The mechanism of adenosine production in rat polymorphonuclear leucocytes

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Adenosine production in intact rat polymorphonuclear leucocytes was studied during 2-deoxyglucose-induced ATP catabolism. A cell-free system containing the cytosolic 5'-nucleotidase (EC 3.1.3.5) as the only phosphohydrolase was also studied. The rate of adenosine formation in both intact cells and the cell-free system showed a similar dependence on energy charge $\{([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])\}$, being maximal only at values close to 0.8. Sufficient cytosolic 5'-nucleotidase was present in intact cells to explain the observed rate of adenosine formation. We conclude that the cytosolic 5'-nucleotidase is responsible for adenosine production in rat polymorphonuclear leucocytes. This mechanism provides a direct biochemical link between the energy status of a cell and the rate of adenosine formation.

The role of adenosine as a physiological regulator (Arch & Newsholme, 1978; Fox & Kelley, 1978) was first recognized in relation to the phenomenon of coronary blood-flow autoregulation (Berne, 1963). Berne (1963, 1964) suggested that an excess of ATP hydrolysis over ATP synthesis caused either by an increase in work-load or a decrease in oxygen partial pressure resulted in adenosine formation. This took place in those cells experiencing the energy imbalance, i.e. the myocardial cells. Adenosine so formed could then diffuse to the coronary resistance vessels and exert its already documented vasodilatory action (Drury & Szent-Gyorgyi, 1929). The increased blood flow would then, by increasing oxygen partial pressure, restore the ATP concentration. Biochemically, the simplest formulation of the adenosine hypothesis proposes a direct metabolic link between ATP catabolism and adenosine formation (Scheme 1). It remains to be demonstrated that such a direct link does exist, to characterize the enzymes involved and to explain the regulation of the proposed pathway.

Abbreviations used: 2'-deoxycyformycin, *R*-3-(2-deoxy- β -D-erythro-pentofuranosyl)-3,6,7,8-tetrahydro[4,5-*d*][1,3]diazapin-8-ol; 5-iodotubercidin, 4-amino-5-iodo-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine; h.p.l.c., high-pressure liquid chromatography.

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The most abundant cellular 5'-nucleotidase has been shown to be an ecto-enzyme (for reviews see Evans, 1978; Stanley *et al.*, 1982), and evidence against its participation in intracellular adenosine formation has been obtained by using intact polymorphonuclear leucocytes (Newby, 1980; Newby & Holmquist, 1981) and the isolated perfused heart (Frick & Lowenstein, 1976; Schütz *et al.*, 1981). A cytosolic 5'-nucleotidase was detected in polymorphonuclear leucocytes (Newby, 1980), although its relation to the extensively characterized enzyme from the liver (Itoh *et al.*, 1967; Fritzson, 1969; Van den Berghe *et al.*, 1977; Itoh, 1981*a,b*) was not established. The present studies aimed to test whether the magnitude and changes in the rate of adenosine formation in intact polymorphonuclear leucocytes could be accounted for by the properties of the cytoplasmic 5'-nucleotidase.

Materials and methods

Preparation and incubation of intact polymorphonuclear leucocytes

Polymorphonuclear leucocytes were prepared and metabolite concentrations were measured by h.p.l.c. as described previously (Newby, 1980; Newby *et al.*, 1983).

Intracellular space were determined at 37°C as described by Newby (1980), with the following modification. Cells were incubated for 7 or 14 min with 3-*O*-methyl-D-[1-³H]glucose (37–40 kBq/ml; 74 GBq/mmol; final concn. 50 μ M) in combination

Scheme 1. *Proposed metabolic pathway of adenosine formation*

x is the kinase substrate.

with inulin- $[^{14}C]$ carboxylic acid (5.7 MBq/mmol, final concn. 65 μM). Radioactive compounds were obtained from Amersham International, Amersham, Bucks., U.K.

Measurement of cytosolic 5'-nucleotidase activity

Partially purified rat liver cytosolic 5'-nucleotidase (0.75 unit/mg of protein) was prepared by the method of Itoh (1981a) as described previously (Worku & Newby, 1982). Enzyme assays were conducted at 37°C for 5–15 min in 0.1 ml containing 100 mM-imidazole/HCl, pH 6.5, 75 mM-KCl, 50 mM-NaCl, 1 mM-mercaptoethanol and 0.1% bovine serum albumin. Combinations of ATP, ADP, AMP and IMP were added from concentrated solutions made in assay buffer to final concentrations derived from measurements of nucleotide concentrations inside intact cells. P_i was added to a final concentration of 1.3 mM. $MgCl_2$ was added to give a free concentration of Mg^{2+} of 1.0 mM. The total concentration of Mg^{2+} to be added was calculated as described by Veloso *et al.* (1973), by using pK_a values and stability constants for magnesium complexes of ATP^{4-} , $HATP^{3-}$, ADP^{3-} , AMP^{2-} and $H_2PO_4^-$ determined in 0.1 M-KCl at 25°C tabulated in Sillen & Martell (1971). The stability constant for Mg-IMP was assumed to be the same as for Mg-AMP. The assay medium was supplemented with 2–3 kBq of either $[2-^3H]IMP$ (231 GBq/mmol) or $[2-^3H]AMP$ (714 GBq/mmol). The formation of $[^3H]$ inosine was assessed by ion-exchange chromatography as described previously (Worku & Newby, 1982), except that a 0.5 ml bed volume of resin was used. $[^3H]$ Adenosine formation was assessed similarly, except that the ion-exchange resin used was AG2-X8 (Bio-Rad) and the extraction and elution steps were done with 5 mM-adenosine.

Preparation of and measurement of 5'-nucleotidase in the soluble fraction of polymorphonuclear leucocytes and rat liver

Polymorphonuclear leucocytes (0.2–0.4 g wet wt. of packed cells) or rat liver (1 g wet wt.) were mixed with 3 vol. of 50 mM-Tris/HCl (pH 7.4)/1 mM-sodium EDTA/10 mM-mercaptoethanol and homogenized (Newby, 1980; Pilgis *et al.*, 1974). Homo-

genates were centrifuged at 100 000 g for 2 h at 4°C. Supernatant (10 μl) was diluted with 90 μl of 100 mM-sodium β -glycerophosphate (pH 6.5)/20 mM- $MgCl_2$ /5 mM-ATP/1 mM-mercaptoethanol/3 mM-IMP containing of 2–3 kBq of $[2-^3H]IMP$ and the mixture was incubated for 15 min at 37°C. $[^3H]$ Inosine production was determined as described above, except that the extraction and elution steps were conducted with water alone. Nucleoside exchange was determined as described previously (Worku & Newby, 1982).

Results

Experiments with intact polymorphonuclear leucocytes

The change in concentration of ATP and its metabolites was measured as a time course after poisoning the cells with a mixture 5 mM-2-deoxy-glucose and 0.5 mM-glucose (Lomax & Henderson, 1973). The pattern of metabolite concentration changes observed after this treatment is shown in Fig. 1. The initial rapid fall in ATP concentration (2.8 ± 0.5 nmol/min per 10^7 cells) was accompanied by increases in the concentrations of ADP, AMP and IMP. Whereas the concentrations of ADP and IMP subsequently decreased (1–9 min), the AMP concentration remained elevated throughout the 20 min of incubation. The IMP concentration increased again from 9 to 20 min, perhaps as a result of synthesis *de novo*, since the sum of the metabolites increased from 9 to 12 nmol/ 10^7 cells (see Newby & Holmquist, 1981).

The instantaneous rate of production of adenosine and hypoxanthine was estimated from their concentrations at adjacent time points. Adenosine concentration rose without an apparent lag, at an initial rate of 62 ± 14 pmol/min per 10^7 cells ($n = 5$). This rate subsequently declined, despite the continuously elevated concentration of AMP. Hypoxanthine concentrations rose after an initial lag, at a rate of 0.7 ± 0.1 nmol/min per 10^7 cells. The rate of hypoxanthine formation also declined subsequently, despite the continued availability of IMP. Thus the rate of formation of neither adenosine nor hypoxanthine varied as a smooth function of its substrate concentration. This phenomenon has been pre-

Fig. 1. Time course of ATP catabolism and of adenosine and hypoxanthine formation in polymorphonuclear leucocytes

Polymorphonuclear leucocytes (1.7×10^7 – 6.5×10^7 cells/ml) were incubated with $30 \mu\text{M}$ -2'-deoxycoformycin, $1 \mu\text{M}$ -5-iodotubercidin and $10 \mu\text{M}$ -allopurinol for 30 min at 37°C . Then (5 mM)-2-deoxyglucose and 0.5 mM-D-glucose were added, and incubation was continued for the times shown. Incubations were terminated, and then ATP (●), ADP (▲), AMP (□), IMP (○), adenosine (Δ) and hypoxanthine (■) concentrations were determined by h.p.l.c. as described in the Materials and methods section. Values are means for five experiments.

viously noted for several enzymes involved in ATP homeostasis (Atkinson, 1968; Chapman & Atkinson, 1973; Chapman *et al.*, 1971, 1976; Schramm & Leung, 1973), including the rat liver cytosolic 5'-nucleotidase (Itoh, 1981b). When the rate of formation of adenosine or hypoxanthine was plotted as a function of energy charge, $([\text{ATP}] + \frac{1}{2}[\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$ (Atkinson, 1968), smooth biphasic relationships were observed (Figs. 2a and 2c). Adenosine formation was maximal at an energy charge value of approx. 0.85, and hypoxanthine formation at a slightly lower value of approx. 0.7.

Characterization of the cytoplasmic 5'-nucleotidase from polymorphonuclear leucocytes and comparison with the enzyme from rat liver

Rat polymorphonuclear leucocytes contained a 5'-nucleotidase which was present in the supernatant after centrifugation at $100\,000g$ for 2 h. The enzyme activity measured under optimal conditions with 3 mM-IMP as substrate was 5.8 ± 0.3 nmol/min per 10^7 cells ($n = 12$). This activity was not inhibited by antiserum raised against rat liver ecto-5'-nucleotidase when used at a dilution (1/100, v/v) which completely inhibited the ecto-enzyme from polymorphonuclear leucocytes (Newby, 1980). It was also insensitive to inhibition by 0.5 mM-adenosine 5'-[α,β -methylene]diphosphate, another ecto-enzyme inhibitor (Evans & Gurd, 1973; Newby, 1980).

The 5'-nucleotidase in the soluble fraction from rat liver showed the same inhibitor specificity. The enzymes in rat liver and polymorphonuclear leucocyte supernatants had a similar K_m for IMP, 0.19 mM and 0.21 mM respectively ($n = 3$). When subjected to electrophoresis at pH 9.6 as described by Bailyes *et al.* (1982), both enzymes had the same electrophoretic mobility, $R_F = 0.3$ in a 10%-acrylamide gel. Moreover, both enzymes catalysed exchange of the nucleoside moiety between [^3H]-inosine and IMP (Worku & Newby, 1982). The rate of nucleoside exchange with 6 mM-inosine and 3 mM-IMP was approx. 50% of the 5'-nucleotidase activity on IMP in each case. We concluded that the same enzyme was present in both cell types. Since much larger quantities of enzyme were available from rat liver, the enzyme partially purified from this source was used in cell-free experiments.

Studies with the rat liver cytosolic 5'-nucleotidase in a cell-free system

An important control for the intact-cell studies was to test the effect on the enzyme of the inhibitors used. The adenosine kinase inhibitors 5-iodotubercidin ($100 \mu\text{M}$) and 5'-amino-5'-deoxy-adenosine (1 mM), the xanthine oxidase inhibitor allopurinol ($100 \mu\text{M}$) and the adenosine deaminase inhibitor 2'-deoxycoformycin (1 mM) were without

Fig. 2. Variation in the rates of formation of adenosine and hypoxanthine, and of 5'-nucleotidase activity on AMP and IMP, with energy charge

(a) The data in Fig. 1 were used to replot the rate of adenosine formation as a function of energy charge (●). Nucleotide concentrations from Fig. 1 were also used to determine 5'-nucleotidase activity on AMP in a cell-free system containing the rat liver cytosolic 5'-nucleotidase (○). Further details are given in the Materials and methods section. (b) The rate of adenosine formation (●) and 5'-nucleotidase activity on AMP (○) were determined as in (a) by using data obtained from a single representative experiment. (c) The data in Fig. 1 were used to replot the rate of hypoxanthine formation as a function of energy charge (●) and to determine 5'-nucleotidase activity on IMP in a cell-free system (○). (d) The rate of hypoxanthine formation (●) and 5'-nucleotidase activity on IMP (○) were determined as in (c) by using data from a single representative experiment. Measurements of metabolite concentrations in intact polymorphonuclear leucocytes were made in triplicate for either five experiments or a single experiment as shown. Enzyme activity was determined with 2–3 munits of enzyme in triplicate in three separate experiments. To normalize the data, enzyme activities obtained were adjusted to correspond to the amount of enzyme present in 10^7 polymorphonuclear leucocytes. Values are means ($\pm$ s.e.m. where appropriate).

effect on the enzyme activity determined with 3 mM-IMP.

Plots of activity versus substrate concentration were obtained for the enzyme at its pH optimum (i.e. pH 6.5; Itoh, 1981a) and under conditions of maximal activation by MgATP (5 mM-ATP, 20 mM-MgCl₂). The K_m for IMP was 0.19 mM and for AMP 2.6 mM. The concentrations of AMP and IMP inside intact polymorphonuclear leucocytes at the time points where formation of adenosine or hypoxanthine was maximal were taken from the data in

Fig. 1. To convert these values from nmol/ 10^7 cells into mM, the cytoplasmic volume ($1.25 \pm 0.07 \mu\text{L}/10^7$ cells, $n = 5$) was determined by using 3-O-methylglucose, a non-metabolizable analogue of glucose (Kletzien *et al.*, 1975). The relevant absolute concentration of AMP was 0.46 mM and the corresponding value of 5'-nucleotidase activity on AMP from the activity-versus-substrate-concentration plot was 580 pmol/min per 10^7 cells. This was easily enough to explain the observed rate of adenosine formation of 62 pmol/min per 10^7 cells. The relevant

absolute concentration of IMP was 1.81 mM, which corresponded to a 5'-nucleotidase activity on IMP of 5.7 nmol/min per 10^7 cells. Again this was easily enough to explain the observed rate of hypoxanthine formation of 0.7 nmol/min per 10^7 cells.

We next attempted to simulate the variation in the rate of adenosine formation seen in intact cells. Rat liver cytosolic 5'-nucleotidase was incubated with ATP, ADP, AMP and IMP at concentrations derived from the data in Fig. 1. Either [^3H]AMP or [^3H]IMP was included in the medium and the initial rate of [^3H]nucleoside formation was measured. The data obtained were plotted as a function of energy charge so as to compare them with the rates calculated for the intact-cell experiments.

The 5'-nucleotidase activities on AMP determined in the cell-free system by using the mean metabolite concentrations obtained from five experiments with intact cells (Fig. 1) are plotted in Fig. 2(a). The maximum activity observed occurred at an energy-charge value of 0.78, which was in the range of values giving maximal rates of adenosine formation in intact cells. At energy-charge values below 0.78 the activity fell to a plateau value similar to that seen in intact cells. The maximum activity observed in the cell-free system was within 1 S.E.M. of the mean obtained in the intact cell experiments. Since the between-experiments variation in estimates of metabolites was much greater than that within a single experiment, we also modelled the experiment from those pooled in Fig. 2(a) which gave the highest rate of adenosine synthesis. Fig. 2(b) illustrates that the correspondence between the cell-free model and the intact-cell measurements was improved. The maximal 5'-nucleotidase activity on AMP was obtained in each system at an energy charge close to 0.8. The maximal rate of adenosine formation obtained in the two systems was indistinguishable (Fig. 2b).

The cell-free model was less successful in predicting the rate of hypoxanthine formation in intact polymorphonuclear leucocytes. Both the experiment based on the pooled data in Fig. 1 and that based on the representative experiment showed a peak of 5'-nucleotidase activity on IMP at an energy-charge value of approx. 0.73 (Figs. 2c and 2d), which was close to that giving maximal rates of hypoxanthine formation in intact cells. The 5'-nucleotidase activity on IMP was decreased in a similar proportion to the rate of hypoxanthine formation at lower energy-charge values. The cell-free model, however, predicted approx. 8 times as much 5'-nucleotidase activity on IMP as the observed rate of hypoxanthine formation at all energy-charge values. The reason for this discrepancy is unclear.

Discussion

Sufficient cytosolic 5'-nucleotidase was present inside rat polymorphonuclear leucocytes to explain

the rates of AMP dephosphorylation observed. There was also a striking similarity between the variations in the rate of adenosine formation in intact cells and in the cell-free system containing the cytosolic 5'-nucleotidase as the only phosphohydrolase. These data argue strongly that the cytosolic 5'-nucleotidase is responsible for adenosine formation in this cell type. Sufficient enzyme was also present to explain the observed rates of IMP dephosphorylation.

In the representative experiment shown in Fig. 2(b), a 32-fold increase in the rate of adenosine formation by intact cells was produced by only an 8-fold increase in AMP concentration. This suggests that allosteric factors (Van den Berghe *et al.*, 1977; Itoh, 1981a,b, 1982) were partly responsible for this activation. The subsequent inhibition of the enzyme took place without a fall in AMP concentration, again implicating allosteric regulation.

AMP hydrolysis catalysed by the cytosolic 5'-nucleotidase would result in the formation of adenosine intracellularly. This appears to occur in polymorphonuclear leucocytes (Newby & Holmquist, 1981) and in the heart (Frick & Lowenstein, 1976; Schütz *et al.*, 1981). Rat heart supernatants after centrifugation at 100000g for 1 h also contained a cytosolic 5'-nucleotidase which catalysed nucleoside exchange, showed the same inhibitor specificity and K_m for IMP and displayed the same electrophoretic mobility at pH 9.6 as did the enzymes from rat liver and polymorphonuclear leucocytes. The activity of the enzyme was 409 ± 22 nmol/min per g wet wt. ($n = 4$).

ATP catabolism necessarily results in a more-than-proportional increase in AMP concentration, owing to the myokinase equilibrium (Newsholme & Start, 1973). Owing to its high K_m for AMP, the cytosolic 5'-nucleotidase would respond to this increase with a proportional rise in the rate of adenosine production and may be further activated by allosteric regulation. Thus the mechanism proposed here not only provides a direct biochemical link between ATP breakdown and adenosine formation but also suggests how this may be regulated in response to changes in the energy status of the cell.

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