cardiac myocytes are still poorly understood.

Adenosine can be formed by dephosphorylation of AMP and by hydrolysis of S-adenosylhomocysteine (SAH). Dephosphorylation of AMP (Fig 1), either intracellularly by cytosolic 5'-nucleotidase(s) or extracellularly by ecto-5'-nucleotidase, is a major source of adenosine under conditions that provoke an increase in AMP, such as ischemia and hypoxia, although the participation of the various 5'-nucleotidases remains unclear.³⁻⁵ Besides dephosphorylation, catabolism of AMP can also proceed by deamination into IMP by AMP deaminase, followed by dephosphorylation of IMP into inosine by 5'-nucleotidase. In the guinea pig heart, hydrolysis of SAH, a product of the transmethylation pathway, has been reported to contribute to the production of adenosine in control conditions but not to the hypoxia-induced elevation of adenosine.⁶

The concentration of adenosine is determined not only by the rate of its formation but also by the rate of its utilization. Two enzymes can utilize adenosine: adenosine deaminase, which produces inosine, and adenosine kinase, which rephosphorylates adenosine into AMP, using ATP as the phosphate donor (Fig 1). The latter process results in recycling of adenosine in a so-called “substrate cycle.” We have reported previously that recycling of adenosine to AMP proceeds at a high rate in normoxic isolated rat hepatocytes.⁷ Recently, interruption of this recycling has been shown to play a major role in the elevation of adenosine induced by anoxia in these cells.⁸

In the present study, we investigated the existence of a substrate cycle between AMP and adenosine in isolated rabbit cardiomyocytes and its role in regulating adenosine concentration in control conditions and in ATP depletion induced by inhibition of glycolysis. The contribution of the hydrolysis of SAH to adenosine formation was also evaluated.

Methods

Isolation of Cardiomyocytes

Cardiac ventricular myocytes were isolated from male New Zealand White rabbits weighing 1.7 to 2.3 kg by a modified
procedure of Altschuld et al.\textsuperscript{9} Hearts were perfused by the Langendorff method and myocytes isolated by the addition of collagenase (Wako). After isolation, the preparations were enriched with viable myocytes by three sequential sedimentations in 4% bovine serum albumin (Serva). Before incubations, the myocytes were counted in a Nageotte cell chamber, and their viability was assessed by trypan blue exclusion. Myocytes were adjusted to \pm 400 000 cells/mL. Contamination of the preparation with endothelial cells was ruled out by measuring hypoxanthine, a catalytite that is formed only in the latter cells and not in myocytes.\textsuperscript{10} The protein content of cell suspensions was determined according to Lowry et al,\textsuperscript{11} 220 000 cells being equivalent to 1 mg of Lowry protein. Myocytes were incubated in HEPES-buffered Krebs-Henseleit solution (pH 7.4) containing (in mmol/L): NaCl 118, KCl 4.8, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, HEPES 25, and CaCl\textsubscript{2} 1, supplemented with 11 mmol/L glucose and 1% bovine serum albumin (fatty acid free, Sigma Chemical Co) and gassed with 100% O\textsubscript{2}. Incubations were performed at 37°C in a gently shaking water bath. Cells were preincubated for 20 minutes before the experiments were started.

\section*{Inhibitors}
Iodotubercidin 2 \textmu mol/L (ITu) (Research Biochemicals) and deoxycoformycin 2 \textmu mol/L (dCF) (Warner-Lambert) were used to inhibit adenosine kinase and adenosine deaminase, respectively.\textsuperscript{12,13}

Preliminary studies, performed as in Bontemps et al,\textsuperscript{14} had shown that these concentrations inhibited by 94\% and 99\% the phosphorylation and the deamination, respectively, of \textsuperscript{14}C-labeled adenosine by the cardiomyocytes. When used, dCF was added at the beginning of the preincubation, owing to the time required for this tight-binding inhibitor to penetrate the cells and bind to its target. ITu was added at time 0. Adenosinediealdehyde 10 \textmu mol/L (Sigma), also added during preincubation, was used to inhibit SAH\textsuperscript{15}; the concentration used was verified to inhibit by 99\% the formation of SAH from endogenous adenosine in the presence of a saturating, 1 mmol/L concentration of L-homocysteine thiolactone. ATP depletion was induced by addition at time 0 of 5 mmol/L iodoacetate, an inhibitor of glycolysis acting at the level of glyceraldehyde 3-phosphate dehydrogenase. Whereas in control conditions, the proportion of viable cells, measured by trypan blue exclusion, decreased from \approx 90\% to 80\% over the duration of the experiments, it decreased to 5\% in the presence of iodoacetate. Extracellular LDH, which increased from about 5\% to 10\% under control conditions, reached \approx 12\% after iodoacetate.

\section*{Determination of Nucleotides and Nucleosides}
Nucleotides and nucleosides were extracted by transferring 500-\textmu L aliquots of cell suspension into 150 \textmu L of ice-cold 10\% HClO\textsubscript{4}, followed by vigorous mixing. After standing on ice for 15 minutes, the extracts were centrifuged with an Eppendorf microfuge, and the resultant supernatants were neutralized with 3 mol/L KOH and 3 mol/L KHCO\textsubscript{3}. Nucleotides were quantified by anion-exchange high-performance liquid chromatography (HPLC) on a 12.5 x 0.46-cm Partisphere 5 SAX column (Whatman) according to the method described by Hartwick and Brown\textsuperscript{16} and nucleosides by reversed-phase HPLC on a 12.5 x 0.46-cm Partisphere C-18 column (Whatman).\textsuperscript{8}

\section*{Statistics}
Results are expressed as mean±SEM, in nanomoles per milligram total Lowry protein for nucleotides and in nanomoles per milliliter of cell suspension for nucleosides. Data obtained at the different time points and accumulation rates were subjected to factorial ANOVA (one-way ANOVA, Fisher test), and a value of P<.05 was considered to be statistically significant.

\section*{Results}
\subsection*{Concentrations of Adenine Nucleotides in Control Myocytes}
After isolation, \approx 90\% of the cardiac myocytes were viable as assessed by trypan blue exclusion, and 75\% to 90\% of the cells were rod-shaped. In the control conditions, ATP content was 28.9±1.2 nmol/mg Lowry protein (n=12) at the beginning of the experiments and did not change significantly over the ensuing 60 minutes of incubation. The ADP content was 5.4±0.3 nmol/mg Lowry protein at time 0 and decreased to 4.3±0.3 after 60 minutes of incubation. AMP content was 1.1±0.1 nmol/mg Lowry protein initially and remained stable over the whole duration of the incubation. IMP was undetectable. These results are in accordance with observations by others in cardiomyocytes isolated from rabbits and rats.\textsuperscript{9,17,18} The sum of adenine nucleotides and the individual contents of ATP, ADP, and AMP were not significantly affected by the addition of either 2 \textmu mol/L ITu, 2 \textmu mol/L dCF, a combination of both ITu and dCF, or the addition of 10 \textmu mol/L adenosinedialdehyde (results not shown).

\subsection*{Influence of Iodoacetate on Adenine Nucleotide Contents}
Addition of 5 mmol/L iodoacetate to the myocyte suspensions provoked a biphasic decrease of the ATP content (Fig 2A). A rapid, \approx 15\% initial diminution was followed by a 10-minute plateau, which was succeeded by a linear decline, up to complete depletion after 60 minutes. The ADP content increased slightly during 40 minutes and decreased progressively thereafter (Fig 2B). The AMP content increased nearly 10-fold within 5 minutes of the addition of iodoacetate (Fig 2C) and remained at that level over the whole duration of the experiment. IMP accumulated to 7 nmol/mg Lowry protein within 5 minutes and to 10 nmol/mg Lowry protein after 60 minutes of incubation (Fig 2D). These results accord with published studies.\textsuperscript{9,18}

Inhibition of adenosine deaminase by dCF and of adenosine kinase by ITu, as well as addition of both inhibitors, had no significant effect on the degradation of ATP (Fig 2A). The inhibitors also did not influence ADP (Fig 2B) and IMP (Fig 2D) content. Addition of ITu or dCF alone had no effect on AMP content (Fig 2C), but the combination of both inhibitors decreased
the latter by 50% after 40 minutes. However, the sum of adenine nucleotides was not significantly affected.

Accumulation of Adenosine and Inosine in Control Conditions

Under control conditions, adenosine accumulated slowly and linearly in the cardiomyocyte suspensions (Fig 3A), at a rate (Table) reaching 4±1 pmol/min per 10^6 cells (n=6). This indicates that the production of adenosine by these cells slightly surpasses its utilization. Inosine is the terminal adenine nucleotide catabolite in cardiomyocytes, owing to the absence or very low activity of purine nucleoside phosphorylase in these cells.10,19,20 Inosine production in normoxic myocytes (Fig 3B) occurred at the rate of 6±4 pmol/min per 10^6 cells (n=6).

The addition of dCF completely suppressed the production of inosine. This indicates that under normoxic conditions, the very slow production of inosine proceeds exclusively by dephosphorylation of AMP into adenosine, followed by deamination of adenosine into inosine. Indeed, if catabolism of AMP had occurred in part via the alternative pathway, deamination into IMP by AMP deaminase followed by dephosphorylation of IMP into inosine (Fig 1), a residual production of inosine would have been observed in the presence of an inhibitor of adenosine deaminase.

The rate of accumulation of adenosine was not significantly increased by the addition of dCF. However, the addition of ITu increased the rate of accumulation of adenosine more than 10-fold, to 53±18 pmol/min per 10^6 cells (n=6) (P<.05). This observation demonstrates that adenosine is rephosphorylatted into AMP by adenosine kinase in the absence of ITu. The preferential utilization of adenosine by adenosine kinase can be explained by its higher affinity for this enzyme compared with adenosine deaminase.21 In the presence of ITu, the rate of production of inosine increased more than 20-fold, to 138±7 pmol/min per 10^6 cells (n=6) (P<.005). This is explained by the fact that under this condition, adenosine can no longer be rephosphorylated into AMP and is consequently further converted into inosine by adenosine deaminase.

In the presence of both inhibitors, the rate of accumulation of adenosine increased more than 50-fold, to 210±32 pmol/min per 10^6 cells (n=6) (P<.005). This high rate reflects the total rate of formation of adenosine by the cells. That this rate was severalfold higher

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Fig 2. Graphs showing time course of ATP (A), ADP (B), AMP (C), and IMP (D) content in isolated rabbit cardiomyocytes incubated in HEPES-buffered Krebs-Henseleit solution after addition of 5 mmol/L iodoacetate, Deoxycoformycin (dCF), an inhibitor of adenosine deaminase, was added during a prior 20-minute incubation; iodotubercidin (ITu), an inhibitor of adenosine kinase, and iodoacetate, an inhibitor of glycolysis, were added at time 0. Values are mean±SEM of three to six experiments.

Fig 3. Graphs showing time course of adenosine (A) and inosine (B) accumulation in rabbit cardiomyocyte suspensions incubated under normoxic conditions in HEPES-buffered Krebs-Henseleit solution. Inhibitors were as in Fig 2. Values are mean±SEM of three to six experiments. **P<.01 and ***P<.005 vs control.
than upon addition of ITu alone confirmed that in the latter condition, a substantial quantity of adenosine was deaminated, owing to the increase of its concentration. In the presence of both inhibitors, the production of inosine was completely suppressed.

**Effect of Iodoacetate on Adenosine and Inosine Accumulation**

Fig 4A shows that the biphasic depletion of ATP induced by iodoacetate was reflected in a similarly biphasic accumulation of adenosine. In the absence of inhibitors, adenosine increased fourfold within 10 minutes (Fig 4A, inset), to a plateau that was maintained during the ensuing 10 minutes; thereafter, adenosine accumulated linearly up to the end of the experiment. The rate of accumulation of adenosine, which was 461 ± 92 pmol/min per 10⁶ cells over the first 10 minutes, reached an overall value of 335 ± 63 pmol/min per 10⁶ cells (n = 3) over 60 minutes, which is 85-fold the rate of accumulation recorded in control conditions in the absence of inhibitors. The effect of ITu was also time-dependent. During the first 10 minutes, addition of ITu significantly increased the rate of accumulation of adenosine by 598 pmol/min per 10⁶ cells, to 1059 pmol/min per 10⁶ cells. This indicates that during this time interval, rephosphorylation of adenosine by adenosine kinase continued. Thereafter, the rate of elevation of adenosine in the presence of ITu evolved in parallel with that recorded in the absence of inhibitors, indicating that adenosine was no longer recycled. Addition of dCF enhanced the rate of accumulation of adenosine approximately twofold during the first 40 minutes of incubation; thereafter, the rate increased about fourfold. Addition of both inhibitors resulted in an even higher rate of accumulation of adenosine, reflecting the total formation of adenosine and reaching 814 ± 16 pmol/min per 10⁶ cells (n = 3). This is a nearly fourfold increase compared with the total rate of production of adenosine in the presence of both inhibitors in control conditions.

In the absence of inhibitors, iodoacetate induced a biphasic accumulation of inosine, resembling that of adenosine (Fig 4B). Over the 20- to 60-minute time interval, the rate of production of inosine reached 1484 pmol/min per 10⁶ cells (n = 3). As in control cardiomyocytes, addition of dCF decreased the rate of accumulation of inosine, but only during the first 20 minutes. The only marginal inhibition of accumulation of inosine by dCF thereafter indicates that the accumulation of AMP induced by iodoacetate provokes catabolism of this nucleotide not only by way of dephosphorylation but also, and to an important extent, by way of AMP
deaminase. This is in accordance with the elevation of IMP under this condition (Fig 2D). There was little or no effect of ITu, alone or in combination with dCF, on the rate of accumulation of inosine, also in accordance with the role of IMP as a source of inosine.

Role of SAH as a Source of Adenosine

In control conditions, the total rate of adenosine formation, as assessed by addition of dCF and ITu, was not significantly decreased by the supplemental addition of adenosine-dialdehyde, an inhibitor of SAH hydrolase (Fig 5A). This indicates that under control conditions, adenosine is nearly exclusively derived from AMP dephosphorylation without significant participation of SAH hydrolysis. After inhibition of glycolysis with iodoacetate, total adenosine production, as assessed with dCF and ITu, was also not decreased by addition of adenosine-dialdehyde, indicating that adenosine was formed exclusively from AMP (Fig 5B).

Discussion

In accordance with experiments by others using isolated rat cardiomyocytes,7,18 we found that isolated rabbit cardiomyocytes can degrade AMP both by dephosphorylation into adenosine followed by deamination into inosine and by deamination into IMP followed by dephosphorylation into inosine (Fig 1). Also in accordance with others, inosine was the terminal purine nucleobase in the cells under investigation, a finding explained by their absent or very low purine nucleoside phosphorylase activity.16,19,20 In addition, our results demonstrate that in isolated rabbit cardiomyocytes, as in isolated rat hepatocytes,7,8 a substrate cycle operates between AMP and adenosine under control conditions and is impaired on depletion of ATP. These two conditions will be discussed separately.

Recycling of Adenosine in Control Conditions

Under control conditions, the catabolism of the adenine nucleotide pool of the isolated myocytes was very slow, as evidenced by the minimal rate of accumulation of adenosine and inosine in the cell suspension (Fig 3 and Table). The complete suppression of the accumulation of inosine by the adenosine deaminase inhibitor dCF indicates, as already concluded,7,18 that this catabolism proceeds only by dephosphorylation of AMP followed by deamination of adenosine.

Substrate cycling between AMP and adenosine, involving reutilization by adenosine kinase of adenosine formed by 5'-nucleotidase(s), was postulated by Arch and Newsholme22 as a mechanism that would permit a rapid regulation of the concentration of adenosine. The first demonstration of an active AMP/adenosine cycle, achieved in normoxic isolated rat hepatocytes, was based primarily on the effect of the adenosine kinase inhibitor ITu to progressively decrease the concentration of ATP, to elevate the concentration of adenosine, and to increase the rate of production of allantoin, the terminal purine catabolite in rat liver.7 The marked, approximately 10-fold increase of the rate of accumulation of adenosine (Fig 3A) and inosine (Fig 3B) induced by ITu in control cardiomyocytes shows that in these cells also, AMP is continuously dephosphorylated and that the resulting adenosine normally is nearly not deaminated but rather rephosphorylated into AMP. Measurement of the absolute rate of adenosine production, achieved by addition of both ITu and dCF, revealed that it reaches 210 pmol/min per 10^6 cells (Table), which is more than 20-fold the rate of accumulation of adenosine and inosine together recorded in the absence of inhibitors (10 pmol/min per 10^6 cells). This shows that 95% of adenosine produced is recycled into AMP under control conditions. The absence of a decrease of ATP on inhibition of adenosine kinase can be explained by the fact that adenosine accumulation after 60 minutes in the presence of ITu and dCF accounted for <10% of the initial adenine nucleotide pool in the cardiomyocytes, compared with 40% in hepatocytes.7,8

Studies in isolated rat hearts have either concluded against the existence of an AMP/adenosine cycle23 or claimed that recycling of adenosine occurs only at a very low rate.24 The latter conclusion was based on a less than twofold increase of total purine nucleoside release on addition of ITu in normoxic hearts. Whether the marked effect of ITu we observed is due to species differences, to the use of isolated cells, or to the great care taken to ensure that the concentration of ITu used was nearly totally inhibitory of adenosine kinase remains to be determined. After submission of this article, rapid recycling of adenosine has, nevertheless, been reported in well-oxygenated isolated guinea pig heart.25

Our observation that the production of adenosine in control conditions was only minimally decreased by adenosine-dialdehyde, an inhibitor of SAH hydrolase, indicates that adenosine was derived nearly exclusively from AMP, with almost no participation of the trans-
methylation pathway. This is in accordance with the latest studies in normoxic perfused guinea pig heart.25

Impairment of Adenosine Recycling on ATP Depletion

When ATP depletion was induced by iodoacetate, degradation of the adenine nucleotides proceeded at high rates by both pathways, as evidenced by the accumulation of IMP (Fig 2D) and by the low inhibitory effect of dCF on the huge accumulation of inosine, particularly during the second half of the experiment (Fig 4B and Table). In the absence of inhibitors of adenosine metabolism, addition of iodoacetate increased the rate of accumulation of adenosine to 335 pmol/min per 10^6 cells (Fig 4A), compared with 4 pmol/min per 10^6 cells in control conditions (Fig 3A). In theory, this 85-fold increase could result from the increase of AMP (Fig 2C), from a decreased recycling into AMP, or from both. During the first 10 minutes after the addition of iodoacetate, ITu enhanced the rate of accumulation of adenosine by 598 pmol/min per 10^6 cells. This indicates that refrosphorylation of adenosine into AMP was still active, even more so than in control conditions. During these 10 minutes, total adenosine production, determined by addition of dCF and ITu, was 1250 pmol/min per 10^6 cells. It can thus be concluded that, in the absence of inhibitors, 50% of the adenosine produced was recycled into AMP during this time interval. The accumulation of adenosine thus results from a large production that exceeds the maximal recycling capacity. During later time intervals, however, no more recycling of adenosine took place, as evidenced by the observation that the concentrations of adenosine evolved in parallel in the presence of ITu and in the absence of inhibitor. The impairment of adenosine recycling at this stage can be explained by depletion of the phosphate donor, ATP, and/or inhibition of adenosine kinase by excess of its substrate, adenosine, or of its product, AMP.26,27 Since the K_m of AMP for adenosine kinase is approximately 0.8 mmol/L in rat heart,28 one would expect recycling of adenosine to be maintained until depletion of ATP to values below 5 to 7 nmol/mg Lowry protein. However, in our experiments, recycling of adenosine was already impaired at ATP levels above 20 nmol/mg Lowry protein. The ATP level is thus not the only determinant of the interruption of the AMP/adenosine cycle. That the K_m of adenosine for adenosine kinase is <1.0 μmol/L in rat heart led to the proposition that adenosine kinase might be nearly saturated under basal conditions.24,28 Nevertheless, Newby et al.29 concluded that adenosine kinase is far from saturated in neonatal rat heart cells, because the basal rate of adenosine formation was only 9% of the maximal activity of adenosine kinase measured in these cells. In our studies, the activity of adenosine kinase in control conditions, calculated from the rate of accumulation of adenosine and inosine on inhibition with ITu, reached 181 pmol/min per 10^6 cells. It was increased threefold, to 598 pmol/min per 10^6 cells, during the first 10 minutes of ATP depletion. This indicates that the enzyme was not maximally active under basal conditions. After 10 minutes, however, the activity of adenosine kinase rapidly declined, probably because of substrate inhibition, provoking a large accumulation of adenosine. Flux toward inosine then became possible owing to the 50-fold higher K_m of adenosine for adenosine deaminase.21

Physiological Significance of the AMP/Adenosine Cycle

The magnitude of the rate of accumulation of adenosine measured in isolated cardiomyocytes under control conditions in the presence of dCF and ITu (Table) demonstrates that the dephosphorylation of AMP proceeds at a substantial rate. This indicates that the enzymes responsible for this process, the cardiac cytosolic IMP-GMP 5'-nucleotidase29 and/or the cytosolic AMPase,30 display appreciable activity under physiological conditions. This finding is somewhat surprising in view of the low affinity for AMP, with K_m in the millimolar range, that has been reported for both enzymes. In addition, the IMP-GMP 5'-nucleotidase is subjected to inhibition by the concentration of inorganic phosphate prevailing intracellularly. Because of this substantial rate of adenosine formation, utilization of adenosine becomes mandatory to avoid its accumulation to vasodilatory concentrations. For maintenance of low concentrations of adenosine, utilization by adenosine kinase will be much more efficient than by adenosine deaminase owing to the severalfold lower K_m for adenosine kinase. In addition, the marked dependence of the activity of this enzyme on a narrow range of ATP, AMP, and adenosine concentrations evidenced in our work results in rapid saturation of the cycle with adenosine. This provides a sensitive and rapid mechanism for the elevation of the concentration of adenosine under conditions of ATP depletion such as hypoxia.

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References

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