Myocardial adenosine cycling rates during normoxia and under conditions of stimulated purine release

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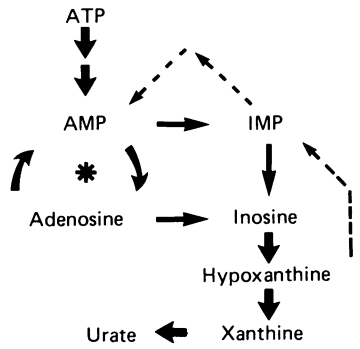
Formation and rephosphorylation of adenosine (adenosine cycling) was studied in isolated rat hearts during normoxia and under conditions of stimulated purine formation. Hearts were infused with an inhibitor of adenosine kinase (5-iodotubercidin, 2 μM). In addition, perfusions were carried out with or without acetate, which is converted into acetyl-CoA, with simultaneous breakdown of ATP to AMP and purines. We found a linear, concentration-dependent, increase in normoxic purine release by acetate (5–20 mM). Differences in total purine release with or without iodotubercidin were taken as a measure of adenosine cycling. In normoxic hearts, iodotubercidin caused a minor increase in purine release (2.7 nmol/min per g wet wt.). Acetate (12.5 mM) increased purine release by 4.9 nmol/min per g, and its combination with inhibitor gave a large increase, by 14.2 nmol/min per g. This indicates a strongly increased adenosine cycling rate during acetate infusion. However, no significant differences in purine release were observed when iodotubercidin was infused during hypoxia, anoxia or ischaemia. The hypothesis that adenosine cycling is near-maximal during normoxia was not confirmed. Increased myocardial adenosine formation appears to be regulated by the availability of AMP and not by inhibition of adenosine kinase. This enzyme mainly functions to salvage adenosine in order to prevent excessive loss of adenine nucleotides.

INTRODUCTION

Regulation of coronary blood flow by adenosine has been extensively studied (for review, see Berne, 1980). Chronotropic and dromotropic influences of adenosine on the heart have also been reported (Belardinelli *et al.*, 1980) as well as anti-catecholaminergic effects (Schrader *et al.*, 1977). Several enzymes are involved in the regulation of myocardial adenosine metabolism. Formation of adenosine can take place via 5′-nucleotidase (EC 3.1.3.5) or *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1) (Schrader *et al.*, 1981; Schütz *et al.*, 1981; Achterberg *et al.*, 1985*a*). The latter enzyme is also known to bind substantial amounts of intracellular adenosine (Ueland & Saebø, 1979). Removal of adenosine occurs by adenosine deaminase (EC 3.5.4.4) or adenosine kinase (EC 2.7.1.20) and by release and washout. One hypothesis for the regulation of adenosine production and concentration (Arch & Newsholme, 1978*a*) assumes that a substrate cycle exists between adenosine and AMP (see Scheme 1) at near-maximal activity of adenosine kinase. Kinetic studies on purified adenosine kinase from rat heart (De Jong, 1977; De Jong *et al.*, 1980; Fisher & Newsholme, 1984) suggest that the above-mentioned hypothesis is feasible. However, Newby *et al.* (1983) infused the adenosine kinase inhibitor 5-iodotubercidin into isolated rat hearts and concluded from adenosine and inosine release that adenosine cycling in normoxic rat heart is virtually absent. However, in the latter study not all adenosine catabolites were determined. The present paper reports the determination of myocardial adenosine-cycling rates during normoxia and under conditions where release of myocardial purines was stimulated, e.g. low-flow ischaemia, hypoxia, anoxia or infusion of acetate during normoxia. Our data demonstrate that in normoxia the adenosine-cycling rate is low, but can be increased by acetate infusion.



Scheme 1. Metabolic pathways involved in cycling and breakdown of adenosine in the heart

* represents adenosine cycling; broken line represents hypoxanthine salvage.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade. Water (used for h.p.l.c. buffers and perfusion media) was purified with the

Abbreviation used: 5-ITu, 5-iodotubercidin {4-amino-5-iodo-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine}.

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Milli-Ro4/Milli-Q system (Millipore Co., Bedford, MA, U.S.A.). 5-Iodotubercidin (5-ITu; NSC 113939) was obtained through the courtesy of Dr. H. B. Wood Jr. (National Cancer Institute, Bethesda, MD, U.S.A.).

Perfusion of hearts

Male Wistar rats (275–350 g), fed *ad libitum*, were used. After anaesthesia of the rats with pentobarbital (intraperitoneally), hearts were quickly excised and immediately arrested in ice-cold 0.9% NaCl or perfusion buffer, a modified Tyrode solution, containing 10 mM-glucose (Harmsen *et al.*, 1984). The Langendorff preparation was used with 100 cmH_2O perfusion pressure. Hearts were paced at 300 beats/min (4 V stimulation for 2 ms). Perfusion temperature was kept at 37.0±0.5 °C. The medium was not recirculated and was gassed with O_2/CO_2 (19:1). Hypoxia was induced by gassing the perfusion medium with $N_2/O_2/CO_2$ (13:6:1), and anoxia by gassing with N_2/CO_2 (19:1). If sodium acetate (5–20 mM) was added to the perfusate, glucose (10 mM) remained present; Na^+ was kept constant by decreasing the concentration of NaCl in the perfusion medium in accordance with the increase in sodium acetate. Ischaemia was induced by lowering perfusion pressure to 15–20 cmH_2O. At the end of the perfusion hearts were taken from the apparatus, blotted dry and weighed.

Normoxic preperfusion (15 min) preceded perfusion with modified medium (15 min) and was again followed by normoxic reperfusion (15 min).

Coronary flow

Mean coronary flow was determined by collection of perfusate in a graduated cylinder over 5 min periods. It was expressed as ml/min per g wet wt. The flow measured between 10 and 15 min preperfusion was taken as 100%.

Myocardial function

Apex displacement was used as an indicator of myocardial performance. It has been shown to correlate well with changes in myocardial pressure development during ischaemia, hypoxia and anoxia (Stam & De Jong, 1977). The displacement observed 15 min after the start of the perfusion was taken as 100%.

Assay of purines

Adenosine, inosine, hypoxanthine, xanthine and uric acid were determined by a slightly modified version of the h.p.l.c. method described by Harmsen *et al.* (1984). In brief, 200 µl of perfusate were injected on to a µBondapak C_{18} column (30 cm × 0.4 cm; Waters Associates, Milford, MA, U.S.A.) and eluted isocratically with 70 mM-KH_2PO_4, pH 4.5, to which methanol was added. Flow rate was either 1 or 3 ml/min (100 or 30 ml of methanol added to 1 litre of buffer respectively). Peak heights, detected at 254 nm, were compared with those of standards. Perfusates were collected on ice and transferred to the h.p.l.c. equipment, where temperature was kept below 5 °C.

Statistics

Results are presented as means ±S.E.M. Statistical significance was evaluated by using Student's *t* test for paired or unpaired observations, and *P* values were calculated. $P \geqslant 0.05$ was considered not significant.

RESULTS

Normoxia

Purine release from isolated normoxic rat hearts decreased gradually from about 5.5 nmol/min per g after 15 min preperfusion to 2–3 nmol/min per g at the end of the 45 min perfusion period. When the adenosine kinase inhibitor 5-ITu (2 µM) was infused, the basic purine release was increased by 2.7 nmol/min per g ($P < 0.05$). Acetate (5–20 mM) was infused because it can increase intramyocardial AMP and purine release, which amounted to 4.9 nmol/min per g at 12.5 mM-acetate. The combination of acetate and 5-ITu, however, caused a very strong increase in myocardial purine release (by 14.2 nmol/min per g), which was significantly greater ($P < 0.05$) than the combined increases that were caused by acetate and 5-ITu alone. This reflects increased adenosine kinase activity (adenosine cycling) during acetate infusion. In Fig. 1 the total purine release over the entire perfusion period is given for control hearts and hearts infused with acetate, 5-ITu, or the combination. The contribution of adenosine, inosine, hypoxanthine, xanthine and uric acid to the total release in these experiments is shown in Fig. 2. Uric acid plus xanthine contribute 60–80% to the total release and therefore also to observed increases in release. Acetate infusion (5–20 mM) gave a linear, concentration-dependent, increase in normoxic purine release (by 7 nmol/min per g at 20 mM-acetate). All increases in purine release were computed for individual hearts by subtracting the average of pre- and post-experimental control release from the release during the last 5 min of experimental infusion.

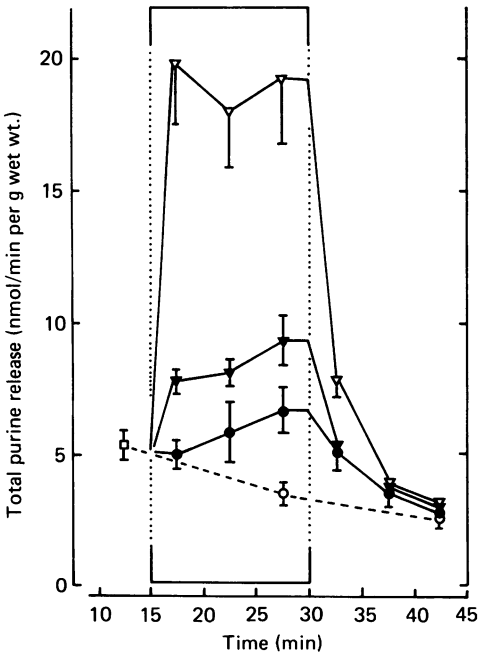

Fig. 1. Total purine release from isolated rat hearts perfused with iodotubercidin, acetate or both

Purine release (nmol/min per g wet wt.) is the average of total release over 5 min periods. From 15 to 30 min, acetate, iodotubercidin or both were infused. ○, Controls; □, combined controls; ▼, 12.5 mM-acetate; ●, 5-ITu (2 µM); ▽, acetate plus 5-ITu. Vertical bars indicate S.E.M. for four to six experiments.

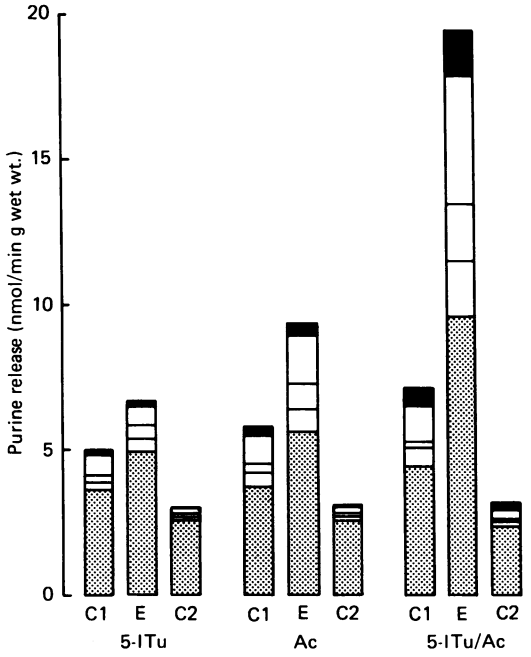

Fig. 2. Composition of total purine release during perfusion with iodotubercidin, acetate or both

Release of uric acid, xanthine, hypoxanthine, inosine and adenosine (subdivision of bars from bottom upwards) is given at the end of preperfusion (C1), at the end of reperfusion (C2) and during the last 5 min of infusion of acetate (Ac), 5-ITu or the combination (E) (see also the legend to Fig. 1 and the Materials and methods section).

Acetate caused a concentration-dependent increase in coronary flow, ranging from 130 to 165% of control flow. Infusion of 5-ITu gave a very strong vasodilation, both in the presence of acetate (220% of control flow; $P < 0.05$) and when given alone (240% of controls; $P < 0.05$). Apex displacement, which was used as an index of myocardial contractility, was not significantly changed by infusion of acetate with or without 5-ITu.

Hypoxia, anoxia, ischaemia

Perfusion of hearts with medium gassed with $O_2/N_2/CO_2$ (6:13:1) (hypoxia) caused an increase in normoxic purine release (to 9 nmol/min per g). No significant differences in purine release were observed when 5-ITu (2 μM) was present during hypoxia (Fig. 3*a*). Coronary flow increased to 130–140% of control values ($P < 0.05$) and contractility decreased to 85% of controls $P < 0.05$).

When hearts were made anoxic (N_2/CO_2, 19:1), a very strong increase in purine release was observed (to 72 nmol/min per g). The presence of 5-ITu gave a non-significant decrease in purine release as compared with anoxia alone (to 61 nmol/min per g) (Fig. 3*c*). The only observable effect of 5-ITu on anoxic purine release is the fact that the relative contribution of adenosine to total purine release is higher (28 versus 22% of total; $P < 0.05$) when 5-ITu is infused.

Anoxia caused a significant increase in coronary flow to 150% of controls ($P < 0.05$) and a strong decline in apex displacement (to 15%; $P < 0.05$) at the end of anoxia. Reoxygenation (15 min) gave a 70% return of contractility. No differences in flow or contractility were

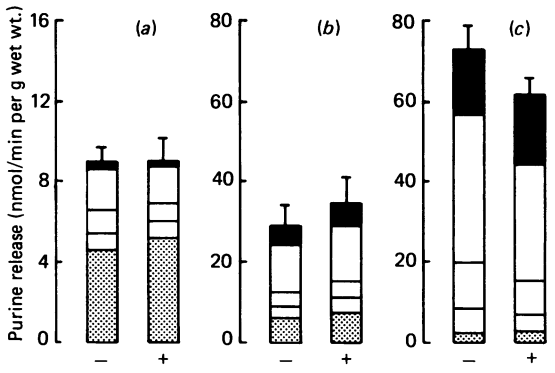

Fig. 3. Influence of iodotubercidin on myocardial purine release during hypoxia (*a*), ischaemia (*b*) and anoxia (*c*)

Total purine release during the last 5 min of hypoxic (30% O_2), ischaemic or anoxic (0% O_2) perfusion was determined in the presence (+) or absence (−) of 5-ITu (2 μM). Subdivisions of bars represent (from bottom up): uric acid, xanthine, hypoxanthine, inosine, adenosine. Vertical bars indicate S.E.M. for three to six experiments. Note the difference in scale between hypoxia and the others.

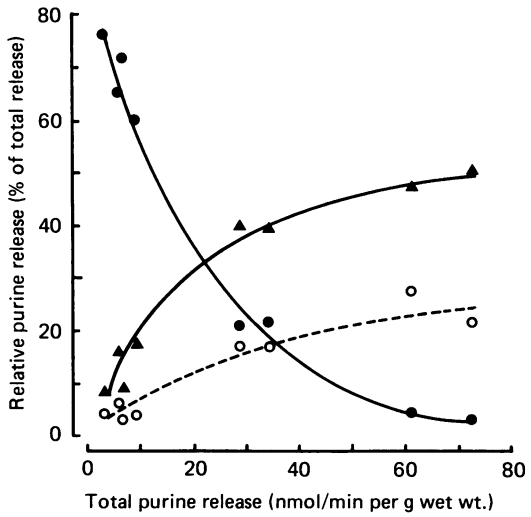

Fig. 4. Contribution of uric acid, inosine and adenosine to total purine release under various conditions

The release of uric acid (●), inosine (▲) and adenosine (○) is plotted as a percentage of total purine release under conditions of varying purine release (i.e. normoxia, hypoxia, anoxia, ischaemia). Each point is the average of three to six experiments.

observed in the presence of 5-ITu during anoxia or hypoxia.

Decreasing the perfusion pressure to 20–25% of control resulted in a strongly decreased coronary flow (to 6–8% of control) and a purine release of 30 nmol/min per g. Infusion of 5-ITu caused a non-significant increase in ischaemic purine release, to 35 nmol/min per g (Fig. 3*b*). In both groups apex displacement declined to 22.4 ± 13.4% of controls ($P < 0.05$). Apex displacement tended to be higher (non-significantly) when 5-ITu was infused during anoxia and ischaemia.

The relative contribution of the various purines to total purine release changed gradually when the total purine

release was increased (Fig. 4). The contribution of uric acid decreased from 70% (normoxic release) to less than 5% (anoxia) and the contribution of inosine increased from 10 to 50% (both $P < 0.05$). The relative release of adenosine increased gradually from less than 5 to about 25% of total purine release ($P < 0.05$).

DISCUSSION

Newby *et al.* (1983) reported that isolated normoxic rat hearts perfused with the adenosine kinase inhibitor 5-ITu do not release increased amounts of adenosine and inosine. It was hypothesized, however (Arch & Newsholme, 1978*a*; Fisher & Newsholme, 1984), that basic adenosine cycling takes place in the heart at near-maximal adenosine kinase activity. This activity is reported to be in the range of 100–200 nmol/min per g wet wt. in rat heart homogenates (Arch & Newsholme, 1978*b*) and to be about 30–40 nmol/min per g when adenosine is infused into isolated rat hearts (Aussedat *et al.*, 1984; Newby *et al.*, 1983). It was therefore expected that inhibition of adenosine kinase during normoxia should lead to highly increased formation and release of adenosine and its catabolites. However, because of very rapid breakdown of adenosine, a major part of purines that are released from adult normoxic rat hearts consists of uric acid and xanthine (Ronca-Testoni & Borghini, 1982; Schoutsen *et al.*, 1983; Achterberg *et al.*, 1985*a*,*b*). The reported absence of an effect of 5-ITu on release of adenosine and inosine might therefore be explained by assuming that the expected increase would be expressed mainly in the release of xanthine and uric acid. In our hands, infusion of 5-ITu (2 μM) into normoxic rat hearts indeed gave a small but significant increase (2.7 nmol/min per g wet wt.) in total purine release, of which 60–80% could be accounted for by xanthine and uric acid. The efficacy of 5-ITu as inhibitor of adenosine kinase in this experimental set-up is essential. It was shown (Newby *et al.*, 1983) that 5-ITu (1 μM) inhibits the incorporation of [^{14}C]adenosine (10 μM) into the myocardial adenine nucleotide pool by more than 90% and also that 5-ITu accumulated intracellularly. It still remained to be demonstrated that 5-ITu infusion could indeed be used to measure adenosine cycling, for instance when cycling in normoxic hearts takes place at a higher rate. With this in mind, acetate (5–20 mM) was infused into normoxic hearts, and a concentration-dependent increase in purine release was found. Acetate is readily taken up by the heart (Taegtmeyer *et al.*, 1980) and transformed into acetyl-CoA. In the course of this reaction, AMP is formed from ATP. Elevation of intracellular AMP content by acetate has been described for isolated rat heart (Williamson, 1965) and for dog muscle *in vivo* (Liang & Lowenstein, 1978). The latter authors also report elevation of coronary-sinus purine release and coronary vasodilation during acetate infusion. The hypothesis that adenosine-cycling rate is normally high also predicts a decrease in cycling rate when adenosine formation is increased. However, simultaneous infusion of acetate and 5-ITu gave a much greater increase in purine release (14.2 nmol/min per g) than the sum of increases caused by acetate (4.9 nmol/min per g) and 5-ITu (2.7 nmol/min per g) alone. This clearly demonstrates increased adenosine-cycling activity in the presence of acetate. In addition, these results show that infusion of 5-ITu is useful to determine adenosine-cycling rates in isolated perfused rat hearts. No significant cycling rates could be detected, however, during hypoxia, anoxia or low-flow ischaemia. Relatively high adenosine-cycling rates (20 nmol/min per g of liver) have been reported (Bontemps *et al.*, 1983) in cultured rat hepatocytes during incubation with 5-ITu. However, these cultures continually release substantial amounts of purines. Intercellular adenosine exchange, which is not the same as adenosine cycling, can take place more easily in cell cultures than in the perfused heart, because in the latter system any adenosine formed will be more rapidly washed out. Newby *et al.* (1983) reported that incorporation of infused adenosine into isolated perfused rat heart continued at a high rate (33 nmol/min per g) during severe hypoxia. This suggests that adenosine kinase is not inhibited and that cycling could take place, if adenosine is available at the site of adenosine kinase activity. These results and ours could be explained by assuming that adenosine formation takes place at a site distant from adenosine kinase or that a major part of purine formation takes place via AMP → IMP → inosine, thereby bypassing adenosine formation (Achterberg *et al.*, 1985*a*,*b*). Evidence has been presented (Rovetto & Williams, 1983) that myocardial adenosine formation from AMP is a transmembrane process by which adenosine is formed and released at an extracellular site.

Both the maximal incorporation rates of infused adenosine (Namm, 1973; Reibel & Rovetto, 1979; Newby *et al.*, 1983; Aussedat *et al.*, 1984) and the cycling rates that we found in this study are at least 2-fold lower than the maximal adenosine kinase activities that were reported *in vitro* (Arch & Newsholme, 1978*b*; Newby *et al.*, 1983). This points either to a strong inhibition of intracellular adenosine kinase (but see Newby *et al.*, 1983) or to a rate-limiting effect of adenosine transport in incorporation studies (Bowditch *et al.*, 1985).

Significant effects of adenosine on coronary flow are observed at less than 0.1 μM-adenosine in the perfusion fluid (Schrader *et al.*, 1977). It can be computed that an increase in adenosine formation of less than 2 nmol/min per g wet wt. will be sufficient for significant coronary vasodilation. A minor shift in the adenylate kinase equilibrium (Newsholme & Start, 1973) can probably cause the increase in AMP that is needed for such a low adenosine production. During acetate infusion and hypoxia a good correlation is found between total purine release and coronary flow (results not shown). The fact that no increases in adenosine concentrations were found can be explained by the rapid breakdown of adenosine to uric acid (Achterberg *et al.*, 1985*b*).

The acetate-induced increase in purine release and coronary vasidilation that we observed is presumably adenosine-mediated and similar to the reported coronary vasodilation caused by fatty acids (Hülsmann, 1976). Acetate (20 mM) caused a 65% increase in coronary flow, but a 3–4-fold increase in purine release, which indicates that increased washout is not the cause of increased purine release.

The relative contributions of the various purines to total purine release (Fig. 4) again emphasizes the importance of measuring all catabolites in these kinds of studies.

Re-incorporation of hypoxanthine could theoretically cause an underestimation of total purine formation (see Scheme 1). However, Harmsen *et al.* (1984) have shown that the rate of hypoxanthine salvage is at least one order

of magnitude lower than normoxic purine release, even when this salvage is stimulated by infusion of ribose or after ischaemia.

It appears that the main importance of adenosine kinase is to salvage the myocardial adenine nucleotide pool (Newby, 1985). Our experiments with 5-ITu show that half of the amount of purines produced can be salvaged through adenosine kinase at low and mildly increased rates of purine production. This allows a maximal cellular signal (adenosine concentration) with a minimal waste of ATP. Our final conclusion is that adenosine production, insofar as it is relevant to coronary vasodilation, is regulated not by inhibition of adenosine kinase, but most probably by the intracellular AMP concentration. Adenosine kinase appears mainly to be involved in salvage of the myocardial ATP pool, for example during increased adenosine production caused by high rates of fatty acid activation.

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