The rate of the AMP/adenosine substrate cycle in concanavalin-A-stimulated rat lymphocytes

Zsusa SZONDY and Eric A. NEWSHOLME

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

The effect of adenosine on the metabolism of prelabelled adenine nucleotides was investigated in concanavalin-A-stimulated rat lymphocytes. Adenosine in the presence of the adenosine deaminase inhibitor, deoxycoformycin, caused a 2-fold increase in the ATP concentration. This effect was, in part, counteracted by an increased rate of adenine nucleotide catabolism, which could be explained by a stimulation of AMP deaminase (EC 3.5.4.6). At the same time a continuous rate of labelled adenosine production was found, which was not affected by the increased ATP concentration and which could only be detected by the trapping effect of a high concentration of added unlabelled adenosine. It is concluded that the rate of the substrate cycle between AMP and adenosine is low (1.9 ± 0.2 nmol/h per 10^7 cells) in comparison to the rate of AMP deamination.

INTRODUCTION

Adenosine deaminase (EC 3.5.4.4) is an ubiquitous enzyme in mammalian tissues [1]. It catalyses, *in vitro*, the deamination of both adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine respectively [2]. Despite similar catalytic properties of adenosine deaminase towards adenosine and deoxyadenosine, data from adenosine deaminase-deficient patients suggest that deoxyadenosine rather than adenosine is the main substrate for this enzyme *in vivo* [3–5].

It is considered that in human T-lymphoblastoid cell lines distinct catabolic pathways exist for adenine ribonucleotide and deoxyribonucleotide catabolism. dATP is first dephosphorylated via dADP to dAMP. Subsequently, dAMP is not deaminated, but is exclusively

dephosphorylated to yield deoxyadenosine. Deoxyadenosine is then deaminated by adenosine deaminase. The initial steps of adenine ribonucleotide catabolism are similar to those of deoxyribonucleotide catabolism, but AMP is deaminated to IMP rather than dephosphorylated to adenosine (Schemes 1 and 2). The evidence for this is that very little adenosine is formed by these cells even in the presence of an adenosine deaminase inhibitor [6].

It is not, however, clear from such experiments whether the low rate of adenosine formation in lymphoid cells is due to a low rate of AMP dephosphorylation via 5'-nucleotidase, or whether the net rate of adenosine formation is low due to the phosphorylation of newly formed adenosine by adenosine kinase (EC 2.7.1.20). If the latter explanation is correct, the rate of substrate

Scheme 1. Reactions of deoxyribonucleotide catabolism

Enzymes: 1, 5'-deoxyribonucleotidase; 2, deoxyadenosine (adenosine) deaminase; 3, purine nucleotide phosphorylase.

Scheme 2. Reactions of ribonucleotide catabolism

Enzymes: 1, AMP deaminase; 2, GMP reductive deaminase; 3, 5'-nucleotidase; 4, purine nucleotide phosphorylase.

cycling between AMP and adenosine could be high. The purpose of the present study was therefore to assess the fluxes through the reactions catalysed by adenosine kinase, 5'-nucleotidase and AMP deaminase in stimulated mesenteric lymphocytes of the rat.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (300–350 g) were obtained from Bantin and Kingman, Grimston, Hull, U.K.

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London), Lewes, Sussex, U.K., except for the following: concanavalin A, bovine serum albumin and poly(ethyleneimine)-cellulose sheets were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K., cellulose thin-layer sheets were obtained from Eastman Kodak Co., Kirkby, Liverpool, U.K., the scintillant and inorganic compounds were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K., and [^{14}C]adenine (sp. radioactivity 100 $\mu\text{Ci}/\mu\text{mol}$) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Deoxycoformycin was kindly given by Dr. J. R. S. Arch, Beecham Pharmaceutical Research Division, Epsom, Surrey.

Preparation of lymphocytes

Lymphocytes were prepared from rat mesenteric lymph nodes as described by Ardawi & Newsholme [7]. Cells were suspended in phosphate-buffered saline [8] oxygenated for 30 min under 100% O_2 .

Incubation procedures

Incubations were performed at 37 °C in 10 ml silicone-treated Erlenmeyer flasks in a total volume of 1 ml of phosphate-buffered saline [8] oxygenated previously and supplemented with 1% (w/v) defatted bovine serum albumin at pH 7.2: each incubation contained 5×10^7 cells and the incubation time was 2.5 h under 100% O_2 . The final concentration of phosphate in the incubation medium was approx 2.2 mM. The incubation medium also contained (final concns.) 5 mM-glucose, 30 μg of concanavalin A/ml and 50 μM -deoxycoformycin. The deoxycoformycin concentration was chosen as the lowest concentration which completely inhibits adenosine deaminase. Preliminary experiments were carried out under conditions identical with those described above to determine the concentration of deoxycoformycin required to inhibit the deaminase. Lymphocytes ($5 \times 10^7/\text{ml}$) were preincubated in the presence of 30 μg of concanavalin A/ml and various concentrations of deoxycoformycin for 2 h. Then 500 μM -[^3H]adenosine (8×10^5 c.p.m./nmol) was given. The reaction was stopped with ice-cold 25% (v/v) HClO_4 at 5, 10 or 15 min after adenosine addition. The pellet was removed by centrifugation for 2 min in an Eppendorf centrifuge, and the supernatant was neutralized for further analysis. Adenosine deaminase activity was estimated from the sum of radioactivity in inosine and hypoxanthine. Inosine and hypoxanthine were separated by the two-dimensional cellulose chromatography which was used for the separation of adenosine. The concentration of deoxyco-

formycin that produced 50% inhibition was 6 μM , and 25 μM produced 95% inhibition.

After preincubation of the cells for 1 h, 5 μCi of [^{14}C]adenine was added to the incubation medium in order to label the adenine nucleotide pools. After a further 1 h, 500 μM -adenosine was added to the incubation medium, and the rate of adenosine uptake, the rate of [^{14}C]adenosine production and the adenine nucleotide concentrations were determined at different time points after adenosine addition, as described below. [^{14}C]Adenine was present throughout the whole experiment. The rate of adenine uptake was constant during this period, and addition of adenosine had no effect. However, the rate of adenine uptake was low (0.58 nmol/h per 10^7 cells), especially compared with that of adenosine (61 ± 0.3 nmol/h per 10^7 cells) or with that of ATP production. Hence this rate does not interfere in the calculation of the rate of AMP deamination.

Determination of adenosine uptake and the radioactivity incorporated in AMP and adenosine

At 5, 10, 15 and 30 min after adenosine addition, 200 μl of ice-cold 25% (v/v) HClO_4 was added to a 1 ml sample of incubation mixture. Precipitated protein was removed by centrifugation. The supernatant was then neutralized with KOH and used for further analysis. The adenosine concentration was determined enzymically [9].

Purine ribonucleotides were separated by one-dimensional t.l.c. on poly(ethyleneimine)-cellulose sheets as described by Crabtree & Henderson [10]. Briefly, a wick of Whatman 3MM paper was taped to the sheet, and sheets were first developed overnight with 4 M-sodium formate buffer, pH 3.4, dried and then developed with methanol/water (1:1, v/v). After drying, 20 μl of neutralized medium and 30 nmol of each purine nucleotide as carrier were applied to the t.l.c. sheet which was developed overnight with methanol/water (1:1, v/v) to wash out purine bases and nucleosides via the wick. For separation of the ribonucleotides, the t.l.c. sheets were then developed with increasing concentrations of sodium formate, pH 3.4, as follows: initially 0.5 M-formate was applied 2 cm above the origin, then 2.0 M-formate was applied 8 cm above the origin, and finally 4.0 M-formate was applied to the top of the sheet. After drying, nucleotides were detected under u.v. light, and nucleotide spots were cut out and the radioactivity was measured in a liquid-scintillation counter.

Purine nucleosides were separated by two-dimensional t.l.c. on Kodak cellulose sheets as described by Crabtree & Henderson [10]. A sample (20 μl) of neutralized medium and 30 nmol of adenosine were applied to the sheets. Development in the first dimension was performed with acetonitrile/ammonium acetate (0.1 M, pH 7.0)/conc. NH_3 (sp.gr. 0.880) (6:3:1, by vol.), and in the second dimension with butan-1-ol/methanol/water/ammonia (60:20:20:1, by vol.). After drying, adenosine was detected under u.v. light, the spot was cut out and radioactivity measured in a liquid-scintillation counter.

The concentrations of AMP, ADP, and ATP in incubated lymphocytes were determined as follows: 10 ml of incubation mixture was centrifuged, and the cells were resuspended in 1 ml of phosphate-buffered saline [8]. This was immediately deproteinized with 200 μl of ice-cold 25% (v/v) HClO_4 and neutralized: the concentrations in the neutralized extract were determined by enzymic methods [11,12].

Fig. 1. Effect of deoxycoformycin on the adenosine uptake by concanavalin-A-stimulated lymphocytes of the rat

Lymphocytes (5×10^7 /ml) were preincubated for 2 h in the presence of $30 \mu\text{g}$ of concanavalin A/ml and 5 mM-glucose, in the absence (●) or presence (▲) of $50 \mu\text{M}$ -deoxycoformycin. Then $500 \mu\text{M}$ -adenosine was added, and the disappearance of adenosine from the incubation medium was determined at various times after addition of adenosine. The results are presented as means of at least three separate experiments: the S.D. was always less than 10% of the mean.

RESULTS

The uptake of [^{14}C]adenine by lymphocytes preincubated in the presence of 5 mM-glucose and $30 \mu\text{g}$ of concanavalin A/ml was linear with time, and addition of $500 \mu\text{M}$ -adenosine did not influence this rate. The ^{14}C was incorporated mainly into the adenine nucleotides: the amount of radioactivity found in nucleotides other than AMP, ADP and ATP was less than 2%. This finding is similar to that reported for other mammalian cells [13–15].

The uptake of adenosine by concanavalin-A-stimulated lymphocytes, in the absence and presence of $50 \mu\text{M}$ -deoxycoformycin and measured after the addition of $500 \mu\text{M}$ -adenosine to the incubation medium, is shown in Fig. 1. The rate of adenosine utilization was 180 ± 2.5 and 61 ± 0.3 nmol/h per 10^7 cells in the absence and presence of the adenosine deaminase inhibitor respectively. This suggests that two-thirds of the adenosine utilized is deaminated, whereas one-third is phosphorylated. However, the rate of 61 nmol/h per 10^7 cells is a minimal estimate of adenosine kinase activity, since additional adenosine may be produced from AMP via the action of 5'-nucleotidase.

The effect of the presence of $500 \mu\text{M}$ -adenosine in the incubation medium on the content of adenine nucleotides in lymphocytes, in the presence of $50 \mu\text{M}$ -deoxycoformycin, is shown in Fig. 2. The total adenine nucleotide content of the lymphocytes increased continuously with time. This increase was due exclusively to an increase in the concentration of ATP. The rate of increase in ATP content was 44 ± 1.6 nmol/h per 10^7 cells during the first 15 min of incubation; this represents 68% of the rate of adenosine utilization (Fig. 1). In the second 15 min, the rate of increase in the ATP content decreased to 23.3 ± 0.8 nmol/h per 10^7 cells. The difference between the rate of adenosine utilization and the increase in ATP content is assumed to represent the rate of AMP

Fig. 2. Effect of adenosine on the concentrations of adenine nucleotides in concanavalin-A-stimulated lymphocytes of the rat in the presence of $50 \mu\text{M}$ -deoxycoformycin

Lymphocytes (5×10^7 /ml) were preincubated for 2 h in the presence of $30 \mu\text{g}$ of concanavalin A/ml, 5 mM-glucose and $50 \mu\text{M}$ -deoxycoformycin. Then $500 \mu\text{M}$ -adenosine was added to the incubation medium, and the concentration of adenine nucleotides were determined at various times after adenosine addition: ▲, AMP; ■, ADP; ●, ATP. The results are presented as means of at least three separate experiments: the S.D. was always less than 10% of the mean.

Fig. 3. [^{14}C]Adenosine production by concanavalin-A-stimulated lymphocytes of the rat

Lymphocytes (5×10^7 /ml) were preincubated for 2 h in the presence of $30 \mu\text{g}$ of concanavalin A/ml, 5 mM-glucose and $50 \mu\text{M}$ -deoxycoformycin. After 60 min, $5 \mu\text{Ci}$ of [^{14}C]adenine was given to label the adenine nucleotides. After 120 min, $500 \mu\text{M}$ unlabelled adenosine was added to the incubation medium, and the [^{14}C]adenosine was determined after separating it by t.l.c. from other ^{14}C -labelled compounds. To calculate the production from AMP, the specific radioactivity of AMP was determined as described in the Materials and methods section. The results are presented as means of at least three separate experiments: the S.D. was less than 10% of the mean.

dephosphorylation plus deamination, that is, 20.6 and 41.1 nmol/h per 10^7 cells in the first and second 15 min of incubation, respectively.

The activity of 5'-nucleotidase can be measured in the cells from the rate of [^{14}C]adenosine formation after pre-labelling the adenine nucleotide pool of the cells, provided that the specific radioactivity of AMP is known. Addition of unlabelled adenosine to the incubation medium acts as a trapping agent for [^{14}C]adenosine. The specific radioactivity of AMP was obtained by measuring the AMP concentration of the cells and the amount of radioactivity present in the AMP pool after separation by t.l.c. There was no detectable production of [^{14}C]adenosine before the addition of unlabelled adenosine; after the addition, the rate of [^{14}C]adenosine formation was $1.8 \pm 0.2 \text{ nmol/h per } 10^7 \text{ cells}$, which was linear with time (Fig. 3). This very low value provides direct evidence that, in lymphocytes, most of the AMP (> 95%) is catabolized by deamination rather than dephosphorylation.

DISCUSSION

Adenosine can be metabolized by the processes of either phosphorylation or deamination, catalysed by adenosine kinase and adenosine deaminase respectively [1,16]. At the concentration of adenosine (500 μM) used in the present work, both these enzymes are likely to approach saturation with adenosine. Hence the fate of adenosine will depend on the V_{max} values *in vivo* of the two enzymes. The present findings suggest that the V_{max} of the kinase is about half that of the deaminase. However, since the K_m of the kinase is very low (1 μM), at physiological concentrations of adenosine (1–10 μM) the phosphorylation of adenosine should predominate. Indeed, it has been shown that, at concentrations of adenosine < 5 μM , phytohaemagglutinin-stimulated human lymphocytes utilize adenosine primarily via phosphorylation [16,17].

A comparison of the rate of adenosine utilization (at 500 μM -adenosine) with that of ATP formation suggests that some of the adenine nucleotide formed from free adenosine is metabolized and that the rate of this metabolism increases with time. If this metabolism involved dephosphorylation, the product would be adenosine, which could then be rephosphorylated by adenosine kinase, producing a substrate cycle between adenosine and AMP.

Using an adenosine deaminase inhibitor and ^{14}C -labelling of the adenine nucleotide pool, we were able to estimate the rate of adenosine formation by the 5'-nucleotidase reaction. This was found to be very low, $1.8 \pm 0.2 \text{ nmol/h per } 10^7 \text{ cells}$, which suggests that most of the AMP (> 95%) that is broken down is converted to IMP. This finding is different from the metabolism of AMP in rat heart [18] or in liver [19], for example, where it is estimated that about half of the AMP is metabolized by the 5'-nucleotidase reaction.

The high V_{max} and the low K_m of adenosine kinase for adenosine explain the fact that there was no detectable [^{14}C]adenosine formation before the addition of unlabelled adenosine, even in the presence of deoxycytidine, since almost all of the adenosine formed would be converted back into AMP, thus forming a substrate cycle between adenosine and AMP. The existence of the substrate cycle could explain the clinical finding that in

adenosine deaminase-deficient patients there is no increase in adenosine concentration in lymphocytes.

The reason for the high rate of adenine nucleotide degradation via deamination rather than dephosphorylation is not known. The concentration of adenosine is maintained as a balance between the activities of 5'-nucleotidase, adenosine kinase and adenosine deaminase, and a low activity of 5'-nucleotidase indicates that the kinase and deaminase will play the more important role in maintaining the adenosine concentration [1]. It is tempting to speculate that these cells are 'protected' from high rates of endogenous adenosine formation from nucleotides because changes in the adenosine concentration in the vicinity of the cells are used to signal the availability of nucleosides for the proliferative response. This signalling would be expected to involve the adenosine receptor.

We gratefully acknowledge financial support from the Higher Studies Fund, Merton College, Oxford, for provision of subsistence and accommodation in Oxford for Z.S.

REFERENCES

1. Arch, J. R. S. & Newsholme, E. A. (1978) *Essays Biochem.* **14**, 82–123
2. Frideriksen, S. (1966) *Arch. Biochem. Biophys.* **113**, 383–388
3. Simmonds, H. A., Sahota, A., Potter, C. F. & Cameron, J. S. (1978) *Clin. Sci. Mol. Med.* **54**, 579–584
4. Mills, G. C., Goldblum, R. M., Schmalstieg, F. & Newkirk, K. F. (1978) *Biol. Med. (Paris)* **20**, 180–199
5. Donofrio, J. F., Coleman, M. S., Hutton, J. J., Daoud, A., Lampkin, B. & Dyminski, J. (1978) *J. Clin. Invest.* **62**, 884–887
6. Barankiewicz, J. & Cohen, A. (1984) *J. Biol. Chem.* **259**, 15178–15181
7. Ardawi, M. S. M. & Newsholme, E. A. (1982) *Biochem. J.* **208**, 743–748
8. Culvenor, S. G. & Weidemann, M. J. (1976) *Biochim. Biophys. Acta* **437**, 354–363
9. Moellering, H. & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1919–1922, Verlag Chemie, New York and London
10. Crabtree, G. W. & Henderson, J. F. (1971) *Cancer Res.* **31**, 985–991
11. Jaworek, D., Gruber, W. & Bergmeyer, H. U. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., p. 2127, Verlag Chemie, New York and London
12. Lamprecht, W. & Trautschold, I. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 2101–2110, Verlag Chemie, New York and London
13. Bagnaram, A. S., Letter, A. A. & Henderson, J. F. (1974) *Biochim. Biophys. Acta* **734**, 255–270
14. Henderson, J. F., Fraser, J. F. & McLay, E. E. (1974) *Clin. Biochem. (Ottawa)* **7**, 339–358
15. Smith, C. A. & Henderson, J. F. (1976) *Can. J. Biochem.* **34**, 341–349
16. Snyder, F. & Lukey, T. (1982) *Biochim. Biophys. Acta* **696**, 299–307
17. Snyder, F., Mendelsohn, J. & Seegmiller, J. E. (1976) *J. Clin. Invest.* **58**, 654–666
18. Fisher, M. N. (1979) D.Phil. Thesis, University of Oxford
19. Bontemps, F., Van den Berghe, G. & Hers, H. G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **50**, 2829–2833